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Molecular Cloning of ssd-Form Neural Cell Adhesion Molecules (N-CAMs) as the Major Form in *Xenopus* Heart

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Different forms of neural cell adhesion molecule (N-CAM) are generated by alternative splicing of primary transcripts and considered to have distinct biological functions. We cloned cDNAs encoding a new form of N-CAMs from the *Xenopus* heart cDNA library. Comparison of the sequences with chicken and mouse N-CAMs revealed that these clones code for ssd-form N-CAM. We demonstrate by Northern blot analysis that the ssd form is the major form expressed in the *Xenopus* adult heart. We obtained two types of ssd-form N-CAM, which are transcripts from N-CAM 1 and N-CAM 2 genes. Both types contain muscle specific domain (MSD) but not π domain. Northern blot analysis also indicated that this form is not expressed in adult brain, in which ld-form N-CAM is the main N-CAM expressed. It is possible that high levels of specific expression of ssd-form N-CAM are related with the differentiation of cardiac muscles.

Neural cell adhesion molecule (N-CAM) is a widely distributed cell-surface glycoprotein, which mediates and regulates various cell-cell interactions through both homophilic and heterophilic binding (1, 2). N-CAM belongs to an immunoglobulin (Ig) superfamily, having a tandem alignment of five Ig-like and two fibronectin (type III)-like domains. Multiple molecular forms of N-CAM are produced from a single copy gene, depending on cellular physiological states (3).

Alternative splicing is a mechanism to generate molecular diversity (4, 5, 6). Posttranslational modifications, such as glycosylation, sulfation and phosphorylation further modify the molecule(7, 8, 9). Polysialylation is a kind of glycosylation specific to N-CAM and is developmentally regulated. In mouse, human and chicken, it has been reported that alternative splicing produces three major forms of N-CAM (10). They differ in the cytoplasmic and transmembrane domains, but identical in extracellular domain (11, 12, 13).

Of the three major forms of N-CAM, two are membrane-binding forms termed ld (large cytoplasmic domain peptide) and sd (small cytoplasmic domain peptide), with a large and a small cytoplasmic domain, respectively. The third one, termed ssd (small surface domain peptide), lacks both transmembrane and cytoplasmic domains, and attaches directly to the outer cytoplasmic membrane by a phosphatidylinositol (PI) linkage (14, 15, 16, 17). Occurrence of soluble form N-CAM is also reported (10), and is considered to be produced from ssd form(16). The relative amounts of these different forms of N-CAM differ from tissue to tissue and depending on developmental stages, suggesting that each form has its distinct biological function.

N-CAM shows one of the most extensive patterns of alternative splicing of any gene yet characterized. Besides the three forms mentioned above, production of more than 100 different N-CAM mRNAs has been reported because of alternatively spliced small exons in the extracellular domain. Among such small exons, 30-base π domain, which is also called VASE domain, has been shown to occur in rat, mouse and *Xenopus* (13, 18, 19). Alternatively spliced exons, called the muscle specific domain (MSD), also produce diversity in the proximal region of the extracellular domain of N-CAM (13, 19, 20, 21).

In *Xenopus*, the major N-CAM is ld form in embryos and adult brain. Two types of cDNA encoding ld-form N-CAM have been so far cloned from a neurula cDNA library (22, 23) and they are considered to be tran-

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Abbreviations used: N-CAM, neural cell adhesion molecule; ld, large cytoplasmic domain polypeptide; sd, small cytoplasmic domain polypeptide; ssd, small surface domain polypeptide; Ig, immunoglobulin; polySia, polysialic acid; PI, phosphatidylinositol; PI-PLC, phosphatidylinositol phospholipase C; mAb, monoclonal antibody; MSD, muscle specific domain; ORF, open reading frame; PCR, polymerase chain reaction; UTR, untranslated region.

