Cardiac and Extracardiac Expression of Csx/Nkx2.5 Homeodomain Protein

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Abstract—Csx/Nkx2.5 is an evolutionary conserved homeobox gene related to the Drosophila tinman gene, which is essential for the dorsal mesoderm formation. Expression of Csx/Nkx2.5 mRNA is the earliest marker for heart precursor cells in all vertebrates so far examined. Previous studies have demonstrated that Csx/Nkx2.5 mRNA is highly expressed in the heart and at lower levels in the spleen, tongue, stomach, and thyroid in the murine embryo. Since some developmental genes are regulated by posttranscriptional mechanisms, we analyzed the developmental pattern of Csx protein expression at the single-cell level using Csx-specific antibodies. Immunohistochemical analysis of murine embryos at 7.8 days post coitum revealed that Csx protein is strongly expressed in the nucleus of endodermal and mesodermal cells in the cardiogenic plate. Subsequently, in the heart, Csx protein was detected only in the nucleus of myocytes of the atrium and the ventricle through the adult stage. During the fetal period, Csx protein expression in the nucleus was also noted in the spleen, stomach, liver, tongue, and anterior larynx. Unexpectedly, confocal microscopy revealed that Csx immunoreactivity was detected only in the cytoplasm of a subset of cranial skeletal muscles. Csx protein was not detected in the thyroid glands. The expression of Csx protein in all organs was markedly downregulated after birth except in the heart. These results raise the possibility that Csx/Nkx2.5 may play a role in the early developmental process of multiple tissues in addition to its role in early heart development. (Circ Res. 1998;82:936-946.)

Key Words: homeobox gene ▪ Csx ▪ Nkx2.5 ▪ cardiac development ▪ immunohistochemistry ▪ NK-2

Recently, a number of genes involved in cardiac development were identified and characterized in several species.① ② The vertebrate heart develops from the paired mesodermal primordia that migrate to the anterior ventral midline. In mice, this migration occurs at ~7.5 dpc. The two primordia fuse into one tube (cardiac tube), which begins to undergo rightward looping at ~8.5 dpc. Beating of the heart also begins at this stage of development. In precardiac mesoderm, several genes encoding tissue-restricted transcription factors, such as MEF2C (a MADS box transcription factor), GATA-4 (a zinc-finger–containing transcription factor), and Csx/Nkx2.5 (a homeoprotein), begin to be expressed at ~7.5 dpc. Targeted disruption of these genes has demonstrated the importance of each to normal cardiac development. MEF2C appears to be required for looping, right ventricular formation, and dHAND (a basic helix-loop-helix transcription factor) expression.③ A similar phenotype was observed in Nkx2.5/Csx-targeted mice,④ which also showed normal heart tube formation and expression of most myofilament genes but displayed an arrest of cardiac development before the completion of looping. GATA4 gene–targeted mice also died between 8.5 and 10.5 dpc as a result of defects in both rostrocaudal and lateroventral folding, which were reflected in a generalized disruption of the ventral body patterning. In these mice, heart precursor cells formed normally but failed to migrate into the heart tube.⑤ ⑥ These data suggest that none of these genes are essential for cardiac myocyte determination. In contrast, Drosophila lacking the tinman gene does not develop any detectable cardiac tissue and visceral mesoderm.⑦ On the basis of cDNA sequence analysis, Csx/Nkx2.5 appears to be one of the mouse homologues of this gene.⑧ ⑨ One explanation for the different phenotype between Nkx2.5/Csx-targeted mice and tinman-mutated Drosophila is that, during evolution, multiple copies of the tinman-related gene were created and diversely evolved to have distinct but overlapping roles in cardiac development. Indeed, several tinman-related genes expressed in the heart have been cloned, such as Nkx2.3 in chick,⑩ Xenopus, ⑪ and zebra fish. ⑫ nkx2.7 in zebra fish,⑫ and cNkx2.8 in chick.⑬ However, mouse Nkx2.3 is not expressed in the heart,⑭ and nkx2.7 and cNkx2.8 homologues have not been found in the mouse.

