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Identification of a Novel, Embryonal Carcinoma Cell–Associated Molecule, Nucling, That Is Up-regulated during Cardiac Muscle Differentiation

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EC cells are characterized by their potent capacity to differentiate into several cell types, such as mesoderm-like cells, endoderm-like cells, or ectoderm-like cells. By subtracting the mRNAs expressed by one EC cell clone, F9 cells, with the mRNAs expressed by another EC cell clone, P19 cells, we identified six novel genes that are expressed selectively by F9 cells. One of these genes (Nucling) encodes a polypeptide of 1411 amino acids containing an ankyrin repeat, aspartyl protease motif, a leucine zipper motif, and two t-SNARE coiled-coil domains. Northern blot analyses revealed the Nucling mRNA to be detected predominantly in heart, liver, kidney and testis, but not in brain or spleen. Immunostaining analyses revealed a unique feature of Nucling that the transiently expressed protein forms aggregates exclusively around nuclear membranes. Moreover, the expression level of the Nucling gene transcript increases progressively during the early developmental stages in mice, and specifically at cardiomuscular differentiation *in vitro* and *in vivo*. These results suggest that Nucling may play some role in the gene regulation of cell differentiation during embryonal development.

Key words: cardiomuscular differentiation, cDNA subtractive screening, cell differentiation, EC cells, embryonal development.

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EC cells, Embryonal carcinoma cells; EtBr, ethidium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MLC2v, ventricular myosin light chain type 2; nt, nucleotides; RA, retinoic acids; SSH, suppressive-subtractive hybridization.

EC cell lines, such as F9 and P19, are widely used as model systems for studies on the regulation of gene expression during the early developmental stages of mammals. There are differences between F9 and P19 in terms of the direction of cell differentiation. P19 cells retain pluripotent properties and can easily be induced to differentiate into several cellular types upon the addition of RA and/or by aggregate formation (*1*). On the other hand, F9 cells differentiate into parietal endoderm-like cells or primitive endoderm-like cells using RA with or without dibutyryl cyclic AMP (*2*, *3*). Early studies carried out in EC cells clearly indicated that these cells possess a number of interesting and unique regulatory features for gene expression. We, therefore, focused on the differences between F9 and P19 in terms of their properties of cell differentiation at the molecular level. The identification and subsequent cloning of factors expressed specifically in either F9 cells or P19 cells represent the first crucial step in unraveling the elusive regulatory mechanisms of the early developmental stages in mammals.

In this report, we describe the molecular cloning of a novel molecule termed Nucling. This molecule was isolated using a subtraction assay system between F9 and P19 cells. Nucling was detected with a perinuclear and reticular distribution in transfected cells. Northern blot analysis revealed that the transcript is expressed widely in several tissues but not in brain or spleen. In addition, the level of expression increases progressively during the embryonic stages of development and also at cardiomusclar differentiation in mammalian cells. Taken together, Nucling may cooperate with gene regulation in embryonal development, especially in cardiac muscle development.

MATERIALS AND METHODS

Cell Culture and Differentiation—COS-7 cells and F9 EC cells were cultured in DMEM (Gibco BRL.) supplemented with 10% fetal calf serum (Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate. F9 cells were maintained in log-phase growth by passaging at 2×10^4 cells per ml every 2–3 d (*4*). P19 EC cells were maintained as described (*5*). P19.CL6 cells (RIKEN GENE BANK) were cultured essentially as described previously (*6*). In brief, the cells were grown in a 100-mm tissue culture grade dish under adherent conditions with α-minimal essential medium (Gibco BRL) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) (growth medium), and were maintained in a 5% CO_2 atmosphere at 37°C. To induce differentiation under adherent conditions, P19.CL6 cells were plated at a density of 3.7×10^5 in a 60-mm tissue culture grade dish in growth medium containing 1% DMSO (differentiation medium). The medium was changed every 2

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d. Days of differentiation were numbered consecutively after the first day of DMSO treatment, day 0.

Isolation of RNA—Poly (A)$^+$ RNA was prepared from undifferentiated F9 cells and P19 cells according to the manufacturer's instructions (Invitrogen Micro-FastTrack™ 2.0). The quality of the isolated RNA was determined via RT-PCR with GAPDH.

Synthesis of cDNA—In a typical subtraction experiment, 2 μg of poly (A)$^+$ RNA was used for first strand cDNA synthesis. First and second strand syntheses were done using a SMART cDNA synthesis kit (Clontech, USA) after thorough optimization of the cycle number. In order to test that the PCR was in the logarithmic phase, one-tenth of the cDNA was labeled and electrophoresed on an alkaline agarose gel. Fifty percent of the poly A$^+$ RNA was subjected to 25 cycles of PCR according to the manufacturer's instructions.

Subtractive Hybridization—Isolation of F9-specific genes was achieved by performing SSH between F9 cells (tester) and P19 cells (driver) cDNA using a PCR-select cDNA subtraction kit (Clontech, USA) (referred to as forward screen). For genes induced by P19 cells, the same procedures were used except that cDNA from P19 cells served as the tester and the F9 cells material was used as the driver (referred to as reverse screen).