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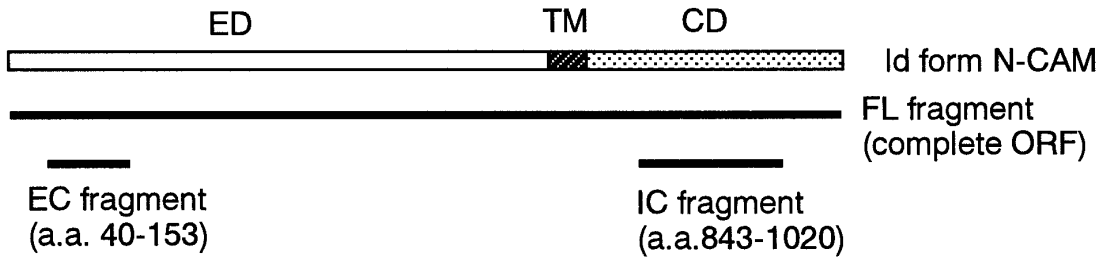

FIG. 1. Probes used for hybridization. EC, TM, and CD are for extracellular domain, transmembrane domain, and cytoplasmic domain, respectively. FL fragment was excised from N1. This contained the complete ORF of ld-form N-CAM. EC fragment and IC fragment were prepared from N1 by PCR. EC fragment contains extracellular domain common to all forms of N-CAM. IC fragment contains cytoplasmic domain specific for ld form N-CAM.

scripts from N-CAM 1 and N-CAM 2 gene, respectively. N-CAM is also expressed in heart (24, 25), however, the form of N-CAM in *Xenopus* heart has not yet been characterized.

Recently we have detected the expression of polysialic acid (polySia) in adult heart that was supposed to be linked to N-CAM (Kudo, unpublished observation). To examine the role played by N-CAM in adult heart especially in relation to polySia, we isolated and characterized the cDNA encoding heart N-CAM. In this paper we report that PI-linked form of N-CAM with MSD is expressed in adult heart as the major form of N-CAM in this tissue.

MATERIALS AND METHODS

Preparation of N-CAM cDNA fragments used as probes. Complete ORF of ld form N-CAM (FL fragment) was prepared from *Xenopus* N-CAM cDNA (N1) (22) using EcoRV and XhoI. Extracellular sequence for a.a. 40 to 153 (EC fragment) common to all forms of N-CAM was prepared from N1 by PCR with 5′-primer, TCCTGTGTCAAGTAAGCGGAC and 3′-primer CTTTGCCTTTATGTGCCAAG. Sequence for the cytoplasmic domain that is specific for ld form, coding for a.a. 843 to 1020 (IC fragment) was also prepared from N1 by PCR with 5′-primer TTTAACTTCTAGCACTGCCCC and 3′-primer AGTAGCAGTTCCAAGAGCTGC (Fig 1). Fragments (2 kb in length) containing 3′-UTR of *Xenopus* heart-specific N-CAM (xhN-CAM, see below) was prepared from xhN-CAM 1 and xhN-CAM 2 by PCR and termed HN1 fragment and HN2 fragment, respectively. Primers used for this PCR were 5′-primers AGCATATGTACAGGGAAGC or GCATATGTACCAGGTAATCC, respectively, and a common 3′-primer CCCGAAATAGCTGATCC.

cDNA cloning. Total RNA was extracted from *Xenopus* heart by the guanidine isothiocyanate method (26). Poly $(A)^+$ RNA was purified from heart RNA by two rounds of chromatography on oligo-(dT) cellulose (Type 3; Collaborative Research) (27). cDNA library was synthesized from heart poly$(A)^+$ RNA using SuperScript Choice System (Life Technologies) with oligo-(dT) primers, ligated with NotI-SalI-EcoRI linkers, and inserted into λgt 10. The library was packaged using a Stratagene Gigapack III Gold packaging extract (Stratagene), plated on *Escherichia coli* NM514, and screened for heart N-CAM with ^{32}P-labelled FL fragment (approximately 2×10^5 plaques). cDNA inserts were isolated from the clones obtained, and subcloned into the NotI site of Bluescript II SK for further analysis. Sequences were determined by dideoxy method using T7 sequencing kit (Pharmacia).

