JUMONJI, a Critical Factor for Cardiac Development, Functions as a Transcriptional Repressor*

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JUMONJI (JMJ) is a nuclear factor that is critical for normal cardiovascular development, evidenced by the analysis of \textit{jmj} homozygous mutant mice. However, the molecular function of JMJ remains to be elucidated. In the present study, we investigated whether JMJ is a transcriptional modulator. Reporter gene assays using the GAL4-DNA binding domain fused to JMJ and a reporter gene consisting of the GAL4 binding sites upstream of a luciferase reporter gene indicated that JMJ functions as a powerful transcriptional repressor. The DNA binding motif of JMJ was determined using CASTing experiments by incubating a random oligonucleotide library with the GST-JMJ fusion protein coupled to agarose beads. Among the selected binding oligonucleotides, the high affinity DNA binding sequences were identified by gel retardation assays. JMJ repressed expression of the reporter genes containing the high affinity JMJ binding sequences, indicating that JMJ is a DNA-binding transcriptional repressor. The domains for transcriptional repression, DNA binding, and nuclear localization signal were mapped by mutational analyses using reporter gene assays, gel retardation assays, and immunostaining experiments, respectively. The present data demonstrate for the first time that JMJ functions as a DNA-binding transcriptional repressor. Therefore, JMJ may play a critical role in transcription factor cascade to regulate expression of heart-specific genes and normal cardiac development.

We have previously reported that JUMONJI (JMJ)\textsuperscript{1} is a nuclear protein that is critical for normal cardiovascular development by characterizing JMJ knockout mice (1). The \textit{jmj} mutant embryos showed heart malformations including ventricular septal defect, noncompaction of the ventricular wall, double outlet right ventricle, and dilated atria. All homozygous \textit{jmj} mutants died soon after birth. Cardiac marker analysis by \textit{in situ} hybridization suggested that cardiomyocytes were differentiated but developmental regulation of chamber-specific genes was defective in late stage embryos.

The \textit{jmj} gene was first identified and described as a developmentally important gene in the nervous system and subsequently in liver, spleen, and thymus development (2, 3), when knockout mice were generated in genetic backgrounds that are different from ours (1). During mouse embryonic development, JMJ is widely expressed, including in the developing heart. In adult mice, it is expressed at a higher level in heart, skeletal muscle, brain, and thymus (1). Continuous expression of \textit{jmj} in the heart suggests that JMJ plays an important role in both the developing and adult heart. Although JMJ may be involved in cell growth when JMJ is overexpressed (4), the molecular function of JMJ remains unknown. The deduced amino acid sequence of JMJ reveals a putative DNA binding domain (DBD) homologous to the DBD of a DNA-binding protein family, AT-rich interaction domain (ARID) (5), suggesting that JMJ is a transcription factor. However, the homology of this domain with the DBDs of other ARID family members is low, with only about 30% amino acid identity in the putative DBD. There is an increasing number of factors that belong to an ARID family, which show diverse functions in vertebrates, plants, and fungi (6).

Cardiac development is a complex biological process requiring the integration of cell specification, differentiation, and morphogenesis. Many factors have been implicated in this process on the basis of their spatial and temporal expression patterns or their phenotypic effects when they are functionally inactivated in flies or mice (for reviews, see Refs. 7–9). Recently, significant advances have been made in understanding the role of transcription factors during heart development. Transcription factors playing critical roles in early cardiac morphogenesis include the homeodomain protein, Nkx2.5 (10), MEF2C, a MADS box factor (11), GATA-4, a zinc finger domain protein (12, 13), and the basic helix-loop-helix factors, eHAND and dHAND (14). Mutations in other nuclear factors, such as KXR (15), RAR (16), TEF-1 (17), Tbx5 (18), and Pitx2 (19), resulted in various abnormal cardiac developments.

Identification of molecular pathways involved in normal heart development led to the discovery of the genetic basis for human congenital heart disease. For example, congenital heart disease characterized by septal defects and abnormal atrioventricular conduction is caused by mutations in the transcription factor Nkx2.5 (20). Another group of human cardiac defects referred to as CATCH-22 (cardiac defects, facial anomalies, thymic hypoplasia, cleft palate, and hypocalcemia, associated with chromosome 22 microdeletion) may be caused by mutation in a downstream gene of dHAND (21). Holt-Oram syndrome, characterized by upper limb malformations and cardiac septal defects, is caused by mutations in the \textit{tbx5} gene (18, 22, 23).

Therefore, it is critical to characterize the molecular function of JMJ. We have used \textit{in vitro} mutagenesis to functionally

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\textsuperscript{1} The abbreviations used are: JMJ, JUMONJI; DBD, DNA binding domain; ARID, AT-rich interaction domain; NLS, nuclear localization signal; GMSA, gel mobility shift assay; CASTing, cyclic amplification and selection of target; SAAB, site selection and amplification binding; HDAC, histone deacetylase; TSA, trichostatin A; CbBP, C-terminal binding protein; GST, glutathione S-transferase; aa, amino acid(s).
characterize the JMJ protein and to better understand the structural/functional relationship of JMJ. The present study indicates that JMJ is a DNA-binding nuclear factor that contains a strong transcriptional repressor domain. JMJ may regulate the expression of cardiac genes, and therefore this study will form a basis to further identify molecular mechanism of cardiac development.