For the first hybridization, a mixture of driver and tester cDNAs was denatured at 100°C for 20 s and then cooled over 1 min to 68°C, and the temperature was maintained for 8 h. For the second hybridization, driver cDNA was denatured at 100°C for 20 s and then added to the pooled mix of the previous hybridization and incubated at 68°C for 20 h.

Subtraction Efficiency—PCR analysis was performed to check the subtraction efficiencies using an Advantage 2 PCR kit (Clontech) according to the manufacturer's instructions for the PCR-Select Differential Screening kit (Clontech). Equal amounts of amplified material were run in an agarose gel and visualized under a UV-transilluminator after staining with EtBr.

Construction of Libraries and Subtracted Probe—The subtracted cDNA pools were amplified by PCR using an Advantage cDNA PCR Kit (Clontech). Unidirectional cDNA libraries were constructed by T/A cloning of the cDNA pools into the pT7Blue vector (Novagen). The RNA was also used for subtractive hybridization with the subtraction kit. All the procedures were performed according to the manufacturer's instructions.

Library Screening and Sequence Analysis—Libraries were plated to a density of 100–500 colonies on 100 cm^2 LB plates containing 40 mg/liter of ampicillin and 40 mg/liter of X-gal. White colonies were selected and checked for the presence of an insert by PCR using the primers provided in the subtraction kit. Colonies containing inserts were rescreened using the PCR-Select Differential Screening Kit (Clontech). Isolated clones were found to contain only cDNA partial sequences. Full-length cDNAs were obtained using the Marathon™ cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. Sequencing was carried out on both strands using overlapping clones with nested deletions (double-stranded Nested Deletion Kit; Pharmacia). Sequence analysis was performed using the Genetyx Computer Group software package (*7*). The BLAST program was used to search the GenBank databases at NCBI.

Virtual Northern—Virtual Northern blots were performed according to the manufacturer's instructions (SMART and PCR-Select subtraction manuals, Clontech, USA). Five hundred nanograms of SMART cDNA derived from the mRNA of both F9 and P19 cells was separated in an agarose gel, stained with EtBr, and transferred onto a nylon membrane (Hybond N, Amersham, UK). The blots were then hybridized to [^{32}-P]dCTP labeled PCR fragments of GAPDH and all the isolated clones under Church buffer conditions.

RNA Analysis—Mouse adult multiple tissue and whole embryo poly (A)$^+$ RNA blots were obtained from Clontech and processed according to manufacturer's instructions. Poly(A)$^+$ RNAs from F9 cells, P19 cells and P19.CL6 cells were extracted using a Micro-Fast Track mRNA Isolation system kit, and Northern blot analysis was performed as described below with 3 μg of poly(A)$^+$ RNA for Nucling, MLC2v and β-actin. Poly(A)$^+$ RNA was subjected to agarose-formaldehyde gel electrophoresis and then transferred onto a Hybond N$^+$ membrane filter (Amersham) (*8*). Hybridization was carried out overnight at 42°C in 40% formamide, 5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA (pH 7.7)], 5× Denhardt's solution, 10% dextran sulfate, and 1% sodium dodecyl sulfate. The probes were labeled with [^{32}-P]dCTP by random priming (Takara) (*9*) at a final concentration of 10^6 cpm/ml. The filters were washed for 1 h under high stringency conditions [65°C, 0.2× SSC, 0.1% (w/v) SDS] and exposed to Kodak Scientific Imaging Film (BioMax MS) at –70°C for 72 h (Nucling), or 24 h (MLC2v, GAPDH and β-actin). The following cDNA fragments were used as probes: the *Eco*RI/*Bam*HI fragment of pBluescript containing murine Nucling cDNA, the *Eco*RI fragment of pCRII containing a PCR product obtained using oligonucleotide primers specific for MLC2v (*10*), and the β-actin cDNA fragment provided from Clontech. For the analysis of Csx/Nkx-2.5 (*11*, *12*) and GAPDH mRNA, RT-PCR was performed. First-strand cDNA was synthesized with Superscript II reverse transcriptase and a random primer (Gibco BRL) from 1 μg of poly(A)$^+$ RNA, and PCRs were performed with 1 μl of cDNA products, a 0.3 μM concentration of each oligonucleotide primer, and 1 U of Taq polymerase (Takara) in 50 μl of buffer containing 200 μM deoxynucleotide triphosphates. For Csx/Nkx-2.5, the PCR primers and regimen used were essentially as described previously (*13*). The sequences of the primers for GAPDH were: sense, 5′-ACCACAGTCCATGCCATCAC-3′ and antisense, 5′-TCCACCACCCTGTTGCTG-3′ with an expected amplification product of 452 bp. PCR products were electrophoresed in 2% agarose gels and visualized by EtBr staining.

Construction of Expression Vectors—The cDNA fragment of Nucling was subcloned into the vector pFlag-CMV2 (Kodak) for expression in mammalian cells.