Construction of expression vectors encoding tagged xhN-CAM. Plasmids encoding tagged xhN-CAM 1 or xhN-CAM 2 consist of antibody recognition domain of mouse 7D-VCAM (38) and C-terminal hydrophobic region of xhN-CAM 1 or 2 were constructed as follows. Sequences corresponding to a.a 688 to 725 plus 3′-UTR of xhN-CAM1 or xhN-CAM2 were prepared, respectively, by PCR with 5′-primer TC(AC)AAGC(AC)CTCGAGTTTATCCTTCAGAACC and 3′-primer GATGTAAAGGTACCATGAGAGTTAATCTAC. The amplified and XhoI-KpnI digested fragments was inserted into XhoI-KpnI site of pBluescript SK II (plasmid A1 and plasmid A2). Mouse 7D-VCAM was cloned from a cDNA library in λgt10 and subcloned into expression vector pcDLSRα296 (39) and the resultant plasmid was termed pTag (Takayama, *et al.*, unpublished data). A PstI-XbaI fragment from pTag corresponding to a.a. 1 to 173 plus 5′-UTR was subcloned into pBluescript SK II (plasmid B). A sequence corresponding to a.a. 174 to 299 was prepared from pTag by PCR with 5′-primer GGAGTTTTCTTCAGAAGAGAGGACA and 3′-primer CTAATTCGAGCACTGCACTCGAGCGAACAATCAGATT. The amplified and XbaI-Sac I digested fragment was inserted into the XbaI-SacI site of the plasmid B. The fragment corresponding to a.a. 1 to 299 plus 5′-UTR of 7D VCAM was isolated from the resultant plasmid with EcoRI-XhoI and inserted into the EcoRI-XhoI site of the plasmid A1 and plasmid A2, respectively. Then the fragments coding entire fusion protein were isolated with PstI-KpnI and subcloned into PstI-KpnI site of pcDLSRα296. The plasmid encoding tagged xhN-CAM 1 and 2 were termed pVN1 and pVN2, respectively.

PI-anchor assay. COS-1 cells were cultured on plastic dishes in RPMI-1640 medium containing 50 μM 2-mercaptoethanol supplemented with 10% FCS. pVN1, pVN2, pTag and pcDLSRα296 were transfected into COS-1 cells, respectively, using a commercial transfection reagent (FuGene6; Boehringer Mannheim) according to the manufacturer's instructions. COS-1 cells were harvested 72 h after transfection and treated with phosphatidylinositol phospholipase C (PI-PLC; Boehringer Mannheim) at 500 mU/ml or mock-treated in RPMI-1640 medium containing 10 mM Tris-HCl (pH 7.5) and 10 mM EDTA at 37°C for 1 h. Then the cells were incubated consecutively with rat mAb for mouse VCAM (RAV-2; Takayama *et al.*, unpublished data) and FITC-conjugated second antibody, and examined by flowcytometry using FACScalibur (Becton Dickinson) with Cell Quest software (Becton Dickinson).

Northern blot hybridization. Mature frogs were chilled in ice and tissues were excised using forceps and scissors. RNAs were prepared by the acid guanidine phenol chloroform procedure(28). Poly $(A)^+$ RNA was purified with oligoTex-dT30 super (Takara). RNAs were fractionated on 0.8 % agarose gels containing 3.7% formaldehyde, transferred to Hybond N^+ membrane, and then probed with gel-purified, ^{32}P-radiolabelled fragments (FL, IC, or HN1 and 2).