EXPERIMENTAL PROCEDURES

**Plasmid Construction**—Cloning of the cDNA encoding jmj was previously described (1). For the GAL4 DBD-JMJ chimera, the wild type and various deletion mutants were constructed as follows. The regions of JMJ were PCR-amplified with primers containing appropriate restriction digestion sites. The PCR products were digested and subcloned in frame to the GAL4 DBD in the pcDNA3 vector (24) and also subcloned in frame to an Xpress tag in pCDNA1/HisB (Invitrogen). The reporter gene consisting of 5× GAL4 binding site upstream of the adenovirus major late promoter TATA box linked to luciferase (pG5Ti-Luc, obtained from Dr. P. Farnham) (24) exhibited low basal activity in vitro (pG5Ti-Luc), which was prepared by cloning oligonucleotide pools comprising a random 30-bp sequence (24) and various deletion mutants were constructed as follows. The various oligonucleotides (Table I) were synthesized and annealed, followed by the reporter gene assays as described previously (24). These oligonucleotides were then subjected to GMSA to select the consensus DNA-binding motif of JMJ that is also a binding site of the high affinity binding site of JMJ upstream of the SV40 promoter linked to luciferase in pGL3. All new constructs were confirmed by restriction enzyme digestion and sequencing.

**Preparation of the JMJ Fusion Protein and Anti-JMJ Antibody**—The bacterially produced JMJ fusion protein was prepared for a gel mobility shift assay (GMSA), cycle amplification and selection of target (CASTing), and production of antibodies. The regions containing a putative DNA-binding domain of JMJ (amino acids (aa) 529–798 and 529–1198) for N-terminal part were amplified by 15 cycles of PCR with the 5′-GTTAATCACAATAAAATATTG-3′. The NP3 oligonucleotide, a tri-mer of the consensus Engrailed binding sequence that is also a binding site of the heterogeneous SV40 promoter linked to a luciferase reporter gene, 5′-GGCTTTGAGATGCCGAGGATCCCG-3′ (Sigma) was designed (25–27). To generate an anti-JMJ antibody against the JMJ fusion protein, GST-JMJ 529–798 was loaded onto SDS-PAGE, and a protein band visualized by Coomassie Brilliant Blue was used to inoculate a rabbit (service provided by Covance). The anti-JMJ antibodies were characterized by immunostaining, immunoprecipitation, and Western blot analysis as described previously (1). Briefly, for immunoprecipitation, in vitro translated and 35S-labeled JMJ (TNT kit; Promega) was incubated with anti-JMJ antibody in NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet P-40) for 2 h at 4 °C followed by incubation with protein A-agarose beads (25). These oligonucleotides were then subjected to CASTing to confirm their binding to JMJ, followed by the reporter gene assays as described above.

**Transfection Assay and Immunostaining**—Transient transfection assays were performed using the calcium phosphate precipitation method as previously described (27) or LipofectAMINE 2000 (Invitrogen). Mouse fibroblast 10T1/2 cells in 60-mm plates were transfected with 1–2 μg of reporter gene and various amounts of JMJ in mammalian expression vectors and 1 μg of CMV-βgal. Two days after glycerol shock, cell lysates were assayed for luciferase activity according to the manufacturer’s recommendation (Promega) using a luminometer. Luciferase activity was normalized to β-galactosidase activity to correct for variations in transfection efficiency. For Western blot analysis, COS cells were transfected with a plasmid encoding JMJ. Whole cell lysate was prepared as described elsewhere (4). The lysates were sonicated and centrifuged to remove the cell debris. Cell extracts were loaded onto 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was incubated with anti-JMJ antibody (1000-fold dilution), and the protein band was detected with the ECL kit (Amersham Biosciences).

**RESULTS**

JMJ Represses the GAL4 Reporter Gene —Although JMJ has been previously identified as a nuclear factor that is developmentally important (1, 2), the molecular function of JMJ remains largely unknown. The deduced amino acid sequence of jmj revealed a homologous region to the DBD of an ARID factor family. In addition, the jmj homozygous mutant embryos showed defective regulation of heart-specific gene expression (1), suggesting that JMJ is a transcriptional regulator.

