Functional Analyses of Three Csx/Nkx-2.5 Mutations That Cause Human Congenital Heart Disease*

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A homeodomain-containing transcription factor Csx/Nkx-2.5 is an important regulator of cardiogenesis in mammals. Three different mutants, Gln170ter (designated A) and Thr178Met (designated B) in the helix 2 of the homeodomain and Gln198ter mutation (designated C) just after homeodomain, have been reported to cause atrial septal defect with atrial ventricular block. We here examined the functions of these three mutants of Csx/Nkx-2.5. The atrial natriuretic peptide (ANP) promoter was activated by wild type Csx/Nkx-2.5 (WT, 8-fold), B (2-fold), and C (6-fold) but not by A. When A, B, or C was cotransfected into COS-7 cells with the same amount of WT, WT-induced activation of the ANP promoter was attenuated by A and B (A > B), whereas C further enhanced the activation. Immunocytochemical analysis using anti-Myc tag antibody indicated that transfected Myc-tagged WT, B, and C were localized in the nucleus of both COS-7 cells and cardiomyocytes of neonatal rats, whereas A was distributed diffusely in the cytoplasm and nucleus in COS-7 cells. Electrophoretic mobility shift assay showed that Csx/Nkx-2.5-binding sequences were bound strongly by WT and C, weakly by B, but not by A. Immunoprecipitation and GST pull-down assay revealed that WT and all mutants interacted with GATA-4. The synergistic activation of the ANP promoter by WT and GATA-4 was further enhanced by C but was inhibited by A and B. In the cultured cardiomyocytes, overexpression of C but not WT, A, or B, induced apoptosis. These results suggest that although the three mutants induce the same cardiac phenotype, transactivation ability and DNA binding ability are different among the three mutants and that apoptosis may be a cause for C-induced cardiac defect.

The cardiac homeobox gene Csx/Nkx-2.5 was first isolated on the basis of its homology to the Drosophila gene tinman (1, 2). Mutation of tinman leads to a complete absence of the formation of the dorsal vessel, the equivalent of the vertebrate heart in insect (3). The expression of Csx/Nkx-2.5 is restricted to the heart and the heart progenitor cells from the very early developmental stage when the two heart primordia are symmetrically situated in the anterior lateral mesoderm (1, 2). Targeted disruption of murine Csx/Nkx-2.5 results in embryonic lethality due to the abnormal looping morphogenesis of the primary heart tube (4), indicating that Csx/Nkx-2.5 plays a critical role in cardiac morphogenesis.

Recently, three different Csx/Nkx-2.5 mutations were found in patients with atrial septal defect and atrioventricular conduction delays (5). A C to T transition at nucleotide 618 is predicted to substitute a termination codon (TAG) for glutamine (CAG) (hereafter designated as A), which would stop translation prematurely at position 33 of the homeodomain. A C to T version of nucleotide 642 is predicted to substitute methionine (ATG) for threonine (ACG) at homeodomain position 41 (hereafter designated as B). A C to T transition at nucleotide 701 is predicted to substitute methionine (ATG) for glutamine (CAG) (hereafter designated as C), which would create a termination signal immediately after the homeodomain. A homeobox is a 180-bp1 conserved sequence motif that encodes a helix-turn-helix DNA binding domain called the homeodomain. It has been reported that the Csx/Nkx-2.5 homeodomain is required for DNA binding and activation of a reporter gene, and a carboxyl terminus outside of the homeodomain composed mainly of clusters of alanines and prolines is an inhibitory domain for Csx/Nkx-2.5 transcriptional activity (6, 7). Thus, it is hypothesized that A and B might impair binding of Csx/Nkx-2.5 to the target DNA resulting in haploinsufficiency and that C would enhance the transcriptional activity (5).

The zinc finger transcription factor GATA-4 also plays a critical role in the early steps of cardiac development. GATA-4 is expressed in the precardiac mesoderm almost simultaneously with Csx/Nkx-2.5 and earlier than any other known transcription factors implicated in cardiogenesis (8). GATA-4−/− mice are embryonic lethal because cardiac primordia fail to fuse at the midline of the developing embryo (9, 10). Recently, GATA-4 haploinsufficiency has been suggested to contribute to the congenital heart disease of patients with 8p23.1 monosomy (11). All these results indicate that Csx/Nkx-2.5 and GATA-4 are essential for the normal development of the heart.