GST Fusion Protein and Polyclonal Antibody Production—The *Nucling* cDNA fragment encoding amino acids 977–1221 was generated by PCR and cloned into pGEX2T (Pharmacia). The fusion protein was prepared and purified on Glutathione-Sepharose (Pharmacia) according to the manufacturer's instructions. The anti-

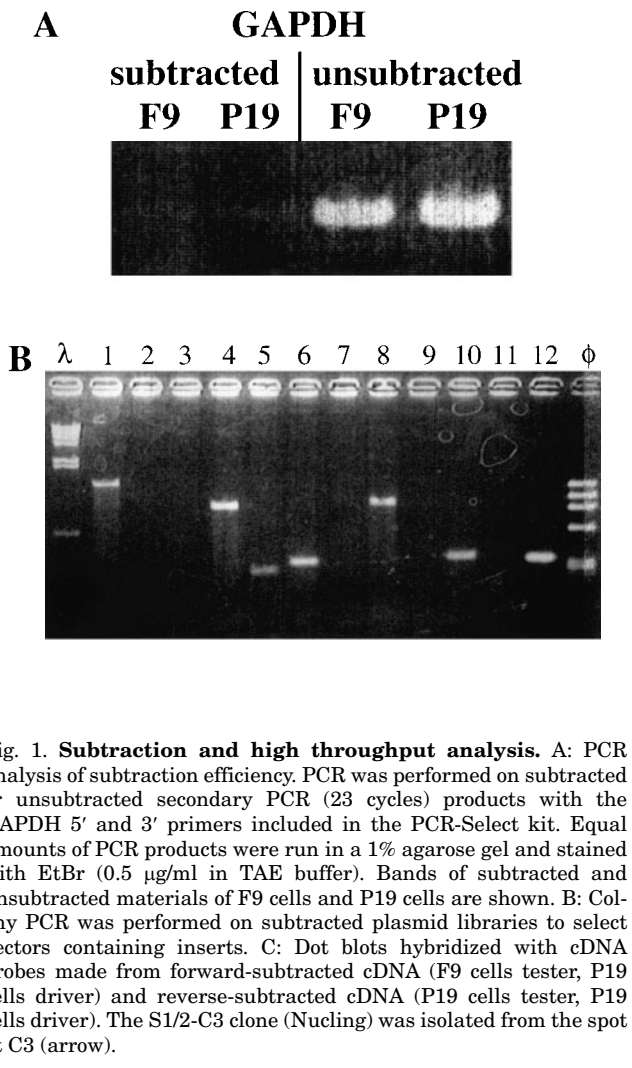

Fig. 1. **Subtraction and high throughput analysis.** A: PCR analysis of subtraction efficiency. PCR was performed on subtracted or unsubtracted secondary PCR (23 cycles) products with the GAPDH 5′ and 3′ primers included in the PCR-Select kit. Equal amounts of PCR products were run in a 1% agarose gel and stained with EtBr (0.5 μg/ml in TAE buffer). Bands of subtracted and unsubtracted materials of F9 cells and P19 cells are shown. B: Colony PCR was performed on subtracted plasmid libraries to select vectors containing inserts. C: Dot blots hybridized with cDNA probes made from forward-subtracted cDNA (F9 cells tester, P19 cells driver) and reverse-subtracted cDNA (P19 cells tester, P19 cells driver). The S1/2-C3 clone (Nucling) was isolated from the spot at C3 (arrow).

Nucl.mid polyclonal antibody was raised by immunizing Japanese white rabbits with this fusion protein.

Immunofluorescence Staining Assay—COS-7 cells grown on 18 × 18 mm glass coverslips were transfected using Effectene (QIAGEN) according to the manufacturer's instructions, then fixed with ice cold 4% paraformaldehyde in PBS for 10 min and permeabilized with methanol for 2 min. In order to detect the product of the transfected gene, cells were washed with PBS(–) twice before incubating them with PBS(–) containing 2% goat serum for 1 h, followed by staining. The anti-Nucl.mid polyclonal Ab was used at 1:200. Texas red secondary antibody conjugates (Cappel) were used at 1:100. Vectashield mounting medium was purchased from Vector Labs. Fluorescence photomicroscopy was conducted on a Zeiss Axiophot, and three-dimensional microscopic images were captured and processed using a confocal Laser scanning microscope and software (Leica TCS NT, Heidelberg, Germany).

Western Blotting for Nucling Transfectant—Protein extract lysates from cells were prepared in ice-cold TNE buffer [10 mM Tris (pH 7.8)/150 mM NaCl/1% NP40/ 1mM EDTA] containing 1 tablet of protease inhibitor cocktail (Complete™ Mini EDTA-free; Boehringer Mannheim)/10 ml. Ten micrograms of protein was used for Western blotting of whole cell lysates. The antibody probes used were the anti-Nucl.mid polyclonal antibody at 1:500 dilution, or anti–β-actin monoclonal antibody (Sigma). Signals were visualized by chemiluminescence (ECL Western blotting detection system; Amersham-Pharmacia).