To investigate whether JMJ functions as a transcriptional repressor, transient transfection assays were performed using a reporter gene containing five tandem copies of the GAL4 DNA binding sites linked to the heterogeneous SV40 promoter upstream of luciferase in the pGL3 vector (5×GpG3L). The reporter gene was cotransfected into 10T1/2 cells with the expression plasmid encoding the GAL4-DNA binding domain fused to the full-length JMJ consisting of 1234 amino acids flanked by 18 bases of termination sequence 5′-GGCGAACCTTGGACTG-3′ (Biotechnology Center at the University Of Wisconsin). BamHI and HindIII sites are underlined. The PCR primers annealing to the 18 bases of each top and bottom strand were synthesized. The 3′ primer was used for a primer extension reaction to make double-stranded oligonucleotides, which was purified by PAGE. The selection procedure was initiated by mixing 10 μg of the purified double-stranded random oligonucleotides with 50 ng of GST-JMJ coupled to glutathione-agarose beads in buffer A (0.5 ml of 20 mM Tris, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 10% glycerol, 20 μg/ml bovine serum albumin, and 2 μg of poly(dI-dC)). The mixture was rotated at 4 °C for 1 h and then centrifuged. The pellet was washed twice with buffer B, resuspended in 85 μl of PCR buffer, boiled for 3 min, and centrifugation was used as template for a 100-μl PCR. The product after 10, 15, and 20 PCR cycles was examined by running 6.0 μl of the PCR on an agarose gel. Then the PCR fraction that was barely visible on the agarose gel was used for the second round of selection. The second generation began when 20 μl of the above PCR was mixed with the GST-JMJ fusion protein exactly as in the first generation. This step was repeated seven times. After the last PCR amplification step, a fraction of the PCR product was cut with BamHI and HindIII and cloned into the pBlueScript vector (Stratagene) and sequenced (Biotechnology Center, University of Wisconsin, Madison, WI). Binding to JMJ was confirmed by GMSA as described above.

**Site Selection and Amplification Binding (SAB)**—To identify the DNA-binding consensus sequences, the SAB method was also carried out as described previously (31, 32) with minor modifications. 1 μg of GST-JMJ eluted from the beads was incubated with 1.6 × 106 cpm of 32P-labeled random double-stranded probe. This probe was generated by PCR using the primer sets as described above. The mixture was electrophoresed on native polyacrylamide gels. After the shifted band was excised, the DNA was eluted from the gel into 0.5 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS for 5 h at 37 °C. The recovered DNA was amplified by 15 cycles of PCR with the 5′ and 3′ primers that were used in the CASTing experiments. The selection procedure was repeated for four rounds. After the last round of SAB, PCR-amplified products were directly subcloned into the pGEMT vector (Promega) and sequenced. These oligonucleotides were then subjected to CASTing to confirm their binding to JMJ, followed by the reporter gene assays as described above.

**GMSA**—To examine whether JMJ binds to DNA in vitro, GMSAs were performed as described previously (25). Briefly, the purified GST-JMJ protein eluted from the glutathione-agarose beads was incubated with 1 μg of poly(dI-dC) in the reaction buffer (10 mM Tris, pH 8.0, 1 mM dithiothreitol, 1 mM NaH2PO4, 5% glycerol, 50 mM NaCl) for 10 min at 37 °C. After 10 min of 32P-labeling by T4 kinase, the ES cell proteins were separated on native polyacrylamide gels. After the shifted band was excised, the DNA was eluted from the gel into 0.5 mM ammonium acetate, 1 mM EDTA, and 0.1% SDS for 5 h at 37 °C. The recovered DNA was amplified by 15 cycles of PCR with the 5′ and 3′ primers that were used in the CASTing experiments. The selection procedure was repeated for four rounds. After the last round of SAB, PCR-amplified products were directly subcloned into the pGEMT vector (Promega) and sequenced. These oligonucleotides were then subjected to CASTing to confirm their binding to JMJ, followed by the reporter gene assays as described above.

**Fluorescence**—The fluorescence of the recombinant GST was visualized using a Zeiss Axiophot microscope and a confocal laser microscope.
(G-J1–1234). The reporter gene will be activated or repressed if JMJ contains a transactivation or repressor domain, respectively. Interestingly, G-J1–1234 markedly repressed the reporter gene activity up to 80% in a dose-dependent manner (Fig. 1), indicating that JMJ contains the repressor domain. The N terminus of JMJ consisting of aa 1–528 (G-J1–528) repressed the reporter gene as efficiently as the full-length JMJ in a dose-dependent manner. In contrast, the C terminus containing JMJ chimera (G-J529–1234) neither repressed nor activated the reporter gene, suggesting the N terminus contains a repressor domain.

This repression is specific to JMJ, because GAL4-DBD alone did not have any effect on the reporter gene activity. In addition, the positive control plasmid, GAL4-DBD, fused to a VP16 activation domain (G-VP16), activated the reporter gene about 1000-fold as compared with the reporter gene alone. Cotransfection assays using the reporter gene containing a different promoter, pG5Ti-Luc, 5× GAL4-DNA binding site linked to the adenovirus major late promoter, yielded the same results as that of 5×G-pGL3 (data not shown), confirming that repression by JMJ is not dependent on the promoter.

