CSX/NKX2.5 is an evolutionarily conserved homeodomain-containing (HD-containing) transcription factor that is essential for early cardiac development. Recently, ten different heterozygous CSX/NKX2.5 mutations were found in patients with congenital heart defects that are transmitted in an autosomal dominant fashion. To determine the consequence of these mutations, we analyzed nuclear localization, DNA binding, transcriptional activation, and dimerization of mutant CSX/NKX2.5 proteins. All mutant proteins were translated and located to the nucleus, except one splice-donor site mutant whose protein did not accumulate in the cell. All mutants that had truncation or missense mutations in the HD had severely reduced DNA binding activity and little or no transcriptional activation function. In contrast, mutants with intact HDs exhibit normal DNA binding to the monomeric binding site but had three- to ninefold reduction in DNA binding to the dimeric binding sites. HD missense mutations that preserved homodimerization ability inhibited the activation of atrial natriuretic factor by wild-type CSX/NKX2.5. Although our studies do not characterize the genotype-phenotype relationship of the ten human mutations, they identify specific abnormalities of CSX/NKX2.5 function essential for transactivation of target genes.
Loss of function and inhibitory effects of human CSX/NKX2.5 homeoprotein mutations associated with congenital heart disease

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CSX/NKX2.5 is an evolutionarily conserved homeodomain-containing (HD-containing) transcription factor that is essential for early cardiac development. Recently, ten different heterozygous CSX/NKX2.5 mutations were found in patients with congenital heart defects that are transmitted in an autosomal dominant fashion. To determine the consequence of these mutations, we analyzed nuclear localization, DNA binding, transcriptional activation, and dimerization of mutant CSX/NKX2.5 proteins. All mutant proteins were translated and located to the nucleus, except one splice-donor site mutant whose protein did not accumulate in the cell. All mutants that had truncation or missense mutations in the HD had severely reduced DNA binding activity and little or no transcriptional activation function. In contrast, mutants with intact HDs exhibit normal DNA binding to the monomeric binding site but had three- to ninefold reduction in DNA binding to the dimeric binding sites. HD missense mutations that preserved homodimerization ability inhibited the activation of atrial natriuretic factor by wild-type CSX/NKX2.5. Although our studies do not characterize the genotype-phenotype relationship of the ten human mutations, they identify specific abnormalities of CSX/NKX2.5 function essential for transactivation of target genes.


Introduction

Homeobox proteins comprise a large family of transcription factors that contain a highly conserved 60–amino acid DNA binding domain (homeodomain [HD]) (1, 2). Csx/Nkx2.5 belongs to the NK2 class of homeobox proteins characterized by a tyrosine residue at amino acid 54 of the HD (54Tyr in HD) and a conserved 23–amino acid NK2-specific domain (3–5). NK2 class HD proteins are expressed in a tissue-specific manner and are important for the determination of cell fate. Csx/Nkx2.5 is the earliest known marker of the heart field mesoderm in Drosophila, zebrafish, Xenopus, chick, and mouse and is shown to be essential for heart formation in Drosophila, Xenopus, and mouse (6–12). In mice, Csx/Nkx2.5 expression starts as early as 7.5 days postcoitum (dpc) in the precardiac mesoderm and its expression continues throughout adulthood (13–15). Csx/Nkx2.5-targeted homozygous mutant mice show normal heart tube formation, but die around 11 dpc before or just after completion of looping morphogenesis (11, 12). Analysis of Csx/Nkx2.5 homozygous mutant embryos showed downregulation of ventricular myosin light chain 2V, atrial natriuretic factor (ANF), and brain natriuretic factor, but most myofilament genes are normally expressed. Transcription factors including eHAND, MEF2C, N-Myc, CARP, Msx2, and Irx4 are downregulated or misexpressed in the mutant embryo (11, 12, 16–19).