Subcellular Fractionation and Western Blotting—To prepare cell extracts for subcellular fractionation, cells (cultured on 10 cm plates for two days after transfection) were washed twice with 5 ml cold PBS, harvested by scraping, divided into two tubes and centrifuged (1,000 rpm for 5 min). Half of the cell pellets were harvested as whole cell extracts. The remaining cell pellets were resuspended in 1 ml of cold hypotonic buffer (42 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$) supplemented with protease inhibitor cocktail (0.1 mM PMSF, 2 μg/ml of leupeptin and aprotinin) and incubated for 30 min on ice. Nuclei were removed by centrifugation at 600 ×*g* for 10 min (crude nuclear fraction), and heavy membrane contents were then removed by centrifugation at 1,000 ×*g* for 10 min. The light membrane and cytosol fractions were separated by additional centrifugation at 100,000 ×*g* for 90 min. All pellets were resuspended in 1 ml of cold extrac-

Table 1. **Summary of the isolation of F9 or P19 cell-specific genes.**

	No. of selected clones/ No. of tested clones		Recovery at each step (%)		Method
	F9	P19	F9	P19	
First screening	53/192	36/192	27.6	18.8	Colony PCR
Second screening	39/53	24/36	73.6	66.7	Dot blot hybridization
Third screening	13/39	8/24	33.3	33.3	Virtual Northern blot

Genes expressed selectively by F9 or P19 cell line were enriched by subtractive hybridization of the F9 or P19 cell cDNA libraries with cDNA prepared from P19 or F9 cell lines, respectively. The 192 clones in the resulting subtractive cDNA libraries were sequentially screened by colony hybridization (first), dot blot hybridization (second), and virtual Northern blotting (third).

tion buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in 2× PBS) supplemented with protease inhibitor cocktail (0.1 mM PMSF, 2 μg/ml of leupeptin and aprotinin), and the cells were broken by passing the suspension through a 26 G needle. Following incubation on ice for 30 min, cell extracts were obtained as the supernatants by centrifugation at 100,000 ×*g* for 5 min. Protein concentrations of the extracts were estimated by the Pierce Protein Assay. The cell extracts were denatured with 1× sample loading buffer for SDS–PAGE. Thirty micrograms of total protein was loaded onto a Tris-Glycine gel at the appropriate percentage, transferred onto nitrocellulose membranes, and incubated with appropriate antibodies. Antibody probes used were the anti-Nucl.mid polyclonal antibody or anti-GRP78 polyclonal antibody (Santa Cruz Biotech). Western blot analysis was carried out according to standard procedures using ECL detection (Amersham-Pharmacia) or AP detection (Roche).

Whole-Mount RNA In Situ Hybridization—A cDNA fragment of mouse *Nucling* spanning nucleotide positions 1–1306 was subcloned into pBluescript II KS(+) (Stratagene, La Jolla, CA) and used to generate digoxigenin-labelled sense and anti-sense transcripts according to the manufacturer's instructions (Roche).

Embryos were recovered from ICR pregnant mice (Clea Japan), fixed in 4 % paraformaldehyde in PBS for 16 h at 4°C, and dehydrated by immersion through a series of ethanol solutions. Whole-mount RNA in situ hybridization was performed essentially as described by Wilkinson (*14*). Proteinase K treatment was performed at a concentration of 10 μg/ml with incubation for 15 min at room temperature.

RESULTS

Identification of Novel Genes Expressed by F9 EC Cells Using a Subtractive Cloning Strategy—In search of factors expressed specifically in either F9 cells or P19 cells, we performed cDNA subtraction analysis between F9 and P19 cells. cDNA was generated and amplified using a SMART cDNA synthesis kit (Clontech). These cDNAs were then used for PCR-based subtraction, the suppressive-subtractive hybridization (SSH) protocol (*15*) (PCR-Select, Clontech). The screening for genes induced by F9 cells is referred to as a "forward"-screen (RNA from F9 cells was used as the "tester", RNA from P19 cells as the "driver"). Genes induced by P19 cells were identified using RNA from F9 cells as the "driver" and RNA from P19 cells as the "tester" in the procedure termed a "reverse"-screen.

After the forward- and reverse-subtracted cDNA libraries were generated, they were tested for efficiency of the subtraction. The degree of subtraction can be determined by monitoring the depletion of transcripts common to both populations after subtraction. GAPDH, a housekeeping gene, should be depleted from the subtracted material. GAPDH was shown to be reduced approximately 1,000-fold in the subtracted material compared to the unsubtracted material (Fig. 1A). The result indicates that the population of cDNA was successfully subtracted and that highly expressed housekeeping genes, such as GAPDH, were eliminated or their amounts greatly reduced. Therefore, we could conclude that the subtraction of cDNA worked at high efficiency and the subtracted cDNA libraries could be transformed into a cloning vector for the screening procedure. Subsequently, we constructed "F9 cell-specific" and "P19 cell-specific" cDNA libraries from the clones and tested these libraries in three rounds of screening. Of 192 independent colonies analyzed first by colony PCR, 53 colonies for F9 cells and 36 colonies for P19 cells contained cDNA fragment inserts (Fig. 1B). We next tested these 89 clones by dot blotting and selected 63 that showed preferential hybridization with radiolabeled cDNA derived from the forward- or the reverse-subtracted libraries (Fig. 1C). Finally, these 63 clones were examined for their expression levels in F9 versus P19 cells by virtual Northern blotting. We were able to confirm F9 cell–specific expression for 13 of the clones and P19 cell-specific expression for 8 (Table 1).