Since Csx/Nkx-2.5 and GATA-4 are coexpressed in the precardiac mesoderm, the Csx/Nkx-2.5-GATA-4 interaction may play an important role in the regulation of gene expressions.  

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1 The abbreviations used are: bp, base pair; ANP, atrial natriuretic peptide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; FITC, fluorescein isothiocyanate.
during early cardiogenesis in the embryo. Several groups including ours (12–16) have reported that many gene promoters such as atrial natriuretic peptide (ANP), A1 adenosine receptor, and serum response factor are activated in synergy with Csx/Nkx-2.5 and GATA-4. ANP is expressed at a very early embryonic stage when cells are committed to the cardiac phenotype. Throughout the embryonic and fetal development, ANP expression is restricted to both atrial and ventricular myocardium, but it is not expressed in other tissues including skeletal and smooth muscle cells. In the present study, we examined the transcriptional activity of the ANP promoter as well as the DNA binding and association with GATA-4 of these mutants in COS-7 cells. In addition, we examined the functions of these mutants in the cultured cardiac myocytes of neonatal rats.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—The plasmids expressing human Csx/Nkx-2.5 and human GATA-4 have been previously described (16). To introduce the A, B, and C mutations into Csx/Nkx-2.5, a two-step polymerase chain reaction method was used. Polymerase chain reaction products were digested with BamHI and EcoRI and subcloned into the BamHI/EcoRI site of pcDNA3. The ANP 200-bp lac reporter gene (which contains the 300-bp 5'-flanking region of the ANP gene) and the 4x(ThF1)-th-luc reporter gene (which contains four tandem copies of Csx/Nkx-2.5 binding elements) have been previously described (16).

**Cell Culture, DNA Transfection, and Reporter Gene Assay**—COS-7 cells and cardiomyocytes of neonatal rats were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum as described before (16). Transient transfections were performed 24 h after plating by the standard calcium phosphate method, and luciferase activity was measured 48 h after transfection with a Berthod Lumat LB9501 luminometers as described previously (16).

**Electrophoretic Mobility Shift Assay (EMSA)**—Double-stranded oligonucleotides corresponding to the TTF-1 (thyroid transcription factor-1) binding sequence were synthesized with AATT overhangs at the 5' terminus of each oligonucleotide. The two complementary oligonucleotides were annealed and labeled with [α-32P]dATP using Klenow enzyme. Labeled probes were incubated with 5 µl of nuclear extracts and 2 µg of poly(dI-dC) in 20 µl of binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol, and 0.05% Nonidet P-40) for 30 min at room temperature. The protein:DNA mixture was resolved on a 5% polyacrylamide gel in 0.5× Tris borate/EDTA buffer at 4 °C for 2 h at 150 V.

**Coimmunoprecipitation Experiments**—The plasmids expressing Myc-tagged Csx/Nkx-2.5 and GATA-4 were transiently transfected into COS-7 cells, and 48 h after transfection, whole cell extracts were prepared as described previously (16). The cell extracts were then incubated with anti-GATA-4 polyclonal antibody (Santa Cruz Biotechnology) in NT2 binding buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 0.5 mM EDTA, 0.05% Nonidet P-40, 150 mM NaCl, 0.5 mM EDTA, 0.5% Nonidet P-40, 10% glycerol, 0.5 mM dithiothreitol, and 0.05% Nonidet P-40) for 30 min at room temperature. The protein:DNA mixture was resolved on a 5% polyacrylamide gel in 0.5× Tris borate/EDTA buffer at 4 °C for 2 h at 150 V.

**RESULTS**

**Intracellular Localization of Csx/Nkx-2.5 Proteins**—We first examined whether and where Csx/Nkx-2.5 mutant proteins are expressed in COS-7 cells by transiently transfecting the Myc-tagged Csx/Nkx-2.5 cDNAs into COS-7 cells. Western blot analysis using whole cell lysates revealed that all mutants as well as WT were expressed in COS-7 cells at comparable levels (Fig. 1A). Immunocytochemistry using anti-Myc antibody revealed that transfected Myc-tagged WT, B, and C were localized in the nucleus of both COS-7 cells and cardiac myocytes of neonatal rats (Fig. 1B). Although A was localized in the nucleus of cardiac myocytes, A was diffusely distributed in the cytoplasm and nucleus in COS-7 cells (Fig. 1B).