In Xenopus and zebrafish, overexpression of wild-type Nkx2.5 causes cardiac enlargement due to an increase in the number of cardiac myocytes (8, 20, 21). On the other hand, dominant inhibitory mutants of Xenopus XNkx2.3 as well as XNkx2.5, created either by fusion to the engrailed repressor domain or by mutating a single amino acid within the HD to abolish DNA binding, causes small hearts or in the most severe case, a complete loss of heart in Xenopus embryos (9, 10).

Recently, heterozygous mutations of human CSX/NKX2.5 were identified in patients with congenital heart disease (22, 23). The most common pheno-
type was progressive atrioventricular conduction delays (AV block) and secundum atrial septal defect (ASD), but other anatomical abnormalities, such as ventricular septal defect (VSD), tetralogy of Fallot (TOF), or tricuspid valve abnormalities including Ebstein’s anomaly, and progressive left ventricular failure were also found (22, 23). These findings strongly suggest that CSX/NKX2.5 is important in the later stages of heart development and maturation in addition to its functions in early cardiac development.

To date, a number of mutations in HD proteins have been identified in a variety of congenital disorders; however, only CSX/NKX2.5 mutations have been identified in the NK2 class associated with congenital disease (24–26). Most of these mutations are point mutations in exons (nonsense or missense) or in RNA splicing signals. Others are small nucleotide insertions or deletions that cause intron-splicing abnormalities or produce frameshifts leading to truncated proteins. Several biochemical studies showed that the mutant homeoproteins may function as either loss of function or dominant inhibitory mutants (25–29). However, the mechanisms by which CSX/NKX2.5 mutations cause congenital heart disease have not been defined. Here, we report the initial biochemical characterization of ten different CSX/NKX2.5 mutations associated with human congenital heart disease.

Methods

Plasmid construct. pBS SK(-)-CSX/NKX2.5 (ref. 30) digested with BglII-blunt-ended and EcoRI was ligated into SacI-blunt-ended and EcoRI-digested pMALC2 (New England Biolabs Inc. Beverly, Massachusetts, USA) to construct maltose binding protein–CSX/NKX2.5 (MBP-CSX/NKX2.5). BglII-blunt-ended and XhoI-digested pBS SK(-)-CSX/NKX2.5 was ligated into EcoRV-XhoI-digested pcDNA3 (Invitrogen Corp., San Diego, California, USA) to construct pcDNA3-CSX/NKX2.5. FLAG epitope tag (gag tag cac aaa gag gac gac aag aag) was inserted at the NH2-terminus of pcDNA3-CSX/NKX2.5. Site-directed mutations were introduced into both plasmids by using Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, California, USA) with the following primers:

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vector and 0.5 µg of Rous sarcoma virus-β-galactosidase (RSV-β-galactosidase) (provided by B. Markham) using
the calcium phosphate method. Total plasmid amount was adjusted to 3 µg with pcDNA3 vector plasmid. For
cotransfection experiments, 1.2 µg of ANF-luciferase reporter construct, 0.3 µg of RSV-β-galactosidase construct, 0.7 µg of pcDNA3-CSX/NKX2.5 expression vector, 0.7 or 1.4 µg of pcDNA3 expression vector encoding mutant proteins and pcDNA3 empty vector to adjust the total plasmid amount 3.6 µg were used. After glycerol shock using 1X HEPES buffer containing 15% glycerol, cells were cultured for another 48 hours, lysed with 300 µL of reporter lysis buffer (Promega Corp., Madison, Wisconsin, USA) and assayed for luciferase activity (Promega Corp.) and β-galactosidase activity.

**Protein-protein interaction.** Bacterially produced MBP-CSX/NKX2.5, MBP, glutathione-S-transferase-GATA4 (GST-GATA4; provided by D. Wilson) and GST protein were made as described previously (31). In vitro-transcribed and translated proteins were generated by using TNT-coupled reticulocyte lysate systems (Promega Corp.). A total of 1 µL of reticulocyte lysate containing [35S]-labeled wild-type or mutant CSX/NKX2.5 proteins was mixed with the fusion proteins bound on the beads in 400 µL of binding buffer (20 mM HEPES [pH 7.5], 100 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100, aprotinin [2 µg/mL], pepstatin [0.7 µg/mL], 0.1 mM PMSF, 1 mM DTT, and 1% BSA) at 4°C for 2 hours. Beads were washed with binding buffer (without BSA) five times and the bound protein complexes were subjected to SDS-PAGE.

