A Novel Myocyte-specific Gene Midori Promotes the Differentiation of P19CL6 Cells into Cardiomyocytes*

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Although several cardiac-specific transcription factors have been shown to play vital roles in various steps during the heart formation, the precise mechanism of the early stage of cardiogenesis has yet to be elucidated. By differential display technique, we tried to identify molecules that are expressed earlier than cardiac transcription factors such as CSX/NKX2-5 and GATA-4 and are involved in cardiomyocyte differentiation using the P19CL6 cell line, which efficiently differentiates into cardiomyocytes when treated with dimethyl sulfoxide. We isolated a novel gene designated Midori. Its deduced amino acid sequence contained an ATP/GTP-binding site, Ig-like domain, and Kringle-like domain. Northern blot analysis revealed that expression of Midori was restricted to the fetal and adult heart and adult skeletal muscle in mice. In whole mount in situ hybridization, Midori was expressed in cardiac crescent and developing heart but not in somites. The MIDORI protein was localized in the nucleus and overexpression of Midori induced expression of endogenous Midori itself, suggesting that MIDORI may act as a transcriptional regulator. Permanent P19CL6 cell lines overexpressing Midori more efficiently differentiated into cardiomyocytes than did parental cells, whereas those overexpressing the antisense Midori less efficiently differentiated. These results suggest that Midori may promote the differentiation of P18CL6 into cardiomyocytes.

Recent genetic studies have demonstrated that several heart-enriched transcription factors play vital roles in various developmental steps during the formation of the heart. Among them, CSX/NKX2-5, GATA-4, and MEF2C have been well characterized and used as early lineage-specific molecular markers of cardiac development. Csx/Nkx2-5 is a murine cardiac homeobox gene that was originally identified as a potential vertebrate homolog of Drosophila tinman (1, 2). Csx/Nkx2-5 is predominantly expressed in the heart and in cardiac progenitor cells from the early developmental stage. The heart does not form at all in the tinman mutant of Drosophila (3), and the development of the heart stops at the looping stage in Csx/Nkx2-5 knockout mice (4). In humans, it has been reported that patients with familial atrial septal defect or other congenital heart diseases have point mutations in the human CSX/NKX2-5 gene in one allele (5–7). Gata-4 and Mef2c are also thought to be involved in the early stage of cardiogenesis (8–10). Both of them started to be expressed in the precardiac mesoderm almost simultaneously with Csx/Nkx2-5. A zinc-finger transcription factor, GATA-4 has been reported to be necessary for differentiation of the pluripotent P19 embryonal carcinoma cells into beating cardiomyocytes. In this system, the inhibition of Gata-4 expression by antisense transcripts interferes with the differentiation of the P19 cells at the precardiac (cardioblast) stage (11). In contrast, the ectopic expression of Gata-4 in P19 cells accelerates cardiogenesis (12). In vivo bilateral cardiac primordia fail to fuse at the ventral midline in Gata-4−/− mice (8, 9). MEF2C is a member of MADS-box transcription factors, and targeted disruption of Mef2c results in right ventricular dysplasia (10). Thus, Drosophila TINMAN, CSX/NKX2-5, GATA-4, and MEF2C are critical regulators of cardiac development and are useful molecular markers for examining effects of inductive signals from other tissues or germ layers.

Although characteristics and functions of these cardiac transcription factors have been well studied, the precise molecular mechanism of the earlier stage of cardiac development, i.e. the mechanism by which these transcription factors themselves are regulated, remains largely unknown at present. It has recently been reported that some growth factors play an important role in initial induction of cardiac differentiation. For example, decapentaplegic (dpp) and bone morphogenetic proteins, which are the members of the transforming growth factor-β superfamily, have been shown to be important candidates that regulate expression of some cardiac-enriched transcription factors such as TINMAN, CSX/NKX2-5, and GATA-4 and induce cardiomyocyte differentiation. We previously demonstrated that bone morphogenetic proteins are indispensable for cardiomyocyte differentiation and that bone morphogenetic proteins induce cardiomyocyte differentiation through cardiac transcription factors CSX/NKX2-5 and GATA-4 (13). As well as bone morphogenetic proteins (dpp), other growth factors such as Wnt/β-catenin (14) and fibroblast growth factors (15–17) have been reported to be essential for normal cardiac development. However, the precise molecular cascades from these growth factors to cardiac-specific genes needed for cardiac specification have yet to be elucidated. From this respect, we tried to identify molecules that are expressed in precardiac cells earlier than cardiac-specific transcription factors and to clarify the molecular mechanism by which induction of cardiac differentiation is controlled.