A homeobox gene product is a transcription factor containing a conserved 60-amino-acid homeodomain that binds DNA in a sequence-specific manner.⑮ To date, >400 homeobox genes have been isolated in various species. In general, their expression pattern has been examined only at the mRNA level by use of in situ hybridization or RNase protection assay. In the several studies in which homeobox protein expression has been examined,⑯ ⑰ ⑱ ⑳ it is clear that protein and mRNA expression do not always correlate, perhaps because of posttranscriptional and/or posttransla-
tional modifications. For example, most of the transcripts of *Xhox36* in the embryo cannot be translated to give a homeodomain protein because of deficient splicing resulting in the accumulation of unspliced RNA or because of alternative splicing resulting in a multiple stop codon. In the case of the *caudal* homeodomain protein, maternally derived *caudal* mRNA is distributed diffusely, but it is translated only in the posterior area, creating a protein gradient. Interestingly, *Csx/Nkx2.5* mRNA is readily detectable in NIH 3T3 cells, but its protein product is undetectable in these cells (authors’ unpublished data). This raises the possibility that *Csx/Nkx2.5* protein expression in vivo may not necessarily correlate with that of mRNA. Two previous studies showed expression of *Csx/Nkx2.5* mRNA throughout cardiac development, but they differed in the extracardiac expression pattern. In the present study, we examined the *Csx/Nkx2.5* protein expression at the single-cell level throughout murine development using immunohistochemical analysis.

### Materials and Methods

#### Fusion Protein Production

(His)5-Csx (160-318) was constructed by insertion of a Kpn-I-HindIII fragment of the full-length cDNA clone for *Csx* gene into the pQE-31 vector (QIAGEN). (His)6-ΔMBP was constructed by BglII-HindIII–digested pMAL-cRI (New England Bio-Labs), cloned into BamHI-HindIII–digested pQE-31.

#### Polyclonal and Monoclonal Antibody Production

The (His)5-Csx(160-138) fusion protein was affinity-purified using the Ni-resin (QIAGEN) and then separated by SDS-PAGE. Coomasie blue–stained Csx fusion protein bands were cut out from the gel and eluted electrophoretically. The purified protein was emulsified either with complete Freund’s adjuvant (day 1) or incomplete Freund’s adjuvant (days 14 and 28) to immunize New Zealand White rabbits for pAb production. Two weeks after the last immunization, the serum was obtained by heart puncture with the animals under deep anesthesia. mAbs were obtained by the standard procedure. Screening for antibody products was performed by ELISA and Western blotting against Csx fusion protein and MBP. Positive clones were expanded and used for further experimentation.

#### Western Blotting

One-dimensional SDS-PAGE was performed using the methods of Laemmli, and after electrophoresis, proteins were electrophoretically transferred to the polyvinylidene fluoride membrane (Millipore). Membranes were treated with the first antibody (Csx mAb [1:1]; Csx pAb [1:1000]) followed by horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham). The localization of peroxidase was detected using either the chemiluminescence Western blotting kit (Boehringer) or the ECL kit (Amersham).

#### Immunoprecipitation

For Western blotting, immunoprecipitation, and immunostaining studies.

#### Immunoblotting

For indirect immunofluorescence microscopic study, cultured cells on the coverslips or the cryosections were fixed with 4% formaldehyde in PBS for 15 minutes at room temperature or with 100% methanol at −20°C. The fixed samples were then washed for 15 minutes with 0.2% Triton X-100 in PBS. After they were washed with PBS three times and soaked in 1% BSA and 0.2% Tween 20/PBS, the samples were then washed three times with 0.5% Tween 20/PBS and incubated with the second antibodies (FITC-conjugated goat anti-mouse or anti-rabbit IgG (Amersham) (1:1000) in blocking buffer (5% FCS, 1% BSA, and 0.2% Tween 20 in PBS) for 20 minutes at room temperature and then with anti-Csx pAb or preimmune sera (1:1000) in blocking buffer for 1 hour at room temperature. They were washed three times with 0.5% Tween 20 in PBS, followed by incubation with biotinylated anti-rabbit IgG (1:200, Vector) for 1 hour at room temperature. Staining was performed using a Vecta-Stain ABC kit (Vector Laboratories) per the manufacturer’s protocol, with diaminobenzidine plus NiCl2 for the peroxidase substrate. The slides were then counterstained with methyl green to visualize the nucleus.