Through partial sequencing and cross-hybridization, we found that the above 21 clones contained 12 distinct genes. Homology search of their nucleotide sequences revealed three genes that encoded currently recognized polypeptides, including GRP78/Bip (78 kDa glucose-regulated protein/immunoglobulin heavy chain binding protein) (*16*), Atox1 (copper transporter protein) (*17*), and ubiquitin (*18*). We have also identified genes that encode the mouse equivalent of human TACC2 (transforming, acidic coiled-coil containing protein 2) (*19*), human HAUSP (herpesvirus associated ubiquitin-specific protease (*20*), and human DPYSL3 (dihydropyrimidinase-like 3) (*21*, *22*). The remaining six genes were judged to be distinct from any nucleotides currently registered in the GenBank™ or EMBL data bank. We focused our subsequent effort on one of these novel genes.

Structual Features of the Novel Polypeptide—Clone S1/2-C3, corresponding to the spot at the C3 position in Fig. 3C, contained 381 nt in its open reading frame, and showed F9 cell–specific hybridization in dot blotting and

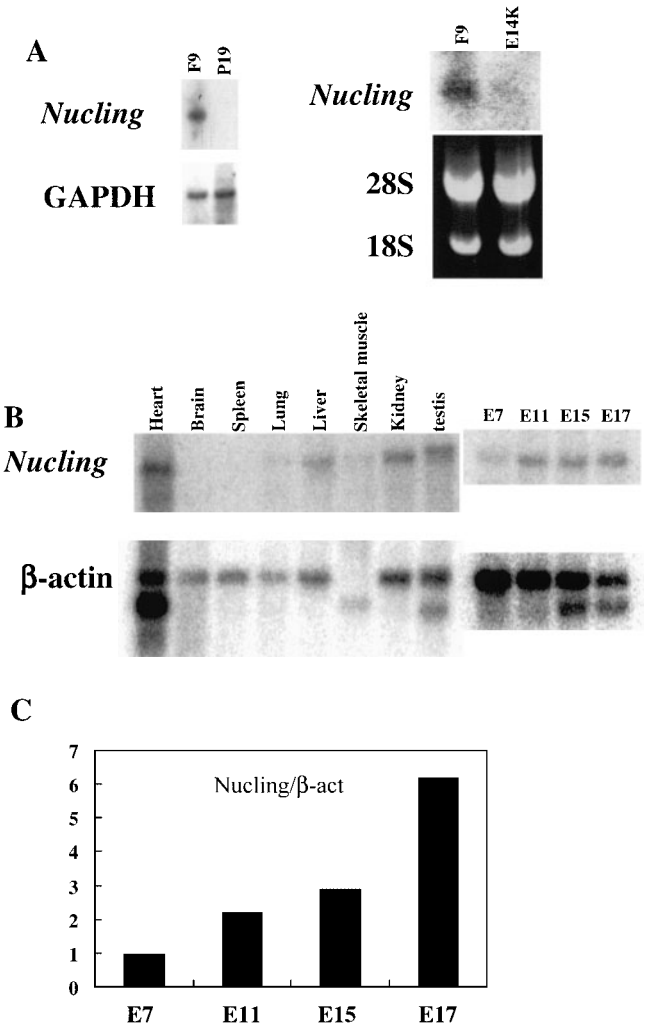

Fig. 2. **Expression pattern of *Nucling* mRNA.** A: Nucling is expressed in F9 cells but not in P19 cells or ES cells. An RNA blot containing 3 μg of poly(A)$^+$ RNA from F9, P19 EC or E14K ES cells was hybridized with a ^{32}P-labeled mouse *Nucling* cDNA probe followed by a ^{32}P-labeled mouse G3PDH cDNA probe. B: Tissue distribution of *Nucling* in mice. poly(A)$^+$ Northern blots of several adult mouse tissues and embryos at various stages of development were probed with a ^{32}P-labeled mouse *Nucling* cDNA probe followed by a ^{32}P-labeled mouse β-actin cDNA probe. C: The expression level of Nucling mRNA increases linearly during development. Band density (B) was quantitated using ImageQuant Version 5.0 software (Molecular Dynamics), and the *Nucling* mRNA level was normalized against the β-actin mRNA level in each lane. Each of the values was divided by the value of E7 dpc and plotted in the graph.