In the investigation of the molecular mechanisms of cardio-
myocyte differentiation, in vitro culture systems present a great advantage in contrast to the complexity in analyzing them in the in vivo situation. In the present study, we used the P19CL6 in vitro cardiomyocyte differentiation system. P19CL6 is a clonal derivative isolated from murine P19 embryonal carcinoma cells by the limiting dilution method (18). Unlike P19 cells (19, 20), whose use is limited because of their quite low efficiency of differentiation into cardiomyocytes, this CL6 subline efficiently (more than 80%) differentiates into beating low efficiency of differentiation into cardiomyocytes, this CL6 carcinoma cells by the limiting dilution method (18). Unlike is a clonal derivative isolated from murine P19 embryonal P19CL6 in vitro are shown at the top of the lane. 0, P19CL6 day 0; 6, P19CL6 day 6.

FIG. 1. Differential mRNA display of undifferentiated and differentiated P19CL6 cells. RNA was extracted from undifferentiated P19CL6 cells (designated day 0) and cells harvested 6 days after the Me2SO treatment (designated day 6), respectively. Reverse transcriptase-polymerase chain reaction with specific primers was carried out, and both samples were electrophoresed. The band present in day 6 and absent in day 0 (arrow, 5A1) was cut out, eluted, and reamplified. The primers used for reverse transcriptase-polymerase chain reaction are shown at the top of the lane. 0, P19CL6 day 0; 6, P19CL6 day 6.

The primers used for reverse transcriptase-polymerase chain reaction and absent in day 0 (MLC2v) are detected from day 6 and from day 10, respectively.

Myocytic induction/differentiation originator, in the present study, we isolated a novel gene named Midori, representing Myocytic induction/differentiation originator, in the present study. Expression of Midori was restricted to the developing heart from the very early stage of cardiogenesis. The MIDORI protein was localized in the nucleus, and overexpression of Midori induced expression of endogenous Midori itself, suggesting that MIDORI may act as a transcriptional regulator. Overexpression of Midori enhanced the differentiation ability of P19CL6 cells into cardiomyocytes, whereas inhibition of Midori suppressed it. These results suggest that Midori may promote the differentiation of P19CL6 into cardiomyocytes.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—P19CL6 cells were cultured essentially as described previously (18). Briefly, the cells were grown in a 100-mm tissue culture dish under adherent conditions with a minimal essential medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (JRH Bioscience), penicillin (100 units/ml), and streptomycin (100 μg/ml) (growth medium) and were maintained in a 5% CO2 atmosphere at 37 °C. To induce differentiation under adherent conditions, P19CL6 cells were plated at a density of 3.7 × 10^5 cells in a 60-mm tissue culture dish with the growth medium containing 1% Me2SO (differentiation medium). The medium was changed every 2 days. Days of differentiation were numbered consecutively with the 1st day of the Me2SO treatment designated as day 0.

RNA Extraction and Analysis—Total RNA was extracted by the acid guanidine method (RNA Zol B, TEL-TEST, Inc.), and Northern blot analysis was performed with 20 μg of total RNA as follows. Total RNA was subjected to 1% agarose/formaldehyde gel electrophoresis and subsequently transferred onto a Hybond-N membrane filter (Amersham Pharmacia Biotech). Hybridization was carried out in 50% formamide, 5× saline/sodium phosphate/EDTA, 5° Denhardt’s solution, 5% dextran sulfate, and 0.5% SDS at 42 °C overnight. The probe was labeled with [32P]dCTP by random priming (Takara).