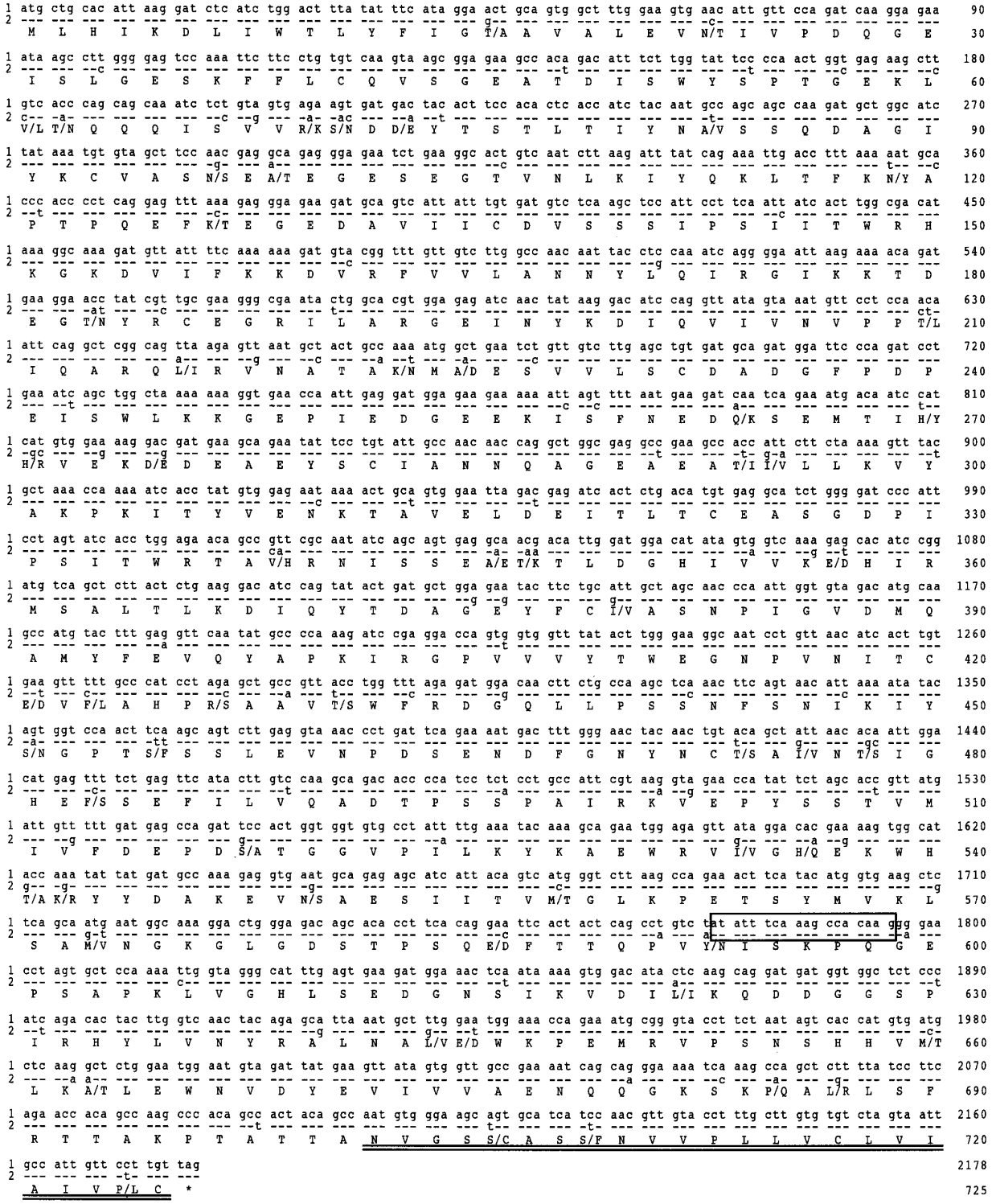

FIG. 2. Nucleotide and amino acid sequences for ORF of xhN-CAM-1 (upper row) and xhN-CAM 2 (lower row). Nucleotides identical between xhN-CAM 1 and xhN-CAM 2 are represented by bars in the sequence of hN-CAM 2. Amino acids which are different between xhN-CAM-1 and xhN-CAM 2 were shown using slash; the former is for xhN-CAM-1 and the latter for xhN-CAM 2. Nucleotides corresponding to MSD is boxed. Amino acids newly found are double-underlined. These sequences will be appeared in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers AB 008162 (xhN-CAM 1) and AB 008163 (xhN-CAM 2).