**Splicing assays, RT-PCR and protein synthesis assay.** Transient transfection of pcDNA3-CSX/NKX2.5 wild-type and various mutants in COS 7 cells was performed by using Lipofectamine (GIBCO BRL, Gaithersburg, Maryland, USA) as described previously (15). Cells on 6 cm plates were harvested approximately 24 hours after transfection, spun down and directly lysed in 200 µL of SDS-PAGE sample buffer (62.5 mM Tris [pH 6.8], 2.3% SDS, 10% glycerol, 5% 2-mercaptoethanol), and 20 µL of protein (~10 µg) was analyzed for protein expression by Western blotting using anti-FLAG mAb (Sigma Chemical Co., St. Louis, Missouri, USA) and anti-CSX/Nkx2.5 mAb (Figure 2a, lanes 1 and 2) and anti-GAPDH mAb (RDI Research Diagnostics Inc., Flanders, New Jersey, USA). Total RNA was extracted from transfected cells (RNeasy kit; Qiagen Inc., Valencia, California, USA), and 0.5 µg were used for RT-PCR (Perkin-Elmer Corp., Norwalk, Connecticut, USA) by the use of a sense primer (5'-CAAGGACCTAGGCAGCGAAAAG-3') and an antisense primer (5'-TTGACCTGCGTGGACGAGGT-AGTTTC-3') for CSX/NKX2.5 that span the intron. In vitro transcription/translation was performed using the TNT-coupled reticulocyte lysate system (Promega Corp.) using 2 µg plasmid/50 µL reaction.

**Results**

**Grouping of ten mutants according to predicted protein structure.** Human CSX/NKX2.5 is a 324- amino acid protein, that includes an HD between amino acids 138 and 197 (30, 32) and an Arg-Lys cluster of a nuclear localization signal at the NH₂-terminus of the HD (Figure 1) (31). Ten mutation sites identified in patients (22, 23) (Figure 1, asterisks) are distributed throughout the CSX/NKX2.5 molecule. Two mutations are located at the NH₂-terminus to the HD, six are within the HD, and two are located COOH-terminus to the HD. As shown in Figure 1, these mutation sites were divided into five groups based on the predicted protein structure: group 1: two nonsense mutations in the HD (M149 and M170); group 2: four missense mutations within the HD [178Thr-Met (M178), 189Asn-Lys (M188), 189Arg-Gly (M189), and 191Tyr-Cys (M191)]; group 3: two truncation mutants occurring COOH-terminal to the HD [Δ198-324 (M198) and Δ259-324 (M259)]; group 4: one missense mutation NH₂-terminal to the HD [23Arg-Cys, M25]; and group 5: one point mutation at the exon-intron splicing site (M112). The corresponding phenotype associated with each of these mutations is indicated in Figure 1 (23).

All mutant proteins were translated and localized to the nucleus, except one splice-donor site mutant protein (M112) that did not accumulate in the cell. To determine whether mutant proteins accumulate in the cell, FLAG epitope tagged mutant cDNAs were subcloned into the mammalian expression vector pcDNA3 and transfected into COS cells. By Western blotting using anti-FLAG mAb (Figure 2a), proteins with expected molecular weight were detected in group 1, 2, 3 and 4 mutants, whereas group 5 (M112) mutant protein was not detected. As shown in Figure 2b, all accumulated proteins were localized in the nucleus.