Differential mRNA Display and Subcloning of Reamplified cDNA Fragments—Differential display was performed with an RNAmap kit (Genhunter Co.) as described previously (21–25) for 0.8 μg each of total RNA from day 0 and day 6 of P19CL6 cells. cDNA bands present in the day 6 lane and absent in the day 0 lane were extracted, reamplified, and subcloned into the TA cloning vector pCR II (Invitrogen). The plasmids

FIG. 2. Midori expression in P19CL6 and in mice. A, Northern blot analysis using the 5A1 fragment as a probe revealed that the single ~6.4-kilobase pair band was not detected in undifferentiated P19CL6 but was present abundantly on day 4 and day 6 of differentiation and was weakly detected thereafter. We designated the gene represented by 5A1 as Midori (arrow). B and C, the expression of Midori in vivo was restricted to the heart of fetal mice (B) and to the heart and skeletal muscle of adult mice (C) among the many organs examined. B, brain; H, heart; K, kidney; L, liver; Lu, lung; C, P19CL6 day 6; I, intestine; Sk, skeletal muscle; Sp, spleen; St, stomach; T, testis.
FIG. 3. cDNA and deduced amino acid sequence of Midori. A conserved Kozak translation initiation sequence, a polyadenylation signal, and an ATP/GTP-binding site motif are shown in bold. Two Ig-like C2-type domains are boxed. Nuclear localization signals are underlined. A proline/alanine-rich region is indicated by a dotted underline. A Kringle-like domain is indicated by a broken underline.
FIG. 3—continued

Midori Promotes Cardiomyocyte Differentiation of P19CL6

381 G I E S T R K T A S V L G I Q D K V Q D
GTCGGCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCTCCCA
1260
391 V P A P A P A P A P A P A P A P V P
GTCGGCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCCCCGCTCCCA
1320
401 V P A P T P V P V S R S S E Q V Y F S L K
GACAGTTTCATGGAGGACCCACCGGAGGAGGGGATCCGGAGAGGAAGAAGAAAACCTCACC
1380
411 D M F M E T R A G R S Q E E E K P P
CAGTACGGGTAGCTGGAGAAAGGTCCCAAGGAAGACACAGATCAAGTCTAGACTG
1440
421 P S T R V A G E S P P G K T P V K S R L
GAGAAGGTACCGATGGTCTCCAGGACCCGCCCCATCTCTGTCCGTTCGCCGCCCATTAAG
1500
431 E K V P M V S Q P T S S M V P P I K
CCTTGAAGAAGGAGAGATTTGGCCTCTCCAAATCTCCAAAGGTGAATCAACTACACCCCT
1560
441 P L N R K F A P P K S K V E S T T S
CTCTCAAGTCGACTTCAGATCTATGCCCCAGAGCTAGGGAAGGCTCTACCTTCAGCC
1620
451 L S S Q T S E M A Q S L G K A L P S A
TCTACCCAGGTCCCCACCACCCCTGCTCGAGGGAGACAGCGACCGCCCCGAGATGCCCCCTTG
1680
461 S T Q V P T P P A R R H G T R D S P L
CAAGAGCAAAACAGGACACAGACTCCGGAGAGGCTCTGGAGTCCCCAGCAAGCGTGGCT
1740
471 Q G Q T S H K T P G E A L E S P A T V A
CCCCAACAGTCTGGCCAACAGCAGTCCGAGATCCGTCCTGTTGATCAGCAAGCTCTGAG
1800
481 P T K S A N S S S D T V S V D H D S S G
AATCAAAGGGCCAGCCAGGCCCAGGATTACAAAGAAGTCGAAGGAGAGACACTTAGT
1860
491 N Q G A T E P M D T E T Q E D G R T L V
GATGGGGAAGCTGGAAGCGGAAAGAAAACACACAGAGATGGGAAAGGACACCTGAG
1920
501 D G R T G S R K K T H T D G K L Q V D G
AGGAGTCGAGGAGGGACAGAGCACAGACAGACAGAGCAGGCTCTTCGACAGCAAGCACGGCAG
1980
511 R T Q E T E H K T E H T L S P R T Q A G
GAGAAGGAGCTTTGATCACAAGGAAAGTGGAGAGGCACAGTCACAGAGAGTTTACATGGAAG
2040
521 E K D V V T Q G S R P O Q D S R S W K
AATTTAGTCACAGAAGAGATGGAATGCGATGGAGAGTGAGCAGGAGGAGGAGGAG
2100
531 N L V T Q R V R D M Q V G Q M Q A G E R
TGGCAGCAAGACCCCTGGAGACGGAAGTACAGGGAAGAAGAAAAGACACAGTCAGCA
2160
541 W Q Q D P G D A R I Q E E E K E T Q S A
GCAGGGCAGCATTTCTGTAGCTTTCCAGAAAACCAATCAGAGCAGCTCTAGTCGTACGGCCAGCTC
2220
551 A G S I P V A F E T Q S E Q L S M A S L
AGCTCATTTCCTGGAGCTCTCAAAGGGTCACCATCGAGATGCCCTAGAGATGCACCGCTCAGG
2280
561 S S L P G A L K G S P S G C P R E S Q A
ATAGAATTTTTTGGAGAAGGACAGACAGAGCACCCTTGTCCTCAAGAAAGATCTGACCTGATG
2340
571 I E C F E K S T E A P C V Q E R S D L M
CTGCGTCTGGAGGAGGACGCTTCCAGAAGCCATGAGAAGGCTGGTCTAGGCTGGCCCACCTCA
2400
581 L R S E E A A F R S H E D G L L G P P S
GGGAAACCTGACCTAACCAACACATGGGCTCCCGAGGGGACACTGACATTGGGAGGA
2460