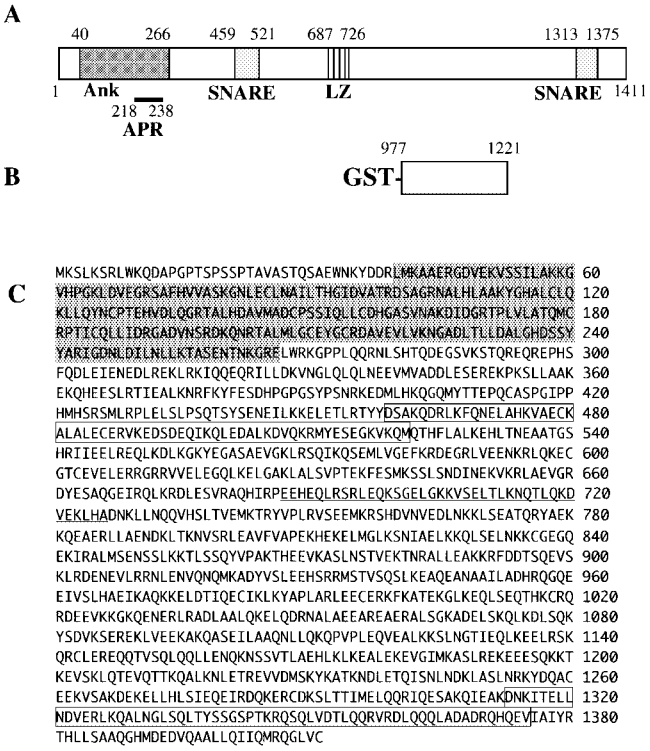

Fig. 3. **Deduced amino acid sequence of murine Nucling.** A: Primary structure of the full-length Nucling is diagrammed with its typical motifs. Ank, APR, SNARE, LZ represent ankyrin repeat region, aspartyl protease motif, t-SNARE coiled-coil domain and leucine zipper motif, respectively. B: A GST fusion protein, GST-Nucl.mid, was used to raise and purify a polyclonal antibody against the middle portion of mouse Nucling (amino acids 977–1221). C: Predicted amino-acid sequence. The nucleotide-sequence data reported have been submitted to the GenBank nucleotide-sequence database with the accession number AB030647. An ankyrin repeat region is indicated in the gray box. Two t-SNARE coiled-coil domains are boxed. The underlined sequence with L in bold indicates a leucine zipper motif.

Northern blotting (Fig. 2A). Undifferentiated ES cells (E14K) failed to exhibit positive hybridization signals with this clone in Northern blotting (Fig. 2A). PCR with primers designed from the S1/2-C3 sequence amplified a product of 250 base pairs, which provided a probe to identify the F9 cell–specific cDNA among RACE products. The overlapping cDNA 5′ and 3′ ends obtained by RACE were combined to construct a 4.5-kb mouse cDNA. This full-length cDNA contained an open reading frame encoding a basic protein (pI = 6.80) of 1,411 amino acids (aa) with a predicted molecular mass of 160.8 kDa. As shown in Fig. 3, A and C, the deduced amino acid sequence of this clone revealed a polypeptide consisting of an ankyrin repeat (aa 40–266), aspartyl protease motif (aa 218–238), a typical leucine zipper motif (aa 687–726), and two t-SNARE coiled-coil domains (aa 459–521 and 1313–1375). Comparison of the full-length cDNA sequence to the GenBank™ database using the BLAST program revealed significant identity (79% for amino acids and 78% for nucleotides) with human KIAA1561 (accession number AB046781), whose function has yet to be described.

Tissue Distribution of the mRNA—Tissue distribution of the mRNA was assessed by Northern blot analysis of several mouse tissues. A transcript of ~4.5 kb was found mainly in the heart, liver, kidney, and testis, and to a lesser extent in the lung and skeletal muscle (Fig. 2B). The transcript was not detected in brain or spleen. Interestingly, the level of gene expression appeared to increase progressively during the later stages of mouse development (Fig. 2, B and C).

Subcellular Localization—In order to determine the intracellular localization of the novel protein, we produced a rabbit polyclonal antibody (Ab) against the middle portion of the polypeptide (mNucl.mid) (Fig. 3B), and