The M112 expression construct contained a mutated intron (G→T transversion) involving the first nucleotide of the splice-donor site and results in a change in sequence from GTTGAGG to TTTGAGG (Figure 2c). To examine whether the failure of protein accumulation in cells is due to transcriptional or translational regulation, we compared mRNA and protein expression in transfected cells. As a control, we inserted the wild-type intron (1,539 bp) in the wild-type cDNA construct used in Figure 2a. RNA isolated from transfected cells was amplified by RT-PCR with two primers spanning the intron. The wild-type construct amplified a major spliced product (240 bp) with a low amount of nonspliced product (1,779 bp). In contrast, RT-PCR of RNA isolated from cells transfected with the M112 construct produced only the nonspliced product (1,779 bp). In wild-type transfec-
tants, CSX/NKX2.5 protein was detected as a 42-kDa band by both anti-FLAG mAb and the anti-CSX/Nkx2.5 mAb (Figure 2d, left, lane 1). However, M112 protein was not detected (Figure 2d, left, lane 2), even though the corresponding mRNA was as easily detected by RT-PCR as that of wild-type (Figure 2c). In vitro transcription and translation of M112 produced a protein with an approximate molecular weight of 29 kDa (Figure 2d, right, lane 2), but in the cell, the translation product did not accumulate.
Thus, it is likely that the M112 allele functions as a heterozygous null allele in vivo.

Assessment of mutant DNA binding using EMSA. We examined DNA binding affinity using the electrophoretic mobility shift assay (EMSA). The ANF promoter, an in vivo Csx/Nkx2.5 target, contains three specific Csx/Nkx2.5 binding sites (TNAAGTG) (3, 33) that are located upstream of the transcription start site at approximately −408, −242, and −87 bp (34, 35). We used the −242 bp binding site as a probe to examine Csx/Nkx2.5 binding affinity, since this site contains two binding sites spaced by 5 nucleotides for the −242 bp binding site as a probe to examine Csx/Nkx2.5 binding affinity (1, 2). 188Asn-Gly (M188) mutant had more than 35-fold (273-fold) lower DNA binding affinity than wild-type. Also, 189Arg-Gly (M189) mutation showed barely detectable DNA binding. The 194 Tyr residue (5′Tyr in HD) is unique for the NK2 class HD proteins (3–5, 8). 191Tyr-Cys (M191) mutant protein also showed a markedly reduced DNA binding affinity by approximately 3-fold (81-fold) (Figure 3c).

Group 3 and group 4 mutants showed similar DNA binding affinity as a monomer but reduced DNA binding affinity as a dimer. In three mutants, the HD was unaffected; two COOH-terminus deletion mutants (M198 and M259) comprised group 3, and an NH2-terminus missense mutation (M25) comprised group 4. All group 3 and 4 mutant proteins bound to DNA (Figure 4), but there were subtle but significant differences in DNA binding characteristics. Csx/Nkx2.5 bound to the dimeric NK2 specific binding sites predominantly as a monomer at a low protein concentration and transitioned into a dimer at a higher protein concentration. In wild-type Csx/Nkx2.5 protein, the monomer-dimer transition was observed in lane 3 (Figure 4a, asterisk in wild-type) at an approximate protein concentration of 0.055 μg/mL or 7.1 × 10−9 M. In contrast, M198 required approximately 9 (32) times higher protein concentration to yield a monomer/dimer ratio similar to that of wild-type (Figure 4b, asterisk in M198; approximate protein concentration 7.7 × 10−8 M). A 31–32 times higher protein concentration was necessary for equimolar monomer-dimer formation in M259 (Figure 4c, asterisk in M259; approximate protein concentration 2.3 × 10−9 (7.0 × 10−9 M). We also detected a subtle defect of dimer formation in the M25 mutant, which required approximately three times higher protein concentration to yield a monomer/dimer ratio similar to that of wild-type (Figure 4d, asterisk in M25; approximate protein concentration 2.1 × 10−8 M).