Fig. 3—continued
FIG. 3—continued

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were digested with EcoRI and electrophoresed on a 2.0% agarose gel. The inserts were extracted from the gel (QIAEX II Gel Extraction Kit, Qiagen) and labeled as described above for Northern blot analysis. If the same expression pattern was obtained in the Northern blot on P19CL6, Northern blot analysis on fetal (17.5 days postcoitus (dpc)) and adult (8 weeks old) mice was performed.

**P19CL6 Day 6 cDNA Library Synthesis**—About 20 μg H9262 RNA of P19CL6 day 6 was separated from 1 mg of total RNA after selecting twice through oligo(dT)-cellulose type 7 (Amersham Pharmacia Biotech), and 5 μg H11001 RNA was used to synthesize a cDNA library using a ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). This manipulation yielded a library consisting of 2.88 μH11003 106 independent clones.

**cDNA Library Screening and DNA Sequencing**—Using the cDNA fragment obtained from the differential screening, Midori was isolated from the P19CL6 day 6 cDNA library prepared as described above. Plasmids were excised from phage vectors in vivo by ExAssist helper phage and were subjected to sequencing with Big Dye Terminator and ABI PRISM 310 (PerkinElmer Life Sciences) using ordinary sequencing primers and four custom-synthesized primers as follows: P1, 5'-TACTCCATGCCAGCTTCGTCTACGATGAGCTGACATGCGAGCTGAG-3'; P2, 5'-GATGCAGGACGCAAAGGATAATGACCAAAGGACGCGA-3'; P3, 5'-GATGCAGGACGCAAAGGATAATGACCAAAGGACGCGA-3'; P5, 5'-GATGCAGGACGCAAAGGATAATGACCAAAGGACGCGA-3'. We also used a mouse skeletal muscle 5'-STRETCH PLUS cDNA library (CLONTECH) to get the full length of Midori. Phage DNA was purified from isolated clones (Lambda DNA miniprep, Qiagen), digested with EcoRI, and subcloned into pBlueScript II SK+(Stratagene). The clones were sequenced and analyzed using the BLAST sequence analysis program.