#### DNA Transfections

A *Csx* cDNA–containing pBluescript SK(−) clone was recloned into pcDNA3 (Invitrogen) to produce a Csx mammalian expression vector (pcDNA3-Csx). Partial *Nkx2.2* 5′-UTR cDNA, which was isolated from a 10.5-dpc mouse embryonic cDNA library screened with an *Tix/Nkx2.6* genomic fragment (I. Komuro, H. Inagaki, and S. Izumo, unpublished data), was recloned into pcDNA3 with an added ATG translation initiation site at the amino terminus (total amino acids, 170). The in vitro–transcribed and –translated product of this clone using an in vitro Express Translation Kit (Strategene) was analyzed by SDS-PAGE. The full-length *Nkx2.2* cDNA clone was isolated from a 9.5-dpc mouse embryonic cDNA library and was recloned into pFLAG-CMV-2 expression vector (Kodak). COST7 cells in a 35-mm dish were transfected with 2 μg of plasmid DNAs using 10 μL of lipofectamine in 1 mL of Opti-MEM1 (GIBCO) for 3 hours. Twenty-four to 48 hours after transfection, these cells were harvested for Western blotting, immunoprecipitation, and immunostaining studies.

#### Immunoprecipitation

COST7 cells transiently expressing Csx were lysed by 1 mL of buffer A (20 mMol/L HEPES, pH 7.9, 100 mMol/L NaCl, 10 mMol/L MgCl2, 10% glycerol, 5 mMol/L dithiothreitol, 0.1% NP-40, 2.0 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.7 μg/mL pepstatin, and 0.1 mMol/L phenylmethylsulfonyl fluoride) and briefly sonicated. Anti-Csx mAb and pAb bound to 20 μL of protein G–Sepharose (Sigma Chemical Co) were incubated with cell lysates for 2 hours at 4°C. The resin was then washed five times with 10 minutes at 4°C with 1 mL of buffer A and then boiled with SDS sample buffer. For detection of the native Csx protein, the hearts of 10 fetal mice at ∼12.5 dpc were isolated and separated from the remainder of the embryos. Cardiac and noncardiac tissues were lysed separately with 1 mL of buffer A and used for immunoprecipitation as described above.

#### Immunohistochemistry

For indirect immunofluorescence microscopic study, cultured cells on the coverslips or the cryosections were fixed with 4% formaldehyde in PBS for 15 minutes at room temperature or with 100% methanol at −20°C. The fixed samples were then washed for 15 minutes in 0.2% Triton X-100 in PBS. After they were washed with PBS three times and incubated with 1% BSA and 0.2% Tween 20/PBS, the samples were then treated with the first antibodies (mAb [1:1] and pAb [1:100]) at room temperature for 1 hour in a moist chamber. The samples were then washed three times with 0.5% Tween 20/PBS and incubated with the second antibodies (FITC-conjugated goat anti-mouse or anti-rabbit IgG, Sigma Chemical Co) and Hoechst dye (Sigma) for 30 minutes. After incubation, the samples were washed with 0.5% Tween 20/PBS three times and examined using a fluorescence microscope (Zeiss) or a confocal microscope (Bio-Rad). Immunohistochemistry was performed as follows: the frozen sections (∼7 μm) mounted on glass slides were fixed by 4% paraformaldehyde in PBS at 4°C for 20 minutes. After they were washed with PBS, the sections were incubated with blocking buffer (5% FCS, 1% BSA, and 0.2% Tween 20 in PBS) for 20 minutes at room temperature and then with anti-Csx pAb or preimmune sera (1:1000) in blocking buffer for 1 hour at room temperature. They were washed three times with 0.5% Tween 20 in PBS, followed by incubation with biotinylated anti-rabbit IgG (1:200, Vector) for 1 hour at room temperature. Staining was performed using a Vecta-Stain ABC kit (Vector Laboratories) per the manufacturer’s protocol, with diaminobenzidine plus NiCl2 for the peroxidase substrate. The slides were then counterstained with methyl green to visualize the nucleus.

#### In Situ Hybridization

In order to detect the transcripts of Csx and other *NK-2* family members, transverse sections from 15.5-dpc embryos were hybridized with a cRNA probe that corresponds to the Csx amino acid 97
to 199 including the homeobox, pcDNA3-Csx plasmid was digested with *Pst*I-*Not*I, blunt-ended, and religated. The construct was linearized at the *Bst*EII site to obtain the anti-sense strand under the direction of the SP6 promoter in an in vitro transcription reaction.