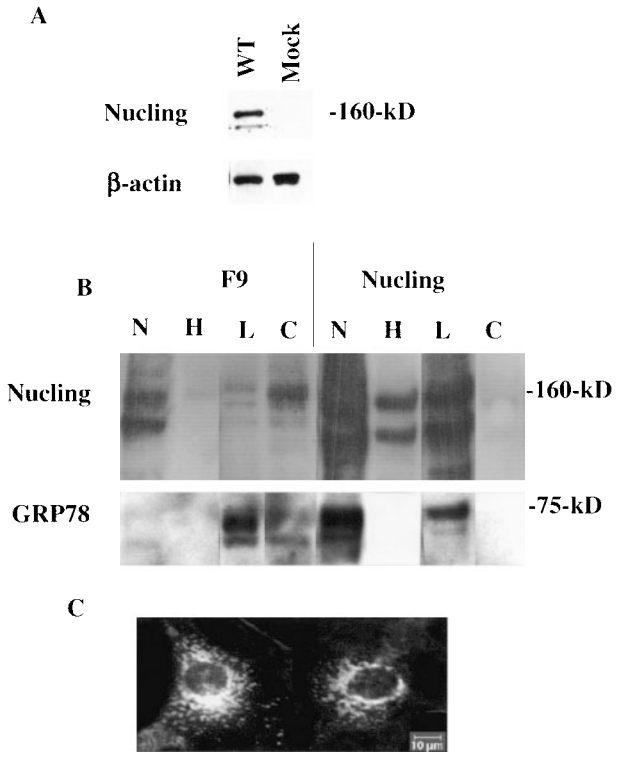

Fig. 4. **The subcellular localization of Nucling.** A: COS-7 cells were transfected with pFLAG-*Nucling* for 24 h (WT). Vector DNA was transfected into COS-7 cells by the same transfection procedure for 24 h as the negative control (Mock). Western blotting was performed to check the immunospecificity of the Nucl.mid Ab (upper panel). Whole cell lysates from transfected cells were used. Anti–β-action Ab was used as an internal control (lower panel). B: F9 cells and transfected COS-7 cells were separated into four fractions (fr): crude nuclear fr (N), heavy membrane fr (H), light membrane fr (L), and cytoplasmic fr (C). The Nucl.mid Ab or anti-GRP78 Ab was used as the first antibody for Western blot analyses. The anti-GRP78 Ab was used as an internal marker for the fraction containing endoplasmic reticulum (ER). Major components in the H fraction are mitochondria and lysosomes, and those in the L are microsomes, including ER and Golgi apparatus. C: Nucling is a nuclear attachment protein. COS-7 cells transfected with pFLAG-*Nucling* were analyzed by indirect immunofluorescence using a Nucl.mid polyclonal antibody. A representative image is shown and, in most cases, aggregations of Nucling were attached to nuclear membranes or deposited around the nucleus in the cytoplasm. A confocal laser microscope (LM) was used for observation.

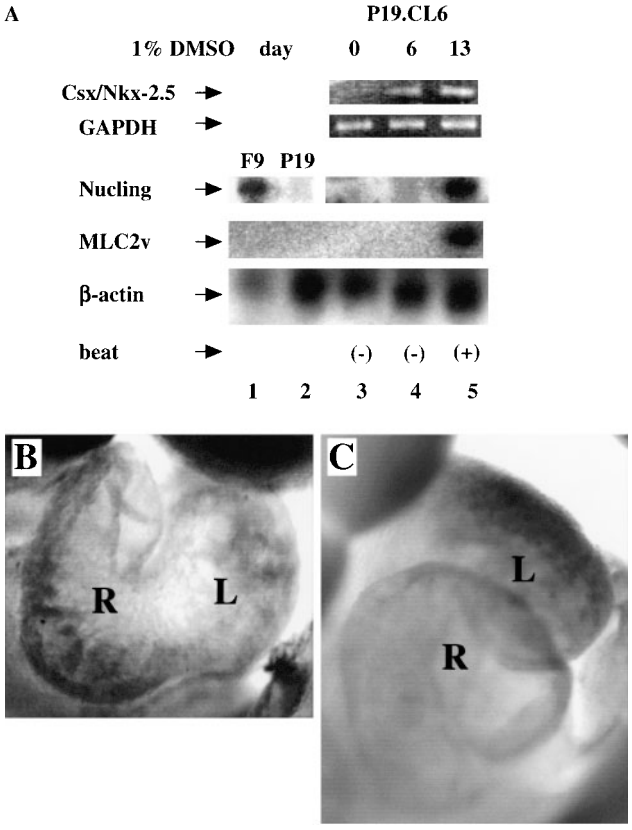

Fig. 5. **Expression of the *Nucling* gene is up-regulated during cardiomyogenic differentiation in P19.CL6 cells and heart development *in vivo*.** A: RNA was prepared from F9 cells (lane 1), P19 cells (lane 2), or P19.CL6 cells on day 0 (before treatment with DMSO) (lane 3), day 6 (lane 4), and day 13 (lane 5). RT-PCR was performed to analyze Csx/Nkx-2.5 and GAPDH mRNA. Three micrograms of poly A^+ RNA from each sample was subjected to Northern blot analysis for other genes. Northern blot data using a β-actin cDNA probe are presented at the bottom to show that the same amount of intact RNA was loaded into each lane. B, C: Expression of Nucling in the developing heart as revealed by whole-mount RNA *in situ* hybridization. The heart region of the embryo is shown in (B) E9.5 at early organogenesis stage and (C) E10.5. Abbreviations: R, right ventricular chamber of the heart; L, left ventricular chamber of the heart.

checked the immunospecificity of the Ab by western blotting using whole cell lysates from COS-7 cells overexpressing Nucling (WT) or mock (Fig. 4A). The results showed that the Ab specifically recognized an ectopic Nucling protein band of 160-kDa, which corresponds to the predicted size for the amino acid sequence of Nucling, while the signal from the band reacting with endogenous Nucling was almost undetectable in the mock. Western blot analyses also revealed that the antibody could detect the endogenous protein in F9 cells (Fig. 4B) but not in P19 cells (data not shown). As shown in Fig. 4B, we detected a 160 kDa band in F9 cell extracts and in a Nucling overexpressing cell extract mainly in the nuclear fraction. However, we also observed the band in several other fractions. Interestingly, there were some discrepancies between the F9 cell fractions and the Nucling transfectant fractions. In F9 cells, large amounts of endogenous Nucling could be detected in the cytosol fraction as well as the nuclear fraction, while quite strong bands were observed in organellar membrane fractions (L and H fr.) of the Nucling transfectant. Based on these observations, we assume that this novel protein may be localized as both nuclear and organellar membrane proteins in the cell. In order to confirm this, we performed immunofluorescent staining assay using mNucl.mid Ab. Although we were unable to stain F9 cells, when the expression plasmid carrying Nucling cDNA into COS-7 cells was transfected, the overexpressed protein was clearly visualized by immunofluorescence assays. As shown in Fig. 4C, the protein was found exclusively localized around the nuclear membrane in clusters, and was also expressed diffusely in cytoplasm. We, therefore, des-

ignate this gene Nucling, a molecule clinging to nuclei, based on its unique subcellular expression profile.