**Whole Mount in Situ Hybridization Analysis**—Pregnant females of mouse strain ICR were killed by cervical dislocation on 7.5–10.5 dpc. Harvested mouse embryos were fixed in 4% paraformaldehyde, phosphate-buffered saline (pH 7.4) and treated with 10 μg/ml proteinase K at 37°C for 3 min. Then samples were incubated with 2 mg/ml glycine, with 0.2% glutaraldehyde, 4% paraformaldehyde, and with 0.1% sodium borohydride subsequently. Digoxigenin (DIG)-labeled cRNA probes were synthesized with in vitro transcription using a DIG RNA labeling kit (Roche Molecular Biochemicals). A part of the cDNA of Midori (not shown) was used for the template of the antisense ribonucleotide probes, and that of MLC2v (not shown) was used for the template of the antisense probe as a positive control for hybridization. Hybridization was done with 2 μg/ml DIG-labeled cRNA probes in 50% formamide, 750 mM sodium chloride, 1 mM EDTA, 100 mM PIPES, 1% SDS, 100 μg/ml yeast tRNA, 0.05% heparin, and 0.1% bovine serum albumin at 63°C overnight. After washing with a low concentration of salt, the RNase reaction was carried out with 100 μg/ml RNase A and 100 units/ml RNase T1 in 0.5 M sodium acetate, 10 mM PIPES, and 0.05% Tween 20. Samples were washed and incubated with a 2000× dilution of anti-DIG alkaline phosphatase conjugate (Roche Molecular Biochemicals) at 4°C overnight. Then mRNA-cRNA hybrids were detected by staining with BM Purple alkaline phosphatase substrate (Boehringer Mannheim) at room temperature for 1 h for the MLC2v probe and for 2–3 h for the others.

**Transfection of Tagged cDNA and Immunochromal Staining**—Two kinds of epitope-tagged cDNAs of Midori in the expression vector were constructed. The HA-pcDNA3 vector was digested and ligated with the open reading frame of Midori to make a HA-tagged MIDORI protein with a 6-amino acid deletion of MIDORI at the amino terminus. Similarly, pcDNA3.1(-)Myc-His B vector (Invitrogen) was used to make Myc-tagged MIDORI protein with a 1-amino acid deletion of MIDORI at...
the carboxyl terminus. COS cells were plated at a density of $4 \times 10^4$ cells in a 35-mm tissue culture dish on the day before transfection. The constructed plasmids were transiently transfected into COS cells by the calcium phosphate method and stained immunochemically on the next day. The cells were fixed in 3% paraformaldehyde, phosphate-buffered saline and subsequently incubated with 50 mM ammonium chloride, 0.2% Triton X-100, and 10% fetal bovine serum. Corresponding to the transfected plasmids, either 500 diluted rabbit anti-HA polyclonal antibody or 20 diluted mouse anti-Myc antibody was mounted as the first antibody. After washing, either 200 diluted fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody or 50 diluted rhodamine-conjugated goat anti-mouse IgG antibody was mounted as the second antibody.

Stable Transformants—Midori cDNA of either sense or antisense orientation was subcloned into pEFSAneo, which harbors the human elongation factor 1-α promoter and a neomycin resistance gene, and the resultant genes were transfected into P19CL6 cells by the calcium phosphate method, respectively. Stable transfectants were selected with 600 μg of neomycin (G418)/ml.

Immunofluorescence—Immunostaining with MF20, a monoclonal antibody against a sarcomeric MHC, was performed on day 8 of differentiation by using anti-mouse immunoglobulin G conjugated with tetramethyl rhodamine isothiocyanate as the secondary antibody. We chose day 8 because thereafter most cells differentiate into cardiomyocytes and are MF20-positive in both P19CL6 and the Midori-overexpressing cell lines, and therefore it might be hard to show the difference among these cell lines.

**Protein Extraction and Western Blot Analysis**—Total protein of the cell lines on day 14.5 of differentiation was extracted using cell lysis buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM sodium chloride, 0.5 mM EGTA, 10 mM sodium pyrophosphate, 1 mM orthovanadate, 10 mM sodium fluoride, and 10 mM okadaic acid. The equal volume of cell lysate was subjected to 5% polyacrylamide gel electrophoresis and subsequently transferred onto a Hybond-ECL membrane filter (Amersham Pharmacia Biotech). MF20 and anti-mouse immunoglobulin G conjugated with horseradish peroxidase were incubated as the first and the second antibody, respectively, in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20. After washing in this buffer, the filter was soaked in ECL Western blotting detection reagents (Amersham Pharmacia Biotech) for 45 s, and subsequently the autoradiogram was developed after a 30-s exposure.