In situ hybridization was carried out as described.26 Briefly, paraffin sections were dewaxed and hydrated, then pretreated with proteinase K and acetic anhydride, and hybridized with [35S]CTP-labeled anti-sense riboprobes as described above. After RNase digestion and exhaustive stringent washings, the slides were coated with Kodak NTB2 emulsion followed by exposure for 14 days. The slides were then developed in diluted D-19 (Kodak).

RT-PCR Assays
RNA was extracted from 15.5-dpc embryos and neonatal hearts using the RNA Easy Kit (Qiagen). RNA (1 μg) was used for the RT with random hexamers using the GeneAmp RNA PCR Kit (Perkin Elmer). Then RT products were used for PCR reaction with Csx-specific primers (forward, 5'-CCCCAAGTGCTCTCCTGTTC-3'; reverse, 5'-ATCTTGACCTGCGTGGACGTGAGC-3'; 314 bp) and with GAPDH primers (forward, 5'-TTCATTGACCTCAACTACAT-3'; reverse, 5'-GTGGCAGTGATGGCAGTGAC-3'; 450 bp). To exclude the PCR products amplified from the genomic DNA, Csx primers were designed to span one intron. After electrophoresis, PCR products were transferred onto the Nylon membrane and then hybridized with the [32P]dCTP random-labeled Csx internal probe.

Culture of Mouse Neonatal Cardiac Myocytes
Hearts from ~15 neonatal mice were used for primary culturing on the 6-cm plates using the methods previously described.27

**Results**

**Antibody Characterization**
Antibodies (pAbs and mAbs) were generated against the fusion protein (His)_6-Csx(160-138) and were characterized by immunostaining, Western blotting, and immunoprecipitation of COS7 cells transiently transfected with a Csx expression vector (Figure 1). About 40% of the cells were noted to express Csx protein when stained with the pAb (Figure 1A-a) or the mAb 2D10 (Figure 1A-c). Immunostaining signals colocalized with the Hoechst dye staining (Figure 1A-b and 1A-d, arrows) indicate that Csx is a nuclear protein. When these cells were used for Western blotting, both 2D10 mAb (Figure 1B, lane 2) and pAb (Figure 1B, lane 4) detected a single band at ~37 kDa. Preimmune pAb does not detect this band (lane 6). C, Immunoprecipitation (IP) of cell lysates with mAb (lanes 1, 2, 5, and 6), pAb (lanes 9 and 10), control mouse IgG1 (lanes 3, 4, 7, and 8), or preimmune sera (lanes 11 and 12). An ~37-kDa band corresponding to Csx is detected when the cell lysate from COS cells transfected with Csx was immunoprecipitated with one of the Csx antibodies and detected with the other (lanes 1 and 9). This band is not detected when preimmune serum is used for blotting (lane 5) or when control IgG1 (lane 3) and preimmune sera are used for immunoprecipitation (lanes 11). That this 37-kDa band is specific for Csx is confirmed by the absence of the band in COS cells transfected with the pcDNA3 parent vector (lanes 2, 6, and 10). Bar=10 μm.

**Cxs Antibody Cross-Reactivity With Other NK-2 Family Proteins**
Because the bacterially expressed Csx fusion protein used as the antigen contains the conserved second and third helix of the homeodomain and NK-2–specific domain, we examined whether anti-Csx pAb and mAb cross-react with other members of NK-2 homeoproteins. The homeodomain and NK-2–specific domain of Csx are most closely related to Nkx2.6/Tix.28 We isolated a partial cDNA encoding the putative specific domain of Csx and subcloned it into the pcDNA3 expression vector. After in vitro transcription and translation,
this cDNA produced an ~18-kDa protein, consistent with the expected size (170 amino acids) encoded by this cDNA (Figure 2A, lane 3). When this Nkx2.6/Tix cDNA was transfected into COS cells, no significant signals were detected after immunostaining with anti-Csx pAb (Figure 2B-a), indicating that Csx pAb does not cross-react with Nkx2.6/Tix protein.