RESULTS

Cloning and analysis of primary structure of the xhN-CAM cDNAs. We screened heart cDNA library with FL fragment (Fig. 1) and obtained 49 positive clones. We selected 24 clones that were also hybridized with EC fragment (Fig. 1) in order to obtain clones encoding complete ORF. Finally we selected two clones of 4.5 kb in length. Partial sequence analysis revealed that both clones corresponded to the transcripts from N-CAM 1 and N-CAM 2 gene, and they were named xhN-CAM 1 and xhN-CAM 2, respectively. Sequence analysis revealed that both had an ORF consisting of 725 amino acids (Fig. 2).

xhN-CAMs did not contain the transmembrane domain but contained 24 hydrophobic amino acids at the C-terminal region (Fig. 2), and thus corresponded to ssd-form, or PI-linked form. Comparison of the amino acid sequences with mouse and chicken N-CAMs suggested that xhN-CAMs were produced by using an exon corresponding to the exon 15 of mouse and chicken N-CAM genes (Fig. 3). Though the similarity of these C-terminal regions of xhN-CAMs to those of mouse and chicken is not so high, a cluster of hydrophobic amino acids are expected to serve in inducing PI-linkage. xhN-CAMs had MSD of 18 nucleotides as reported by Krieg but did not have sequence from π exon (Fig. 2).

xhN-CAMs are attached to cytoplasmic membrane by PI-linkage. To investigate if xhN-CAM 1 and xhN-CAM 2 are actually attached to cytoplasmic membrane by PI-linkage, PI-PLC that specifically hydrolyzes PI-linkage was used. xhN-CAMs were tagged (pVN1 or pVN2; Fig. 4a) as described in Materials and Methods and expressed on COS-1 cells. Tagged xhN-CAMs were used for this assay because we do not have appropriate antibody that recognizes extracellular domain of *Xenopus* N-CAM. After 72 h of culture, transfected cells were treated with PI-PLC or mock-treated, and expression of the tagged xhN-CAMs on the cell surface was assayed with anti-Tag antibody (RAV-2) by flowcytometry. Tag was detected from mock-treated cells, whereas, after cells were treated with PI-PLC, it was not detected (Fig. 4b), indicating that PI-anchor was hydrolyzed by PI-PLC. In contrast, when pTag, that encodes transmembrane protein, was introduced into COS-1 cells, Tag was detected even after PI-PLC treatment. These results indicate that xhN-CAM 1 and xhN-CAM 2 are attached to cytoplasmic membrane by PI-linkage.

Tissue-specific expression of different forms of N-CAM. To examine if there is tissue-specificity in the expression pattern of different forms of N-CAM, we carried out Northern blot analyses using poly(A)$^+$ RNA from adult heart, brain and eye using various N-CAM fragments as probes (Fig. 5). When probed with FL fragment, 9.5-kb, 4.0-kb, and 3.8-kb signals were detected in brain and eye (Fig. 5a) in consistent with the previous report (22). Signals of 9.7 kb and 4.0 kb reportedly code for ld form whereas 3.8-kb signal codes for sd form (22). In heart by contrast neither 9.7-kb nor 4.0-kb signal was detected, and instead, only 4.5-kb signal was detected (Fig. 5a). When probed with IC fragment that is a specific probe for ld form, 9.7-kb and 4.0-kb signals were detected in brain. In heart, however, no signal was detected with IC fragment. Therefore, we concluded that in heart ld form mRNA is not expressed appreciably. When probed with HN1 and HN2 that is specific for 3′-UTR of xhN-CAMs, a distinct signal of 4.5 kb was detected in heart, however, no such signal was obtained in brain. From these results we concluded that ssd-form is the only form or at least the major form of N-CAMs expressed in *Xenopus* heart, though this form was not expressed in brain.