Up-Regulation of Nucling Expression during Cardiomyogenic Differentiation—In order to examine a possible role of Nucling during embryonal development, we evaluated the expression level of Nucling in P19.CL6 cells during differentiation (Fig. 5A). P19.CL6 is a clonal derivative of P19 and can differentiate efficiently into beating cardiac muscle in adherent culture in the presence of 1% DMSO (*6*). Therefore, this cell line is regarded as the most useful cell line for studying myocardial differentiation, and has been reported to express cardiomyocyte markers such as α- and β-cardiac myosin heavy chain (MHC) transcripts and a sarcomeric MHC protein on day 10 after treatment with DMSO, simultaneously with the initiation of contraction (*6*). When cultured in growth medium, P19.CL6 cells grew well and did not differentiate into cardiomyocytes. When 1% DMSO was added to the medium, P19.CL6 cells differentiated into mononucleated, spontaneously contracting cardiomyocytes. As previously reported (*6*), spontaneous beating was first observed in a limited area on day 10–13 (10–13 d after the initiation of DMSO treatment), and subsequently the majority of cells beat synchronously until around day 16. The expression of the Nucling gene and several cardiac-specific markers was examined in P19.CL6 cells during differentiation. As shown in Fig. 5A, cardiac transcription factor Csx/Nkx-2.5 (*11*, *12*) started to be expressed on day 6 in P19.CL6 cells. The expression of the *Nucling* gene was detected in P19.CL6 cells on day 13, the same as the MLC2v gene (*13*). In addition, the expression of the MLC2v gene was not detected in F9 cells while that of the *Nucling* gene was clearly detected (Fig. 5A).

Whole-mount RNA in situ hybridization assay revealed that Nucling expression is highly dynamic during heart development. Expression in heart is observed weakly at the edge of both sides of the common ventricular chamber of the heart at the 9.5 stage (Fig. 5B), and is then progressively up-regulated and restricted to the myocardial wall of left common ventricular chamber of heart (Fig. 5C).

DISCUSSION

We are interested in understanding at the molecular level the unique regulatory mechanisms of cell differentiation that have been observed in pluripotent embryonic stem cells. As a means of isolating genes regulating the direction of cell differentiation, we employed a subtractive cDNA cloning strategy using two EC cell lines that have different profiles of cell differentiation. We identified 12 different genes (including 6 novel genes) that were expressed selectively by either of the EC cells, documenting the efficacy of our approach.

Clone S1/2-C3 encoded a novel type of polypeptide containing an ankyrin repeat, aspartyl protease motif, a leucine zipper motif, and two t-SNARE coiled-coil domains. This polypeptide, termed Nucling, was uniquely expressed in cytoplasm, especially around nuclear membranes in mammalian cells. The ankyrin repeat, leucine zipper motif or t-SNARE coiled-coil domains may play some roles in complex formation.

Another unique feature is the developmental and tissue specific regulation of gene expression of *Nucling*. We investigated its expression in F9 cells, at different embryonic stages in mice, and in several tissues of adult mice by Northern blot analyses (Fig. 2). The expression level of *Nucling* in tissues was very low compared with that in F9 cells. Its endogenous expression level in tissues may be strictly regulated.

There are some discrepancies concerning the distribution pattern in western blot between Nucling transfectants and F9 cells (Fig. 4B). Strong bands were observed in both the nuclear and cytosol fractions of F9 cells. In contrast, a very strong band was observed in organellar membrane fractions (H and L) of Nucling transfectants as well as nuclear fractions. We speculate that this might arise due to the different characteristics of the cell types, especially in the case of transfection with the heavy load of overexpression.

The findings that Nucling is expressed specifically in cardiomuscular tissues (Fig. 2B), that its expression is up-regulated during cardiac differentiation (Fig. 5A), and that the expression level of the transcript increased during the development of the embryo (Fig. 2, B and C, Fig. 5, B and C) suggest the possibility that Nucling may cooperate with gene regulation in embryonal development, especially cardiac muscle development. In addition, the *Nucling* transcript was detected at quite high levels in the heart, but not in the brain. This observation seems to be in good accord with the fact that the gene is expressed in F9 but not in P19 cells. F9 cells are reported to be unable to differentiate into ectodermal cells, but P19 cells, in which *Nucling* is not expressed, can be induced into ectodermal cells, such as neurons and glia. It may be tempting to suggest that Nucling is a negative regulator of ectodermal differentiation from the pluripotent cells such as embryonic stem cells and a positive regulator of cardiac muscle differentiation. Further detailed analyses based on studies on *in vitro* gain- or loss-of-function are necessary to clarify the function of this gene during ectodermal and cardiac differentiation.

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