To confirm that these mutant proteins bound to the monomeric binding site with a similar affinity, we performed EMSA using oligonucleotides in which one of the DNA binding sites was deleted from the ANF-242 site. Group 3 and group 4 mutants bound to the DNA binding sites identified in congenital heart disease. Ten mutation sites (asterisks in Wild) were divided into five groups based on the predicted protein structure: nonsense mutation in the HD (group 1: M149 and M170); missense mutation in the HD (group 2: M178, M188, M189, and M191); premature termination after HD (group 3: M198 and M259); 23Arg-Cys missense mutation (group 4: M25); and mutation at the intron-splicing donor site (group 5: M112). Pheno- types observed in patients are listed on the left. For example, "11/12" indicates that 11 patients show the phenotype among 12 patients examined. These mutation sites were mapped on CSX/NKX2.5 cDNA, which encodes 324 amino acids including 60 amino acids of HD (shaded box). Nuclear localization signal at the NH2-terminus of the HD is indicated (black box). Predicted translated product of M112 mutation in splicing donor site is indicated with a light gray box. AV block, atrioventricular conduction block; ASD, atrial septal defect; VSD, ventricular septal defect; TOF, tetralogy of Fallot; TV, tricuspid valve abnormality; DORV, double outlet right ventricle; NLS, nuclear localization signal; HD, homeodomain.
mutated monomeric site with identical affinity as wild-type CSX/NKX2.5 (Figure 4, e–h). These findings suggest that group 3 and group 4 mutants bound to monomeric DNA binding sites with similar affinity as wild-type CSX/NKX2.5, but the mutations decreased the ability to form dimers on the dimeric site.

Transcriptional activation function of CSX/NKX2.5 mutants. We examined the transcriptional activation function using the ANF-luciferase reporter construct. The mutants subcloned into pcDNA3 were transfected into 10T1/2 cells with ANF luciferase reporter plasmid (Figure 5). Wild-type CSX/NKX2.5 activated the ANF-luciferase reporter gene 23.0 ± 2.2 fold compared with the pcDNA3 parental vector, indicating that human CSX/NKX2.5 is a transcriptional activator as is the mouse Cs/Nkx2.5 (31, 33–35), which shows 87% overall amino acid homology (30).

As expected from the EMSA data, group 1 and group 5 (M112) with truncated HD (Figure 3b) did not activate the ANF-luciferase reporter gene (Figure 5). Group 2 mutants, which have markedly reduced DNA binding, also failed to activate the ANF reporter. M198 in group 3 and M25 in group 4 mutants transactivated the reporter construct as effectively as wild-type, whereas M259 in group 3 had a reduced transcriptional activation function (5.2 ± 0.9 fold). Because the COOH-terminus deletions of the mouse Cs/Nkx2.5 were shown to cause markedly increased transcriptional activity (31, 33), the finding of unchanged or lower transcriptional activities of group 3 mutants was unexpected. Accordingly, we con-
constructed another COOH-terminus deletion mutant, CSX/NKX2.5(1-200) that includes an additional three amino acid COOH-terminus to the HD and corresponds exactly to the murine Csx/Nkx2.5(1-199) mutant and was previously shown to have very high transcriptional activity (240-fold) (31). Similar to the mouse mutant, CSX/NKX2.5(1-200) had markedly increased transcrip-
tional activity (136 ± 10 fold; Figure 5). These results demonstrate that mutants that do not encode the com-
plete HD (group 1 and group 5) or with single missense
mutation in the HD did not transactivate the ANF pro-
moter. Although CSX/NKX2.5(1-200) showed “gain of
function,” neither M198 nor M259 COOH-terminus
deletion mutants were gain-of-function mutations.

Effects of group 1, 2, 3, and 4 mutants on wild-type
CSX/NKX2.5. To examine whether these mutations affect transcriptional activity of wild-type CSX/NKX2.5, we cotransfected the expression plasmid encoding M170 (group 1), M189 (group 2), M191 (group 2), M259 (group 3), or M25 (group 4) with the plasmid encoding the wild-type CSX/NKX2.5 and examined reporter gene activity compared with that of wild-type cotransfected
with pcDNA3 empty plasmid (Figure 6a, open bar, none). Luciferase activation after cotransfection of wild-
type with pcDNA3 empty plasmid was defined as 100%.