**Determination of a Transcriptional Repression Domain in JMJ**—Since the JMJ N terminus (aa 1–528) mediated repression of the target gene expression, serial deletion mutants were examined for their transcriptional activities to map the repressor domain (Fig. 2) in association with their intracellular location (Fig. 3, A and B). Both G-J1–377 and G-J1–222 markedly repressed the reporter gene as efficiently as the full-length chimera G-J1–1234. In contrast, G-J1–130 completely lost repressor activity, indicating that the repressor domain is located between aa 131 and 222. When this reporter domain was deleted from the full-length JMJ, this mutant (G-J1–130/225–1234) lost most of its repression function, exhibiting about 80% activity as compared with the reporter gene alone. These results indicated that the region between aa 131–222 is necessary. This region between aa 131 and 222, together with the nuclear localization domain (aa 1–130; see Fig. 3A), is sufficient to mediate major transcriptional repressor activity as indicated by maximum repression by G-J1–222. Therefore, this region (aa 131–222) was designated as a transcriptional repressor domain. All of these N terminus-containing chimeras were localized in the nucleus (Fig. 3, A and B).

None of the C terminus-containing JMJ chimeras, G-J529–1234 (Fig. 2), G-J529–806, and G-J807–1234 (data not shown), significantly repressed the reporter gene activity. However, these C-terminus-containing G-JMJ chimeras were not localized in the nucleus but rather in the cytoplasm, as visualized with immunostaining experiments (Fig. 3A). Therefore, the lack of transcriptional activity of these C-terminus-containing mutants could be attributed to their inability to localize in the nucleus. To determine whether there is an effector domain in this C terminus, the nuclear localization signal (NLS) domain (aa 1–130; see below) was fused to the C terminus. This NLS domain alone does not have any transcriptional regulatory activity, as indicated by lack of transcriptional activity of G-J1–130 (Fig. 2). These mutants, G-J1–130/529–1234 and G-J1–130/529–792, localized in the nucleus (Fig. 3A) but showed very weak repression if any (Fig. 2). These data indicate that a strong transcriptional repressor domain is located between aa 131 and 222, and perhaps a weak repressor domain exists between aa 529 and 798. The repressor domain deletion mutant G-J1–130/225–1234 was located in the nucleus but lost most of its repressor activity (Fig. 2). These data confirm that the strong repressor domain is situated between aa 131 and 222.

**Identification of a Nuclear Localization Signal in JMJ**—Transcription factors should enter the nucleus where they regulate transcription of target genes. Many nuclear factors are actively transported into the nucleus, which requires an NLS (33). When the NLS is deleted or blocked by steric hindrance, nuclear proteins will either accumulate in the cytoplasm or be rapidly degraded. Therefore, it is critical to examine intracellular location of various mutants.

JMJ contains several putative NLS similar to that of the SV40 T-antigen at aa 104–110, 147–154, and 433–438. To examine intracellular location of various JMJ mutants, immunostaining experiments were performed on COS cells transfected with JMJ mutants in the mammalian expression vectors (Fig. 3, A and B). Depending on which mutant was used, fixed cells were incubated with either anti-JMJ polyclonal antibody or anti-Xpress antibody (Invitrogen). The JMJ antibodies raised against the peptide were described elsewhere (Fig. 3C) (1), and the GST-JMJ fusion proteins were characterized as described below.
activity was normalized with G-J529–1234, and G-J131–1234 ([Ag]
meras that contain cytoplasmic JMJ mutants, G-J131–222, intracellular location. Unexpectedly, all GAL4 DBD-JMJ chimera were examined for their DBD. Since some JMJ mutants were observed in the cytoplasm. These data suggest that JMJ contains a strong transcriptional repressor domain between aa 1 and 130.

Between aa 1 and 130 is sufficient for nuclear localization. J131–1234, where the region between aa 1–130 was deleted, was observed in the cytoplasm (Ad), indicating that an 1–130 is necessary for nuclear localization of JMJ. When aa 1–130 was fused to the cytoplasmic mutants, J1–130/225-1234 (Af) and J1–130/529-1234 (data not shown) were observed in nuclei. Therefore, the region between aa 1 and 130 was designated as an NLS domain. When point mutations were introduced into the putative NLS in the NLS domain (aa 104, -PSRKRPR to -PSAARPR) of the full-length JMJ, this mutant (J106RK-AA) was detected as a 150-kDa band (lane 2). No band was detected in the control lane that does not contain the JMJ cDNA in the reaction (lane 1). JMJ was incubated with anti-JMJ antibodies followed by incubation with protein A-agarose. This anti-JMJ fusion protein antibody immunoprecipitated the in vitro translated JMJ, whereas the preimmune serum did not (lanes 3 and 4, respectively), indicating the specificities of anti-JMJ antibodies. In Western blot analysis, this anti-JMJ fusion protein antibody recognized the JMJ protein in COS cell extracts overexpressing JMJ (lane 5) but not in control extracts (lane 6) in Western blot analysis. These data together with immunostaining results (Fig. 3, A and B) indicated that the anti-JMJ antibodies raised against the JMJ fusion protein recognize both the soluble and denatured form of JMJ.