**RESULTS**

**Cloning of the Muscle-specific Midori cDNA**—To isolate novel molecules expressed in the very early stage of cardiac development, we performed the differential display method using the P19CL6 cell line as an *in vitro* model for cardiomyocyte differentiation. Because expression of cardiac transcription...
tion factors such as CSX/NKX2-5, GATA-4, and MEF2C are detected on day 6 (6 days after the initiation of Me₂SO treatment) in P19CL6 cells (13), we compared mRNA extracted from the differentiating cells of day 6 and from the undifferentiated cells of day 0 (i.e. before the Me₂SO treatment). We obtained several differentially expressed cDNA fragments that were detected on day 6 but not on day 0. These cDNA fragments were therefore thought to represent parts of genes whose expression was induced in the early stage of differentiation. Among them, a fragment primarily amplified using the prepared primers in the RNAmap kit, AP5 and T12MA, was denoted as 5A1 (Fig. 1). Northern blot analysis using the 5A1 fragment as a cDNA probe revealed that the transcripts represented by 5A1 were detected as a single 6.4-kilobase pair band abundantly on days 4 and 6 and faintly thereafter but not at all on day 0 (Fig. 2A), indicating that the mRNA expression was induced by the treatment with Me₂SO and preceded expression of cardiac transcription factors such as CSX/NKX2-5, GATA-4, and MEF2C. Such temporal expression patterns imply the possibility that this molecule represented by the 5A1 fragment might play an important role in cardiomyocyte differentiation of P19CL6 at the relatively early developmental stage. We next examined tissue distribution of this mRNA using 20 μg of total RNAs from various tissues of fetal and adult mice. Northern blot analysis revealed that the expression was restricted to the heart of fetal mice and to the heart and skeletal muscle of adult mice among the various organs examined, suggesting that expression of this gene was myocyte-specific (Fig. 2, B and C). We screened the originally prepared cDNA library of P19CL6 day 6 and a random and oligo(dT)-primed cDNA library of murine skeletal muscle using the subcloned band as a probe and obtained a corresponding cDNA ~6.4 kilobases in length. It contained the Kozak consensus sequence (26) in the 5'-region and 5-kilobase pair open reading frame. A polyadenylation signal was situated at 32 base pairs upstream of the poly(A) tract. Two Ig-like C2-type domains, several nuclear localization signals, a proline/alanine-rich region, an ATP/GTP-binding site, and a Kringle-like domain were present in the deduced amino acid sequence (Fig. 3). Considering these results and its possible functions as described below, we designated this novel gene Midori representing Myocytic induction/differentiation originator.

**Expression of Midori in Murine Embryos**—To examine the precise spatial and temporal expression pattern of Midori in mouse embryogenesis, whole mount in situ hybridization was performed. Murine embryos at 7.5, 8.5, 9.5, and 10.5 dpc were prepared and hybridized with Midori sense and antisense and MLC2v antisense ribonucleotide probes. The probes were synthesized using DIG-labeled UTP. The Midori expression was first detected in the cardiac crescent at 7.5 dpc when the developing heart became visible and continued through 10.5 dpc. The expression of Midori was localized within the heart at least until 10.5 dpc as well as that of MLC2v used as a positive control (Fig. 4). Although Northern blot analysis revealed that Midori was abundantly expressed in the skeletal muscle of adult mice (Fig. 2C), the expression was not detected in somites where differentiation into skeletal muscle took place. These results suggest that Midori is expressed specifically in the heart.
Midori Promotes Cardiomyocyte Differentiation of P19CL6