We next examined whether Csx Ab cross-reacts with Nkx2.2. We isolated the full-length murine Nkx2.2 cDNA and subcloned it into the pFLAG-CMV-2 expression vector. When FLAG-tagged Nkx2.2 was overexpressed in COS cells, nuclear FLAG signals were observed in ~60% of the cells (Figure 2C-a). In the strongly FLAG-positive cells, we detected Csx-positive staining when the cells were double-immunostained with anti-Csx antibody at the same dilution ratio used for detecting the native Csx protein in situ (Figure 2C-b, arrow). However, it was hard to detect the Csx nuclear staining in the moderately FLAG-positive cells (Figure 2C-b, arrowheads). Thus, in this COS cell overexpression system, Csx pAb weakly cross-reacted with Nkx2.2. In the mouse embryo, Nkx2.2 mRNA is expressed only in the forebrain. However, Csx pAb did not detect any signals in the forebrain, suggesting that it does not cross-react with the native (non-overexpressed) Nkx2.2 in situ (see below). 2D10 mAb, whose recognition epitope was mapped at the carboxyl terminus to the NK-2-specific domain (data not shown), did not cross-react with either Nkx2.6/Tix or Nkx2.2 (data not shown).

**TTF-1/Nkx2.1** mRNA is specifically expressed in the thyroid, bronchial mucosa, lung, and hypothalamus in the murine embryo. Targeted disruption of the TTF-1/Nkx2.1 gene caused severe defects in formation of thyroid, lungs, and hypothalamus, suggesting that TTF-1/Nkx2.1 mRNA is translated in these organs as well. However, Csx pAb did not detect signals in thyroid, lungs, and hypothalamus in 12.5- and 15.5-dpc embryos (see below), suggesting that Csx pAb does not cross-react with TTF-1/Nkx2.1 protein in situ.

The murine Nkx2.3 gene was recently isolated, and its mRNA was detected only in the inner ring of the gut mesoderm, the epithelium of the tongue, and jaw in 14.5-dpc embryos. Csx Ab did not show significant immunostaining in these tissues (see below), suggesting that Csx pAb does not cross-react with Nkx2.3 protein, if one assumes that Nkx2.3 proteins are expressed where its mRNA is expressed. The expression pattern of the Nkx2.4 gene has not been reported yet.

Recently, nksx2.7 cDNA was isolated from a zebra fish, and nksx2.8 cDNA was isolated from the chick embryonic library. However, it is not clear at present whether murine Nkx2.7 and Nkx2.8 homologues exist, since they have not been isolated despite efforts by several laboratories.

Thus, available evidence indicates that Csx pAb does not cross-react with other known members of the murine NK-2 family homeoproteins in situ, although it weakly cross-reacts with some members of the NK-2 family (ie, Nkx2.2) only when overexpressed in COS cells. In the following experiments, we used pAb for immunostaining and Western blotting and 2D10 mAb for immunoprecipitation of tissue samples.

**Expression of Csx in Cardiac Myocyte**

We examined Csx protein expression in primary cultured mouse neonatal cardiac cells. Cultured cardiac cells were stained with anti-Csx Ab (Figure 3A-a). Hoechst dye for nuclear staining (Figure 3A-b), and MF20 antibody to detect...
sarcomeric myosin heavy chain (Figure 3A-c). Csx protein was detected in the nucleus of MF20-positive cardiac myocytes (Figure 3A-a, long arrow), whereas it was undetectable in the MF20-negative cells (Figure 3A-a, short arrows).

Next, we analyzed Csx protein expression in murine fetal hearts (≈12.5 dpc) using Western blotting (Figure 3B). After lysis, each supernatant (embryonic cardiac tissue and noncardiac tissue) was immunoprecipitated with Csx mAb (Figure 3B, lanes 1 and 2) and control mouse IgG1 (Figure 3B, lanes 3 and 4). Csx Ab precipitated a protein with a molecular size of ≈37 kDa from the cardiac lysate (Figure 3B, lane 1) but not from the noncardiac tissue lysate (Figure 3B, lane 2). When control mouse IgG1 was used for immunoprecipitation (Figure 3B, lane 3), 37-kDa protein was not detected. Csx protein expression was also detected in neonatal mouse hearts when a stronger detergent such as 0.1% SDS was used (data not shown).

Expression of Csx in Cardiogenic Plate and Primitive Pharyngeal Endoderm
In the transverse sections of an ≈7.8-dpc embryo (Figure 4), Csx protein was detected in the nucleus of two cardiogenic plates at the anterolateral areas. These cardiogenic plates are separated in the caudal regions (Figure 4b through 4d) but fuse together in the midline at the cranial end (Figure 4a). Higher magnification clearly identified two morphologically distinct layers of Csx-positive cells in each cardiogenic plate (Figure 4b’ and 4c’). Csx was present in both the precardiac mesoderm, characterized by larger columnar nuclei, and the pharyngeal endoderm cells, identified by their smaller rounder nuclei.