Fig. 2. Determination of a transcriptional repression domain in JMJ. Transient transfection assays were performed using 10T1/2 cells as described in Fig. 1. Schematic diagrams of JMJ mutant proteins fused with the GAL-DBD are shown at the left. The reporter gene 5xG-pGL3; 2 µg) was cotransfected with a series of G-JMJ mutant chimeras (1 µg). Luciferase activity by reporter gene alone was set at 100%. Luciferase activity was normalized with β-galactosidase activity to correct transfection efficiency. Bars indicate means with S.E. from at least four separate experiments with duplicate plates.

Characterization of Anti-JMJ Antibodies—The GST-JMJ fusion protein containing aa 529–792 was used to generate polyclonal anti-JMJ antibodies. To characterize these antibodies, immunoprecipitation was performed as described elsewhere (27) (Fig. 3C). The in vitro translated 35S-labeled full-length JMJ was detected as a 150-kDa band (lane 2). No band was detected in the control lane that does not contain the JMJ cDNA in the reaction (lane 1). JMJ was incubated with anti-JMJ antibodies followed by incubation with protein A-agarose. This anti-JMJ fusion protein antibody immunoprecipitated the in vitro translated JMJ, whereas the preimmune serum did not (lanes 3 and 4, respectively), indicating the specificities of anti-JMJ antibodies. In Western blot analysis, this anti-JMJ fusion protein antibody recognized the JMJ protein in COS cell extracts overexpressing JMJ (lane 5) but not in control extracts (lane 6) in Western blot analysis. These data together with immunostaining results (Fig. 3, A and B) indicated that the anti-JMJ antibodies raised against the JMJ fusion protein recognize both the soluble and denatured form of JMJ.

Neither HDAC nor C-terminal Binding Protein (CtBP) Mediates the Repression Activity of JMJ—It has been shown that histone deacetylase (HDAC) mediates transcriptional repression (34–36). We then asked whether HDAC activities are involved in JMJ-mediated repression. After transfected cells were treated with 300 or 600 nM trichostatin A (TSA), a repressor of HDAC activities (37, 38), repression activities of JMJ were compared with those from untreated cells (Fig. 4). TSA treatment did not block repressor activity of G-JMJ on the GAL4-Luc reporter gene. Therefore, it seems unlikely HDAC mediates transcriptional repression by JMJ.

The CtBP family has been reported to mediate repressor function of several transcription factors by binding to the consensus sites, PXDLSRK (39). It was of interest that JMJ contains a variant CtBP binding motif in the transcriptional repression domain (aa 144ISDLSRK; conserved amino acids are
JMJ antibody (1000-fold dilution), and the protein was visualized by dene difluoride membrane. The membrane was incubated with anti-(a–f) or G-JMJ chimeras (g–i). Prior to Western blot analysis, whole COS cell extracts (50 μg/lane) were loaded onto 7.5% SDS-PAGE (lanes 1–4) and Western blot analysis (lanes 5 and 6). For immunoprecipitation, control reticulocyte lysate and in vitro translated and 35S-labeled JMJ and (2 μl each) were loaded onto 7.5% SDS-PAGE (lanes 1 and 2, respectively). JMJ (5 μl of programmed reticulocyte lysate) was incubated with the anti-JMJ antibody raised against GST-JMJ fusion protein or preimmune serum (lanes 3 and 4, respectively). The binding complex was then resolved by SDS-PAGE and autoradiographed. For Western blot analysis, whole COS cell extracts (50 μg/lane) either overexpressing the full-length JMJ 1–1234 (lane 2), or control extract (lane 1), were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was incubated with anti-JMJ antibody (1000-fold dilution), and the protein was visualized by ECL (Amersham Biosciences).

underlined). To determine whether repression function of JMJ is mediated by CtBP, we made a deletion mutation of JMJ, where two amino acids, 146DL, were deleted. This mutant (Δ146DL) retains its repressor activity, indicating that CtBP does not seem to mediate repressor function of JMJ (Fig. 4).

Determination of DNA Binding Sequence of JMJ in Vitro—CASTing experiments were performed to identify the DNA binding sequence of JMJ in vitro as described under “Experimental Procedures.” The GST-JMJ fusion protein (aa 529–798) was coupled to glutathione-coated agarose beads and incubated with random oligonucleotides, and the bound oligonucleotides were amplified by PCR. After this cycle was repeated a total of seven times, the selected oligonucleotides were subcloned into the pBluescript vector and sequenced. Among 38 oligonucleotides sequenced (data not shown), 25 (66%) contain a/T-rich sequences, suggesting that JMJ prefers to bind to A/T-rich sequences. To confirm the DNA binding sites of JMJ, among 48 sequences we sequenced (data not shown), 25 (66%) contain A/T-rich sequences and one does not.

These results indicate that JMJ binds to A/T-rich sequences but can also bind to non-A/T-rich sequences in vitro, suggesting that JMJ can bind to more than one DNA sequence, since homeobox proteins bind to different DNA sequences in vitro (31, 40).