**Transcriptional Activity of Csx/Nkx-2.5 Mutants**—We and
ANP promoter with 0.1 cate. *, into COS-7 cells with the 300-bp ANP promoter (0.2 μg) as the reporter gene. Luciferase activity was assayed as described under "Experimental Procedures," and the activity was normalized to β-galactosidase activity for each sample. The activity was presented as fold relative to the activity of the ANP promoter alone (−1). Values are the mean ± S.E. of data from three independent experiments performed in triplicate. *, p < 0.01 versus the ANP promoter alone; **, p < 0.05 versus the ANP promoter with 0.1 μg of WT.

Fig. 2. Transcriptional activity of Csx/Nkx-2.5 mutants. WT alone and/or each of the three mutants (0.2 or 0.1 μg) were transfected into COS-7 cells with the 300-bp ANP promoter (0.2 μg) as the reporter gene. Luciferase activity was assayed as described under "Experimental Procedures," and the activity was normalized to β-galactosidase activity for each sample. The activity was presented as fold relative to the activity of the ANP promoter alone (−1). Values are the mean ± S.E. of data from three independent experiments performed in triplicate. *, p < 0.01 versus the ANP promoter alone; **, p < 0.05 versus the ANP promoter with 0.1 μg of WT.

others (12, 14, 16) have demonstrated that Csx/Nkx-2.5 activates the ANP promoter. To examine the transcriptional activity of the mutants, we transfected WT and each of these mutants with the 300-bp ANP-luc reporter into COS-7 cells. WT strongly activated the ANP promoter (Fig. 2) as previously reported (16). WT (0.2 μg) activated the ANP promoter by ~8-fold (Fig. 2). The ANP promoter was also activated by B (~2-fold) and C (~6-fold) but not by A (Fig. 2). When the same amounts of mutant cDNA (0.1 μg) were cotransfected with WT (0.1 μg), WT-induced activation of the ANP promoter (~6-fold) was modulated in different ways. The luciferase activity was attenuated by coexpression of A (~6-to ~3-fold) and by B (~6-to ~4-fold) (Fig. 2). In contrast, C enhanced the activity by ~12-fold (Fig. 2). Similar results were obtained with the artificial reporter construct containing multimerized Csx/Nkx-2.5-binding sites (4X(TTF-1)-tk-luc) except for stronger activation by C (~15-fold) than by WT (~5-fold) (data not shown). These results suggest that the transcriptional activities of these three Csx/Nkx-2.5 mutants are different.

DNA Binding Activity of Csx/Nkx-2.5 Mutants—We next examined the DNA binding activity of each mutant using EMSA. We used nuclear extracts prepared from COS-7 cells, which were transfected with WT and the mutants, and the TTF-1-binding sequences, which are favorable binding sequences of Csx/Nkx-2.5 proteins (7). The amount of each expressed protein was estimated by Western blot analysis, and the same amounts of proteins were used for EMSA. WT bound to TTF-1 binding sequences, and addition of the nonlabeled TTF-1 attenuated the WT binding, indicating that the binding was specific (Fig. 3A). C showed a stronger shifted band than WT, suggesting that the DNA binding affinity of C was stronger than that of WT. B very faintly bound to the DNA, and A showed no shifted band. The whole cell lysate as well as nuclear lysate of A-transfected cells did not show TTF-1 binding, suggesting that A did not bind to the DNA sequence. To assess if these mutants affected WT binding to TTF-1 binding sequences, we mixed the same amounts of nuclear extracts of WT and of each mutant, and we performed EMSA. The DNA bind-

Fig. 3. DNA binding activity of Csx/Nkx-2.5 mutants. The DNA binding activity of Csx/Nkx-2.5 proteins was examined by EMSA using TTF-1 binding sequences. WT and each mutant cDNA were transfected into COS-7 cells, and nuclear extracts were prepared. A 32P-labeled oligonucleotide probe corresponding to the TTF-1 binding sequences was incubated with the nuclear extracts and subjected to electrophoresis on a 5% polyacrylamide gel. A, the binding affinity of the WT protein was reduced by the presence of unlabeled TTF-1. The TTF-1 binding sequences bound strongly to C, weakly to B, but not to A. B, the binding affinity of the WT protein was not affected by the presence of the mutant proteins. The binding affinity of the C protein was slightly enhanced by the presence of the WT protein. FP indicates free probes.ing of WT was barely inhibited by any mutants (Fig. 3B). WT slightly enhanced the binding of C to TTF-1-binding sequences. These results suggest that these mutants have different DNA binding ability.