Overexpression of Midori Up-regulated Expression of Endogenous Midori and Enhanced the Ability of P19CL6 to Differentiate into Cardiomyocytes—We next performed gain-of-function experiments by overexpressing Midori in P19CL6 cells. Five independent neomycin-resistant clones that stably overexpressed Midori were isolated, and two of them (designated as S1 and S2, Fig. 6) were differentiated and analyzed. Midori-overexpressing cells did not show any morphological difference with wild-type P19CL6 cells when cultured without Me₂SO (data not shown). However, unlike in wild-type P19CL6 cells, mRNA levels of endogenous Midori (Fig. 6, arrow) were more abundant than those of exogenous Midori (Fig. 6, large arrowhead) in the undifferentiated cells, suggesting that expression of Midori was induced by itself. When treated with Me₂SO, Midori-overexpressing P19CL6 cells differentiated into cardiomyocytes more efficiently than did wild-type P19CL6. Highly aggregated noncardiomyocytes seen in wild-type or in the antisense cell lines were only weakly detected in the Midori-overexpressing cell lines (Fig. 7A). Immunocytochemical staining of MHC on day 8 of differentiation (Fig. 7B, stained in red) and Western blot analysis of MHC on day 14.5 (Fig. 7C, arrow) revealed that the Midori-overexpressing cells more efficiently differentiated into cardiomyocytes than did parental P19CL6 cells, suggesting the potential role of Midori in the induction of cardiomyocyte differentiation.

**DISCUSSION**

Cardiac-specific transcription factors such as CSX/NKX2-5, GATA-4, and MEF2C have recently been clarified to play important roles in the formation of the heart. These cardiac transcription factors are critical regulators of normal cardiac development and are useful molecular markers to examine effects of inductive signals from other tissues or germ layers. However, little is known about the genetic cascades that induce expression of these transcription factors themselves in the earlier stage of cardiac development. Therefore, we tried to identify transcriptional regulators that exist upstream of these cardiac transcription factors and control their expression using the P19CL6 in vitro culture system and obtained several results as follows. (i) We isolated a novel myocyte-specific gene Midori that was expressed earlier than cardiac transcription factors such as CSX/NKX2-5, GATA-4, and MEF2C in P19CL6 cells. (ii) Expression of Midori was localized in the primitive heart in early embryogenesis and was detected in cardiac and skeletal muscle in the adult mice. (iii) The MIDORI protein was localized in the nucleus and induced by Midori itself. (iv) Overexpression of Midori enhanced the differentiation of P19CL6 into cardiomyocytes, whereas the blockade of Midori expression inhibited it.

The cDNA sequence of Midori revealed that MIDORI contained two Ig-like C2-type domains (Fig. 3, boxed), several nuclear localization signals (Fig. 3, underlined), a proline/alanine-rich region (Fig. 3, dotted-underlined), an ATP/GTP-binding site motif A (Fig. 3, bold), and a Kringle-like domain (Fig. 3, broken-underlined). Transfection experiments using the epitope-tagged cDNA of Midori revealed that the MIDORI protein was localized in the nucleus and that exogenous Midori induced expression of endogenous Midori itself in undifferentiated P19CL6 cells, suggesting its possible role as a transcriptional regulator. Several transcription factor domains contain proline-rich regions, which are known to act as transcriptional repression sites (27, 28). Vascular endothelial growth factor receptor (29–32) and platelet-derived growth factor receptor (33, 34) have regions homologous with the Ig-like domain of MIDORI. These domains consist of a heparin- or agonist-binding site and are thought to be essential for receptor dimerization. Hepatocyte growth factor contains domains similar to the

**FIG. 6. Isolation of P19CL6-derived cells overexpressing the sense or antisense Midori transcripts.** Three independent P19CL6-derived cell lines overexpressing antisense Midori and five lines overexpressing Midori were isolated, and two lines of each group are shown. Endogenous Midori (arrow) was expressed more abundantly than exogenous Midori (two large arrowheads) in both of the sense Midori-overexpressing cell lines (S1 and S2) without Me₂SO treatment, whereas endogenous Midori was not detected in any antisense cell lines (A1 and A2).

from the very early stage of cardiac development also in vivo.