In order to further confirm the DNA binding motif of JMJ, we performed the SAAB experiments as described under “Experi-

FIG. 4. Repression by JMJ involved neither HDAC nor CtBP activities. To examine whether HDAC mediates the repression activity of JMJ, the cells were cotransfected with the reporter vector, 5×GEGalLuc, and G-JMJ in the expression vector and treated with 300 nm (+) or 600 nm (+ +) TSA, a HDAC inhibitor. The repression activities of JMJ were compared with those from untreated cells. Luciferase activity by reporter gene alone set as 100%. Since TSA alone can change the luciferase and β-galactosidase activities, the luciferase activity by reporter gene alone treated with TSA was set at 100% for TSA-treated groups. To determine whether repression function of JMJ is mediated by CtBP, a deletion mutant, J146DL, was cotransfected with 5×GEGalLuc. All culture dishes received a β-galactosidase plasmid (0.5 μg) to normalize transcription efficiencies.

FIG. 3. JMJ contains the nuclear localization signal. A, the immunostaining experiments with JMJ antibody. COS cells were transfected with various JMJ mutants in pcDNA3.1/HisB (a–f) or G-JMJ chimeras (g–i). Fixed cells were incubated with anti-JMJ antibodies or anti-Xpress antibodies followed by anti-rabbit IgG conjugated to fluorescein isothiocyanate or anti-mouse IgG-Texas Red, respectively. Subcellular localization of JMJ mutants was examined under epifluorescence or confocal laser microscope. B, the same field of 4′,6-diamidino-2-phenylindole staining, which stains the nuclei. C, characterization of the anti-JMJ antibody. Polyclonal antibody against GST-JMJ fusion protein was raised in rabbit and characterized by immunoprecipitation (lanes 1–4) and Western blot analysis (lanes 5 and 6). For immunoprecipitation, control reticulocyte lysate and in vitro translated and 35S-labeled JMJ and (2 μl each) were loaded onto 7.5% SDS-PAGE (lanes 1 and 2, respectively). JMJ (5 μl of programmed reticulocyte lysate) was incubated with the anti-JMJ antibody raised against GST-JMJ fusion protein or preimmune serum (lanes 3 and 4, respectively). The binding complex was then resolved by SDS-PAGE and autoradiographed. For Western blot analysis, whole COS cell extracts (50 μg/lane) either overexpressing the full-length JMJ 1–1234 (lanes 2, or control extract (lane 1), were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane. The membrane was incubated with anti-JMJ antibody (1000-fold dilution), and the protein was visualized by ECL (Amersham Biosciences).
JUMONJI Is a Transcriptional Repressor

We have sequenced 35 oligonucleotides (data not shown), of which 26 oligonucleotides (75%) have A/T-rich sequences, which is similar to the result of the CASTing experiments. The four highest binding sites, S11, S15, S36, and B5, were identified by GMSA (Table I), and three of these contained A/T-rich sequences.

In summary, JMJ prefers to bind to A/T-rich sequences, as evidenced by the fact that 66−88% of the selected oligonucleotides contain A/T-rich sequences from three different sets of experiments. In addition, among 10 high binding sites, eight contain one or more A/T-rich sequences. However, JMJ also seems to bind to non-A/T-rich sequences.

It was of interest that the B-cell-specific protein termed Bright (B-cell regulator of IgH transcription) (28) and the dead ringer (Dri) of Drosophila (5), which belong to the ARID factors, bind to the A/T-rich sequences similar to the JMJ binding sites. To test whether JMJ binds to the A/T-rich DNA sequence that is identified in the endogenous target promoter region of other ARID members, GMSAs were performed using two DNA binding sequences (Fig. 5B) as described previously (25). The duplicated oligonucleotides for Bright (Tx2) and the trimer for the Dri binding site (NP3) were labeled with 32P and incubated with GST-JMJ containing aa 529−798. As noted earlier, the JMJ region between aa 529 and 798 contains a putative DNA-binding domain that was determined by amino acid sequence analysis. Binding mixtures were loaded onto nondenatured PAGE and autoradiographed. The single band was observed with Tx2 (lanes 2−4), which was competed by an excess amount of its own nonradiolabeled oligonucleotide (lanes 5 and 6) but not by nonspecific oligonucleotides (lanes 7 and 8). GST alone did not show any binding (lane 9), indicating that JMJ bound specifically to this oligonucleotide. GST-JMJ containing aa 529−1109 showed a slow migrating binding complex as indicated by an arrowhead (lane 10). JMJ bound to NP3, a Dri binding site with much weaker affinity (data not shown). It should be noted that JMJ seems to bind to the selected oligonucleotides (C15, C29, and C31) with much higher affinity than to the Tx2 oligonucleotide. This result suggests that JMJ is a DNA-binding protein with specificity to certain DNA sequences.