When the M170 protein expression plasmid was cotrans-
fected with wild-type CSX/NKX2.5 expression plasmid,
we noted an approximately 23% decrease in activation of
ANF-luciferase reporter gene compared with wild-type alone at a plasmid ratio of 1:1 (0.7 µg) (Figure 6a, hatched bar, M170) and 2:1 (1.4 µg) (Figure 6a, black bar, M170). Reduction of luciferase activity was observed in M189; 34% reduction at 1:1 ratio, and 35% reduction at 2:1 ratio, as well as in M191 (34% and 44% reduction, respectively) and M259 (18% and 44% reduction, respec-
tively). In contrast, when the plasmid encoding the M25
mutant was cotransfected, we noted 39% increase in
luciferase activity at 1:1 ratio, and 91% increase with 2:1
ratio. These data demonstrate that cotransfection of the
plasmid-encoding groups 2 and 3 inhibited transactiva-
tion function of wild-type CSX/NKX2.5 moderately, and
further reduction of luciferase activity was observed with
cotransfection of plasmid encoding M189, M191 (group
2), and M259 (group 3) mutants, suggesting that these
mutants act in a dominant inhibitory manner on wild-
type CSX/NKX2.5 in transient transfection assays. How-
ever, these mutants did not act in a typical dominant
inhibitory manner (see Discussion). Interestingly, the
M25 mutant did not exhibit an inhibitory effect, rather
it transactivated the ANF promoter.

Protein-protein interaction of mutants with wild-type
CSX/NKX2.5. As shown in Figure 3b and Figure 4, we
found that CSX/NKX2.5 bound to the ANF-242 site as
a monomer as well as a dimer. Our recent studies
demonstrated that the mouse Csx/Nkx2.5 physically interact with each other in vitro as well as in the cell in the absence of DNA (Kasahara et al., unpublished data). Because DNA binding of group 2 mutants was totally abolished or markedly reduced (Figure 3, b and c), and these mutants themselves did not activate the ANF promoter (Figure 5), it is of interest to examine whether the inhibitory effect (Figure 6a) is due to dimer formation of the mutant proteins with wild-type CSX/NKX2.5. Accordingly, MBP-fused CSX/NKX2.5 or MBP alone were mixed with in vitro translated [35S]-labeled mutant proteins. After extensive washing, the protein complexes were resolved on SDS-PAGE and autoradiographed (Figure 6b). [35S]-labeled wild-type CSX/NKX2.5 bound to MBP-CSX/NKX2.5 fusion protein was detected in lane 1 (Figure 6b, top), but not with MBP alone (data not shown). In contrast, group 1 (M149 and M170) and group 5 (M112) mutants did not interact with wild-type CSX/NKX2.5 (asterisks in lanes 2, 3, and 11), whereas group 2 (M178, M188, M189, and M191), group 3 (M198 and M259), and group 4 (M25) mutants interacted with CSX/NKX2.5 (lanes 4–10). Therefore, group 1 and 5 mutants, which completely or partially lack the HD, did not associate with wild MBP-CSX/NKX2.5, whereas mutants with the HD associated with MBP-CSX/NKX2.5. These data demonstrate that the HD region is necessary for dimerization with wild-type CSX/NKX2.5, but the amino acid residues mutated in group 2 mutants (amino acids 178, 188, 189, and 191) did not significantly affect the dimer formation with wild-type CSX/NKX2.5.