Synergistic Transcriptional Activity between Csx/Nkx-2.5 Mutants and GATA-4—Csx/Nkx-2.5 and GATA-4 activate several cardiac gene promoters including the ANP promoter in a synergistic manner (12–16). We examined whether these mutants affect the synergistic effects of Csx/Nkx-2.5 and GATA-4 on the ANP promoter. Consistent with our previous results, cotransfection with WT and GATA-4 showed much stronger activation (~15-fold) of the 300-bp ANP promoter than single transfection with WT (~8-fold) or GATA-4 (~2-fold) (Fig. 4). The synergistic activation by WT and GATA-4 was inhibited by A and B, whereas it was further enhanced by C (Fig. 4). C alone had a stronger synergistic effect with GATA-4 on the ANP promoter (~20-fold) than WT did (~15-fold), whereas A or B had no effect on GATA-4-induced activation of the ANP promoter (~2-fold) (Fig. 4). These results suggest that these mutants modulate the synergistic effects of WT and GATA-4 on the ANP promoter in different ways.

Association of Csx/Nkx-2.5 Mutants with GATA-4—Csx/
Nkx-2.5 associates with GATA-4 and shows synergistic action on the ANP promoter (12, 14, 16). To elucidate the mechanism of how these mutants affect the synergistic effects of WT and GATA-4 on the ANP promoter, we examined whether these mutants also interacted with GATA-4. The GST pull-down assay revealed that WT and all mutants interacted with GATA-4 in vitro in the following order: C > WT > B > A (Fig. 5A). The coimmunoprecipitation assay also showed similar results. All Csx/Nkx-2.5 proteins, including WT and the mutants, were also associated with GATA-4 in vivo in the same order as in vitro (Fig. 5B, top).

C-Induced Apoptosis in Cultured Cardiomyocytes of Neonatal Rats—Since mutant C has enhanced function, it is unknown how mutant C induces the same cardiac defects as A and B. To address this question, we examined whether mutant C induces cell death in cardiomyocytes. We transfected the Myc-tagged WT and mutants into the cultured cardiomyocytes of neonatal rats and stained the cells by TUNEL methods. As shown in Fig. 6, although overexpression of WT, A, and B did not change the number of TUNEL-positive cells as non-transfected cells (~3%), overexpression of C significantly increased the number of TUNEL-positive cells (10~15%), suggesting that mutant C may induce apoptosis of cardiac myocytes during cardiac development.

DISCUSSION

Intracellular Localization of Csx/Nkx-2.5 Mutants—Transfected Myc-tagged cDNAs of WT, B, and C were localized in the nucleus, whereas that of A was diffusely distributed in the cytoplasm and nucleus of COS-7 cells. This result suggests that the nonsense mutation of A (Gln170ter) loses a nuclear localization signal. Since mutant C that lacks 3’ outside of the homeodomain was localized in the nucleus, there may be a nuclear localization signal between the 170 and 198 amino acids in the homeodomain, although there are no typical sequences of nuclear localization signals in that region. When transfected in the cultured cardiac myocytes, A was also local-
ized in the nucleus (Fig. 1), suggesting that A might be dragged into the nucleus by other factors that exist in the nucleus of cardiac myocytes but not of COS-7 cells.

**Transcriptional Activity of Mutants**—Transient transfection analysis revealed that WT increased the ANP promoter activity by ∼8-fold as reported before (16). Mutant A did not show any transcriptional activity on the ANP promoter, and B only weakly activated the ANP promoter (∼2-fold). Since the 60-amino acid homeodomain plays a critical role in DNA binding of homeoproteins (17), we examined the DNA binding ability of A and B by EMSA. B weakly bound to the Csx/Nkx-2.5-binding DNA sequences but A did not bind at all. Many studies on the homeoprotein-DNA complex have shown that the third helix of the homeodomain contacts with the major grooves of DNA (18–20). Mutant A, which lacks the third helix, was thought not to bind to DNA. Although the role of the threonine codon at homeodomain position 41 between helix II and helix III is not clear, the threonine at 41 is highly conserved in NK class homeoproteins (17), suggesting that the threonine is also critical for DNA binding at least of NK family proteins.

As reported by Schott et al. (5), the three different Csx/Nkx-2.5 mutations found in patients with atrial septal defect and atrioventricular conduction delays are all heterozygous mutations. Thus, to elucidate whether the induction of congenital heart diseases by these mutations is mediated by haploinsufficiency or by dominant negative manner, we cotransfected the heart diseases by these mutations is mediated by haploinsufficiency or by dominant negative manner, we cotransfected the

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