**JMJ Represses Transcription via the DNA-binding Sequence**—We next address the question of whether JMJ regulates expression of the reporter gene containing its own binding site by reporter gene assays. Reporter plasmids were constructed by inserting the oligonucleotide sequences of high binding affinity selected by CASTing and SAAB experiments into the pGL3 vector. For example, the reporter gene containing the C15 oligonucleotide was designated as C15-pGL3. The reporter gene was cotransfected with JMJJ/pcDNA3.1/HisB into 10T1/2 cells and luciferase assays were performed. Fig. 6A shows the representative results of these reporter gene assays. JMJ significantly repressed all three reporter genes, C15-, C29- (Fig. 6A), and C31-pGL3 (data not shown) up to 50%.
JUMONJI Is a Transcriptional Repressor

Fig. 6. JMJ represses the transcription via DNA-binding sites. A, the pGL3 reporter gene containing one JMJ high binding affinity site (2 μg), C15, C29, or S15 (designated as C15-, C31-, or S15-pGL3, respectively) or Tx2-pGL3 was cotransfected with 1 μg of JMJ/pcDNA3/HisB into 10T1/2 cells as described in Fig. 1. Relative luciferase activity was calculated when luciferase activity with reporter gene alone was set at 100%. Luciferase activity was normalized with β-galactosidase activity. Values indicate means with S.E. from four separate transfection assays with duplicate plates. B, various JMJ mutants (1 μg) were cotransfected with the reporter gene, C29-pGL3 (2 μg), into 10T1/2 cells. Bars indicate means from two experiments. S.D. was less than 10% of the reporter gene activity.

DNA binding domain deletion mutant, J1–528, lost most of the repression activity as compared with that of the wild type JMJ when cotransfected with the C29-pGL3 reporter plasmid. The transcriptional repression deletion mutant, J1–130/225–1234, also lost its repression activity as compared with the wild type JMJ. The C-terminal containing mutant, J529–1234, did not exhibit any transcriptional activity as expected due to its cytoplasmic localization. These results demonstrated that JMJ represses target gene expression by binding to the target DNA sequence, and this repression requires the repression domain of JMJ. Identical results were obtained when various JMJ mutants were cotransfected with the C15-pGL3 and C31-pGL3 reporter genes (data not shown).

These data together with GAL4-JMJ chimera assays indicate that JMJ is a novel transcriptional repressor, whose function is probably mediated by the DNA binding activity. The structural/functional analyses of JMJ have indicated that the repression domain is located between aa 131 and 222, the NLS domain between aa 1 and 130, and the DNA binding domain between aa 529 and 798. A schematic diagram of the structural and functional analyses is shown in Fig. 7.

DISCUSSION

The phenotype analyses of jmj mutant mouse embryos revealed a critical role of JMJ in heart development (1). JMJ is essential for normal morphological development of the outflow tract, ventricular septum, and the ventricular wall. In addition, JMJ plays a critical role in developmental regulation of heart-specific genes in late stage embryos. Here, we demonstrated that JMJ functions as a transcriptional repressor and a DNA binding factor.

JMJ Contains a Transcriptional Repression Domain—The present study demonstrates that JMJ contains a strong transcriptional repressor domain by a mammalian one-hybrid system using a GAL4-JMJ chimera and a reporter gene containing the GALA binding site. JMJ contains a functional polypeptide element that can mediate efficient transcriptional repression of the wild type JMJ as well as when it was fused to a heterologous GAL4-DNA binding domain. In this study, we have shown the following. 1) The region between aa 529 and 798 bound to the target DNA in vitro and, therefore, was designated as a DNA-binding domain. This is a homologous region to the DNA binding domain of an ARID factor family. 2) A nuclear localization signal domain is located between aa 1–130, which is both necessary and sufficient for nuclear localization. 3) Most importantly, we have identified a novel transcriptional repressor domain between aa 131 and 222, which is sufficient and necessary to confer the repressor activity of JMJ. JMJ contains a strong transcriptional repression domain between aa 131 and 222. There seems to be a weak, if any, repressor domain between aa 529–792. A GAL4-JMJ chimera containing aa 529–792 or aa 529–1234 does not enter the nucleus and, therefore, did not significantly repress reporter gene expression. When the NLS domain is fused to this region, the GAL4-JMJ chimera 1–130/529–792 entered the nucleus and showed weak repression of the GAL4-reporter gene.

In contrast to the DNA-binding domains, analysis of the structure or targets of effector domains has been hampered by the lack of amino acid sequence homology or structural motifs common among them. Nevertheless, some effector domains can be loosely categorized according to the primary amino acid content. It has been reported that activation domains are often rich in acidic amino acids and/or proline and glutamine (41, 42). Less is known about repression domains. However, some of them are rich in alanine, proline, and charged amino acids (43, 44). Although no common signature has been found within the repression domain of JMJ when the data base was searched,
binding consensus sequences (5, 28, 31). Among a total of 86 JMJ, we performed the CASTing and SAAB experiments, both (aa 1/H11002 130; The sequence present study demonstrates that JMJ can bind to the DNA nuclear export signal, which dominate in the absence of NLS. contain a cytoplasmic docking protein interaction domain or a general assumption that GAL4 fusion proteins would be this is the functional NLS of JMJ (data not shown). Contrary to this mutant showed the cytoplasmic localization, indicating mutation was introduced at the NLS (SKRKPK to SAAKPK), and necessary for nuclear localization of JMJ. When the point intracellular location of JMJ mutants indicated that the NLS dependent on protein context. Our mutational analyses on NLS should be tested by mutational assays, since the NLS rich cluster flanked by the helix breakers, proline, or hydro-phobic amino acids (33, 47, 48). The amino acid sequence of JMJ contains several putative NLSs. However, the functional NLS should be tested by mutational assays, since the NLS sequence is heterogeneous and the function of the NLS is dependent on protein context. Our mutational analyses on intracellular location of JMJ mutants indicated that the NLS domain is located between aa 1–130. This region is sufficient and necessary for nuclear localization of JMJ. When the point mutation was introduced at the NLS (SKRRPKP to SAAKPK), this mutant showed the cytoplasmic localization, indicating this is the functional NLS of JMJ (data not shown). Contrary to a general assumption that GAL4 fusion proteins would be located in the nucleus, some GAL4-JMJ chimeras localized in the cytoplasm. Therefore, subcellular location of GAL4-JMJ chimeras was determined by JMJ, suggesting that JMJ may contain a cytoplasmic docking protein interaction domain or nuclear export signal, which dominate in the absence of NLS.