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          702                    725
Xenopus 1 NVG*SSASSNVVPLLVCLVIAIVPC
Xenopus 2 ---*-C--F----------L-
Chick     TL-SP-T--SF-S--LSA-TLLLL-
Mouse     TL-G--T-YTL-S--FSA-TLLLL*
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FIG. 3. An alignment of the C-terminal region of *Xenopus*, chicken and mouse N-CAM deduced from nucleotide sequences of exon used for ssd-form (exon 15 of chicken and mouse N-CAM). Identical amino acids are represented by bars, and amino acids deletions are represented by asterisks. The numbering of amino acids for *Xenopus* is as in Fig. 2.

DISCUSSION

We have cloned a new type of *Xenopus* N-CAMs (xhN-CAMs) from adult heart and showed that this is the major form of N-CAM expressed there. We obtained two clones xhN-CAM 1 and xhN-CAM 2 that represents transcripts from N-CAM 1 gene and N-CAM 2 gene, respectively. xhN-CAMs have a cluster of hydrophobic amino acids at C-terminal region, and comparison of their sequences with those of mouse and chicken N-CAMs revealed that both clones code for ssd form (PI-linked form) N-CAMs. We examined that xhN-CAMs are attached to cytoplasmic membrane by PI-anchor using PI-PLC. xhN-CAMs expressed on the cell surface of COS-1 cells which were transfected with tagged xhN-CAMs were cleaved by PI-PLC. xhN-CAMs have MSD sequences of 18 nucleotides as previously reported by Zorn (19), but do not contain π domain. We analyzed the expression of ld-form and ssd-form N-CAMs in adult brain and heart by Northern blot analysis using specific probes for these two forms. The results showed that ld form but not ssd form is expressed in brain, whereas, to the contrary, ssd form but not ld form is expressed in heart.