**Nuclear Localization of the MIDORI Protein in Transfected COS Cells**—The existence of a nuclear localization signal in the amino acid sequence of MIDORI protein (Fig. 3) suggested its localization in the nucleus. To examine the intracellular localization of MIDORI, HA- and Myc-tagged MIDORI were separately transfected into COS cells. Immunocytochemical staining with both anti-HA and anti-Myc antibodies revealed that the MIDORI protein was expressed only in the nucleus (Fig. 5).

**Blockade of Midori Expression Inhibited the Differentiation of P19CL6 into Cardiomyocytes**—To elucidate the role of Midori in cardiac differentiation, we isolated the stable P19CL6 cells that overexpressed the antisense Midori and examined the differentiation ability of the cells. Three independent neomycin-resistant clones containing the Midori antisense sequence were isolated, and two of them (designated as A1 and A2, Fig. 6) were differentiated and analyzed. Expression of the Midori antisense sequence had no effect on the morphology and the proliferative ability of the undifferentiated cells (data not shown). When treated with Me₂SO, however, the cell line A1, which expressed antisense transcripts more abundantly than A2 (Fig. 6, large arrowhead), differentiated much less efficiently than A2 and parental P19CL6 cells. In P19CL6 and antisense cell lines, some parts of the cells formed aggregations (Fig. 7, A and B, arrowheads) that were not immunostained by MP20 and did not beat at all. More aggregations were observed in A1 than in A2 (Fig. 7A). More MP20-positive beating foci were seen at day 8 in A2 than in A1 (Fig. 7B, stained in red). In Western blot analysis on day 14.5 of differentiation, a lesser amount of MHC protein was detected in the line A1 than in A2 and P19CL6 cells, suggesting that inhibition of Midori partially blocked cardiomyocyte differentiation of P19CL6 in a dose-dependent manner.
Kringle-like domain of MIDORI. This domain is necessary for receptor binding (35–38). No other nucleus-localized genes with these motifs have been reported to date, and possible interactions with other transcription factors and the transactivating/repressing ability of this novel gene have yet to be investigated.

To elucidate the physiological roles for Midori in cardiomyocyte differentiation, we performed transfection experiments to enhance or inhibit the function of Midori in P19CL6 cells. Stable transfectants overexpressing either antisense or sense Midori transcripts were differentiated with Me\(_2\)SO. A, on day 11 of differentiation, phase-contrast photomicrographs of live cultures showed that the Midori sense clone (S1) more efficiently differentiated into beating cardiomyocytes than did parental P19CL6 cells, whereas antisense clones (A1 and A2) less efficiently differentiated. Highly aggregated cells (arrowheads) appeared in wild type or more often in the antisense clones and did not beat at all. B, each cell line was double-stained with anti-sarcomeric MHC antibody (MF20) and Hoechst dye on day 8 of differentiation. MF20-positive cells are shown in red. In A1, many more aggregations (arrowheads) were seen than in any other cell lines. In contrast, many more MF20-positive cells were observed in S1 than in parental P19CL6 cells, whereas less cells survived in S1 than in wild type because of the extensive cell death during differentiation. C, Western blot analysis of MHC was performed on day 14.5 of differentiation. The bands corresponding to MHC are indicated by an arrow. The amount of the protein was less in A1 than in P19CL6, whereas more protein was detected in S1 than P19CL6.

Recently another group cloned a part of a potential human homolog of Midori. The cDNA fragment, designated KIAA1330, was cloned from human brain but was expressed mainly in the heart and skeletal muscle (39) as we showed in mice (see Fig. 2C). The genomic locus was assigned to human chromosome 15.
(39) KIAA1330 contains an ~2.7-kilobase pair open reading frame corresponding to the 3’-part of the open reading frame of Midori. In the 3’-part of the open reading frame, the amino acid sequence of MIDORI is 74% identical and 79% homologous to that of KIAA1330. However, no information about the function of this gene is available at present.

Here we have first demonstrated potential important roles of this novel gene in cardiomyocyte differentiation. Further investigation of the precise molecular functions of MIDORI will provide new insights into the understanding of the complicated mechanisms of heart development.

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