**JMJ Is a DNA-binding Transcriptional Repressor—**The present study demonstrates that JMJ can bind to the DNA sequence *in vitro*. To determine the DNA binding sequence of JMJ, we performed the CASTing and SAAB experiments, both of which have been used successfully to identify many DNA-binding consensus sequences (5, 28, 31). Among a total of 86 oligonucleotides selected by CASTing, 67 oligonucleotides (78%) contained the A/T-rich sequence. From GMSA, JMJ bound to all selected oligonucleotides but with different affinities. We have identified the six oligonucleotides of high JMJ binding affinity from the 86 oligonucleotides by GMSA (Table 1). It would be interesting to determine the exact nucleic acids that JMJ contacts within these selected oligonucleotides. The mutations in the selected JMJ binding sites would lead us to identify exact nucleic acids that are critical for DNA binding and transcriptional repression of JMJ. However, this study is impossible to perform, since JMJ can bind multiple DNA sequences including non A/T-rich sequences. The apparent heterogeneous binding sites, including those without an A/T-rich sequence, may represent *bona fide* DNA-binding characteristics of JMJ. For example, homeo-domain proteins bind to a broad range of DNA sequences *in vitro* but have specific physiological roles *in vivo* (40). Heart-restricted Nkx-2.5 homeobox protein binds to a novel binding site, 5′-TNNAGTG, with high affinity and binds to 5′-CAAT/TTAATT-, which contains a typical 5′-TAAAT core required by most homeodomain factors, with lower affinity (31). The JMJ binding A/T-rich sequence consists of 5′-(CG)(AT)4–6 (CG)-, 5′-TAAAT-, or 5′-TAAAT-

These results were confirmed by SAAB experiments, where 75% of selected oligonucleotides contained A/T-rich sequences. However, we cannot exclude the possibility that the *in vitro* selected DNA binding motif in this study may not reflect the *in vivo* DNA binding motif. The DNA binding site or affinity could be altered depending on the nearby cis-acting elements and interacting cofactors. Binding of JMJ to DNA suggests that JMJ directly regulates target gene expression by binding to the enhancer/promoter region of the target gene. However, due to the loose consensus DNA binding sites of JMJ, it is difficult to identify the JMJ target gene by searching the JMJ binding motif in the promoter region. The high affinity binding sites selected by CASTing and SAAB experiments followed by GMSA mediate the repressor function of JMJ, since the reporter genes consisting of the high binding affinity sites were repressed by cotransfection with JMJ.

Although JMJ contains a homologous region to the DNA binding domain of the ARID transcription factor family, the homology is low. The B-cell-specific transactivator, Bright (28), and the dead ringer gene product of *Drosophila* (5) are members of this multigene family that bind to A/T-rich DNA and regulate target gene expression. The other motif in JMJ is homologous to yeast SWI1 that mediates transcriptional activation (49) and two retinoblastoma binding proteins (50) that bind to pRb, an important cell cycle regulator.

These data so far indicate that JMJ is a DNA-binding transcriptional repressor. The mechanisms by which JMJ mediates transcriptional repression and endogenous target genes of JMJ remain to be elucidated. JMJ may recruit co-repressor(s) and/or interfere with the activity of transcriptional activators or basal transcription factors. The expression pattern of sarcomeric protein and atrial natriuretic factor gene is tightly regulated in a tissue- and developmental stage-specific manner and is responsive to hormonal, physiological, and pathological stimuli (for a review, see Ref. 51). We showed that regulation of several cardiac genes including atrial natriuretic factor and
cardiac α-myosin heavy chain was disrupted in late stage jmj homozygous mutant embryos (1). These genes that exhibit altered expression patterns in jmj mutant hearts may be direct or indirect target genes of JMJs.

By defining the functionally important domains of JMJs, this report forms the foundation for studies of JMJs-regulated cardiac gene expression and transcription factor cascade in cardiac development.

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JUMONJI, a Critical Factor for Cardiac Development, Functions as a Transcriptional Repressor

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