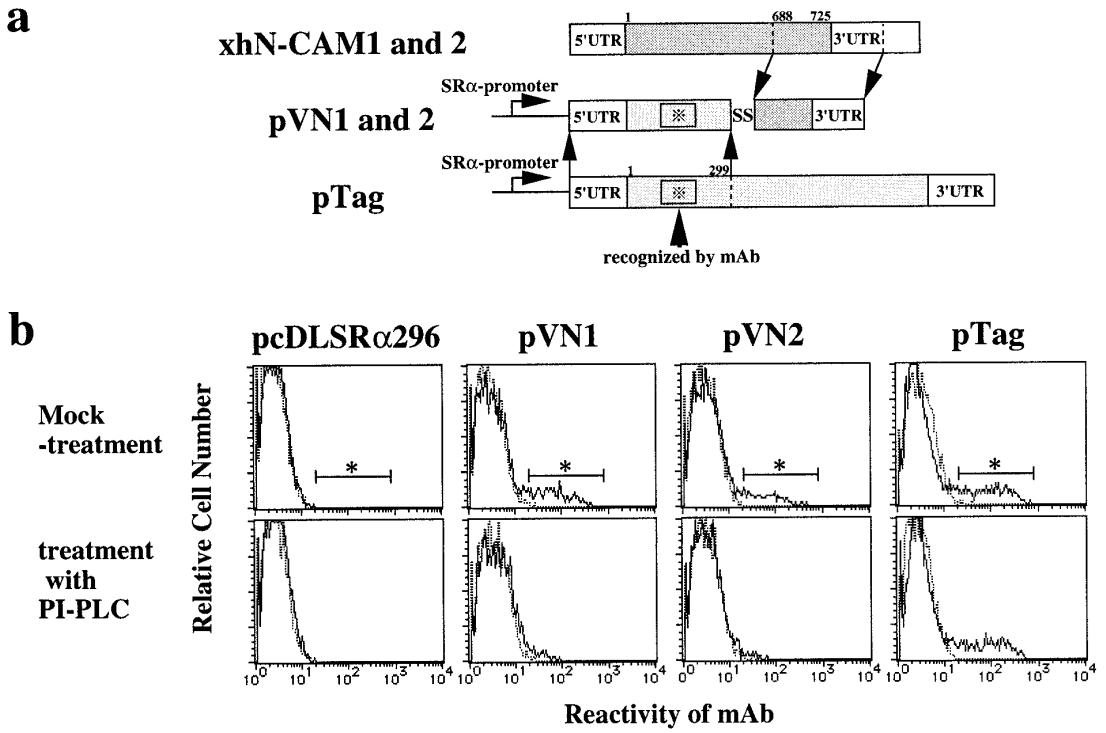

FIG. 4. (a) Structure of cDNA transfected into COS-1 cells. xhN-CAM 1 and 2 were tagged as described in Materials and Methods and subcloned into pcDLSRα296 (pVN1 or pVN2). (b) pVN1, pVN2, pTag and pcDLSRα296 were transfected into COS-1 cells. Expression was detected by anti-Tag antibody (RAV-2) after treatment (lower panels) or mock-treatment (upper panels) with PI-PLC. Cells detected by RAV-2 were marked with asterisks. pTag was transfected into COS-1 cells as positive control for transfection and negative control for PI-PLC treatment.

N-CAM was reported to be expressed in cardiac muscles during metamorphosis and remains to be expressed in adult heart of *Xenopus* (25). N-CAM is associated with polysialic acid (polySia), and polySia on N-CAM is considered to negatively regulate the homophilic interactions between cells though a vast, voluminous hydration sphere and the polyanionic nature of the polySia structure (29, 30, 31). We had previously immunohistochemically detected the expression of polySia in *Xenopus* adult heart, and found it widely distributed in cardiac muscles (Kudo, unpublished observation). This observation suggested that N-CAMs in *Xenopus* adult heart are polysialylated as N-CAM is the only candidate for polysialylation. Expression of polySia and N-CAM in heart was studied in rat, mouse and chicken (32, 33, 34), though the form of N-CAMs expressed in heart of these animals is not yet identified. During heart development of rat, expression of polysialylated N-CAM in cardiac muscles is transient, and the expression of N-CAM in adult heart is weak and restricted to nerve fibers. On the contrary, our data suggests that polysialylated N-CAM remains to be expressed in cardiac muscles even in *Xenopus* adult heart.

N-CAM expressed in *Xenopus* adult heart is ssd form with MSD and polysialylated. In mammals N-CAM also have important roles in myoblast interaction during myogenesis (35, 36). The ssd-form N-CAM with MSD is reported to be transiently expressed in myo-

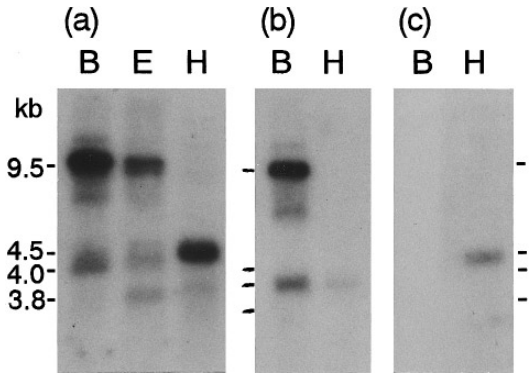

FIG. 5. Northern blot analyses detecting N-CAMs in brain (B), eye (E), and heart (H) with various probes. Poly(A)$^+$ RNAs (0.2 μg for brain or 2 μg for eye and heart) were loaded on gels. (a) Signals for all forms of N-CAM were detected when probed with N1 fragment. (b) Signals for ld-form N-CAM were detected when probed with IC fragment which is specific for ld-form. (c) Signals for ssd-form was detected when probed with the mixture of HN1 and HN2, which are specific for ssd-forms.

tubes and implicated in myoblast fusion (37), however, in the differentiated skeletal muscles no N-CAM is detected except at neuromuscular junction. Skeletal muscles are multinucleated whereas cardiac muscles consist mononuclear cells, but indifferent to such differences, N-CAM appears to be involved in development of cardiac muscles. The expression of ssd-form N-CAM that is polysialylated and contains MSD suggests that *Xenopus* cardiac muscles are not fully differentiated even in adult heart. The role of N-CAM in *Xenopus* heart must be further analyzed together with polySia.

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