Group 2 mutants can associate with GATA4 protein. Previous studies demonstrated that Csx/Nkx2.5 transactivates the ANF promoter synergistically with the zinc-finger transcription factor GATA4 (34–37). We also demonstrated that the second zinc finger of GATA4 is necessary and sufficient in the specific interaction with Csx/Nkx2.5, and the third helix is required for
Because three of group 2 mutation sites are within in the third helix (Figure 3c), we examined whether these group 2 mutation sites affect the association with GATA4. As demonstrated in Figure 7, lane 1, [35S]-labeled wild-type CSX/NKX2.5 associated with GATA4, but not with GST alone (lane 6). Similar protein-protein interactions with GATA4 were observed in all four group 2 mutants (lanes 2–5), but none of these proteins associated with GST protein alone (lanes 7–10). These results demonstrate that the missense mutations found in group 2 patients do not significantly affect the interaction with GATA4. Therefore, they potentially sequester GATA4 from wild-type CSX/NKX2.5.

Discussion

Heterozygous mutations of the homeoprotein CSX/NKX2.5 are associated with progressive AV block with secundum ASD as well as several other cardiac malformations that are transmitted as an autosomal dominant trait (22, 23). In this study, we examined the function of ten known mutations of CSX/NKX2.5 found in patients in order to gain insights into the nature and mechanism of the molecular defects responsible for the clinical abnormalities. We were able to categorize these mutation sites into five groups (Figure 1).

In spite of the extensive in vitro characterization of these CSX/NKX2.5 mutations presented here, it is not yet possible to define clear genotype-phenotype associations. However, some generalizations are possible. For example, the M112 mutation (group 5) abolished intron splicing (Figure 2c) and is likely to function as loss of function allele in vivo, as the mutant protein did not accumulate in transfected cells (Figure 2d). Patients with M112 mutation show second-degree AV block without morphological defects, which is similar to the phenotype of hemizygous Csx/Nkx2.5-null mice, who showed a high penetrance of AV block and low incidence of ASD (20%) without other morphological defects (M. Tanaka et al., unpublished data). These findings suggest that a loss of function of one allele may cause AV conduction delay but additional dominant effects of “hypo-morphic” mutant proteins (groups 2 and 3) may cause various anatomical anomalies such as ASD, VSD, TOF, and left ventricular failure. (Note that group 2 and 3 mutants have altered DNA binding but preserved protein-protein interactions). In contrast, the patient with the M25 mutation (group 4, 25Arg-Cys missense mutation in the NH2-terminus), exhibited VSD with TOF without AV block (Figure 1). In EMSA as well as in transient transfection assays, M25 appeared to function similarly to the wild-type CSX/NKX2.5 (Figures 4 and 5). Cotransfection of plasmid encoding M25 and wild-type
CSX/NKX2.5 increased the ANF luciferase activity, which is different from other mutants examined (Figure 6a). Although, M25 is genetically a dominant mutation vivo, our in vitro assays of CSX/NKX2.5 function is limited in explaining the nature of the molecular defect of the M25 mutation.

Patients with the other eight mutations showed both AV block and cardiac malformations (Figure 1). Six mutations were found in the HD either as a nonsense (M149 and M170 in group 2) or a missense (M178, M188, M189, M191 in group 3) mutation. All six HD mutant proteins had markedly reduced DNA binding affinity (Figures 3 and 4). Even in the presence of two repeated CSX/NKX2.5 binding sites, M149 and M170 (group 1) and M189 (group 2) did not show any shifted bands (Figure 3, b and c). M178, 178Thr-Met mutation in between the second and third helix, is likely to change the angle of the helix III, resulting in the reduction of contact to the major groove of DNA (10, 38, 39). Other group 2 mutations were mapped in the third helix. Interestingly, two conserved amino acids in the HD were mutated in patients. 188Asn (51Asn in HD), which is conserved in all members of the homeoprotein family and is known to directly contact adenine in the major groove of DNA (1), was mutated into Lys in M188 mutant, resulting in markedly reduced DNA binding affinity by more than 243-fold. Also, 191Tyr (54Tyr in HD), which is conserved in all NK2 class homeoproteins and is speculated to specify the DNA binding (4), was mutated into Cys in M191, which reduced its DNA binding affinity by 81-fold. Consistent with absent or markedly reduced DNA binding, group 1 and 2 mutants had markedly reduced transcriptional activity on the ANF-luciferase reporter (Figure 5).

Previous studies in Xenopus demonstrated that non–DNA-binding missense mutants act in a dominant inhibitory manner on wild-type homeoproteins, Mix1, XVent2, XNkx2.3, and XNkx2.5 (10, 38, 39). In Mix1 HD proteins, the dominant inhibitory effect of mutant proteins is likely due to their ability to homo- or heterodimerize with other HD proteins, and these mutant–wild-type homeoprotein complexes may change the transcriptional activity (38). Accordingly, we examined the homodimerization of CSX/NKX2.5 and found that wild-type CSX/NKX2.5 homodimerize on DNA that contains two binding sites (Figure 3b). We also demonstrated that CSX/NKX2.5 can homodimerize without DNA by in vitro pulldown assay (Figure 6b). All the group 2 mutations with a single missense mutation in the HD had markedly reduced DNA binding (Figure 6c) but preserved dimerization ability (Figure 6b). The mutants themselves did not transactivate or suppress the ANF promoter (Figure 5) but inhibited the transcriptional activity of wild-type CSX/NKX2.5 (Figure 6a). However, the degree of inhibition increased little by the increase in the wild-type versus mutant plasmid from 1:1 to 1:2. This suggests that the mutant may not simply act as a dominant inhibitor of wild-type CSX/NKX2.5, but may inhibit CSX/NKX2.5 function in a more complex manner.

The COOH-terminus deletion mutant of CSX/NKX2.5(1-200) had a significantly increased transcriptional activity on ANF promoter (136-fold) compared to wild-type (23-fold). In contrast, two COOH-terminus deletion mutants found in patients (group 3, M198 and M259) exhibited similar (22-fold in M198) or reduced (fivefold in M259) transcriptional activity. This suggests that the region COOH-terminus to the HD may contain several transcriptional activation and repression domains, or the deletion mutations may change the conformation of CSX/NKX2.5 to increase or decrease transcriptional activity. Considering that no mutations seen in patients showed higher transcriptional activity than wild-type, it is possible that constitutively active mutations may cause a different phenotype or may even cause an embryonic lethal phenotype, therefore escaping clinical detection.

In summary, although nuclear localization was intact in all mutants except M112, the mutations in the HD (groups 1 and 2) results in the loss or severely impaired DNA binding with the concomitant loss of their trans-activation function. Mutations located outside of the HD had normal DNA binding to the monomeric target site but had reduced DNA binding to the dimeric target site compared with wild-type CSX/NKX2.5. Group 2 mutations had preserved protein dimerization function and exhibited an inhibitory function on wild-type CSX/NKX2.5. Group 4 mutant with a single missense mutations NH2-terminal to the HD acted very similarly as the wild-type CSX/NKX2.5 except for subtle DNA binding defect to the dimeric binding site. CSX/NKX2.5 is likely to form multifactor complexes to transactivate target genes (34–37, 40), and these factors in each complex could be different depending on the context of the target sites. Through a complex mechanism, CSX/NKX2.5 seems to regulate precisely target gene activation and repression at each developmental stage. Further analysis of target genes of CSX/NKX2.5 may aid in the understanding of the role of the CSX/NKX2.5.

Figure 7
Interaction of group 2 mutants with GATA4 protein. [35S]-labeled wild-type CSX/NKX2.5 and four group 2 mutant proteins were mixed with GST-GATA4 protein (lanes 1–5) or GST alone (lanes 6–10). Bound labeled proteins were resolved on SDS-PAGE and autoradiographed (top panel). Fifty percent input of [35S]-labeled proteins is also shown. Coomassie blue-stained GST-GATA4 (lanes 1–5) or GST (lanes 6–10) fusion proteins are shown (bottom panel).
in cardiac development as well as the genotype-phenotype associations resulting from its mutations.

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