Csx Expression During Heart Development
At 9.0 dpc (Figure 5a), the myocardial wall was uniformly stained with Csx Ab, except for the endocardium (marked by an asterisk). By 10.5 dpc (Figure 5b), the endocardial cushions were clearly visible but were not stained with Csx Ab. By 15 dpc, both the heart and the vascular system have completed their basic structural development. In the 15.5-dpc murine heart, Csx-positive cells were restricted to the myocardium (Figure 5c). Since Csx mRNA is expressed in the sinus venosus, the sinus venosus derivatives in the structurally complete heart were examined for Csx expression. The inferior vena cava and venous valve (VC and VV in Figure 5c), which are derived from the right sinus venosus, did not have significant levels of Csx protein expression.

In the neonatal heart (Figure 5d), both atrium and ventricle were positively stained with Csx Ab. However, the endocardial cushion derivatives, such as the tricuspid valve (Figure 5e) and atrioventricular bundles (Figure 5f), were negative for Csx staining. Epicardium (Figure 5g), which is derived from the mesothelium on the external surface of the sinus venosus, did not express Csx. The vascular structure, including the aorta (Figure 5c and 5d) and small coronary vessels, was negative for Csx expression (blue nuclei in Figure 5h, arrows). In the adult heart, we noted Csx expression in the nuclei of myocytes; however, endocardial cells protruding into the lumen were negative for Csx staining (Figure 5i, large arrows). As shown in Figure 5e through 5i, Csx Ab lightly stained the cytoplasm of cardiac myocytes. This is likely to be nonspecific staining because the preimmune sera also lightly stained the cardiac cytoplasm (Figures 5j and 6d) but never stained the nuclei (Figure 5j, arrows).

Csx Expression in Tongue and Cranial Skeletal Muscles
At 10.5 dpc, Csx expression was observed in the center of the developing tongue primordium (Figure 6a and 6a’) before differentiation of tongue muscle cells. At 15.5 dpc, Csx expression was clearly observed in three layers of the intrinsic muscle and in the extrinsic muscle as well (Figure...
The staining intensity was comparable to that noted in the heart at this stage. In the newborn mouse tongue, Csx-positive cells were observed under the epithelium (Figure 6c). Faint stainings in the cytoplasm of the tongue muscle cells and the desquamating epithelium (Figure 6c) were probably nonspecific, since it was seen only after long incubation with substrate. Its expression markedly decreased in the adult mouse tongue: only few positive nuclei were observed after long incubation with the substrate (data not shown). When the preimmune serum was used, the 15.5-dpc

Figure 5. Csx expression during heart development. a, In sagittal sections of heart at 9.0 dpc, Csx is detectable in the primitive atrium (A), ventricle (V), and bulbus cordis (BC). Inner layer cells (*endocardium) do not express Csx. b, In the sagittal section of a 10.5-dpc embryo, endocardial cushions (ECs [atrioventricular and aorto-copulmonary septal]) are clearly visible but are negative for Csx staining. c, In the sagittal section of a 15.5-dpc embryo, the trabeculated right ventricle (RV) and right atrium (RA) are positively stained. Inferior vena cava (VC), venous valve (VV), atrioventricular bulbar cushion tissue (EC), and aorta (AO) are not stained. d, In the sagittal section of the neonatal heart, Csx is expressed in RA, RV, and left atrium and ventricle (LA and LV, respectively, e through h. Negatives for Csx staining are the tricuspid valve (TV) (e), atrioventricular bundle (AV) (f), endocardium (arrows) (g), and coronary vessels (short arrows) (h). i, In an adult heart section, flat nuclei of cardiac myocytes are positively stained with Csx Ab (short arrows); however, endocardium does not express Csx protein (long arrows). j, Preimmune serum lightly stains the cardiac cytoplasm but does not stain the nuclei (arrows). Bar = 100 μm.

Figure 6. Csx expression in tongue. In the sagittal section of a 10.5-dpc embryo, Csx-positive cells (arrowheads) are localized to the center of the first brachial arch (a and a'). Staining is weaker than that noted in the heart (H). At 15.5 dpc (b, b', and b''), Csx expression is clearly observed in three layers of the tongue under the epithelium (Ep). The staining intensity is comparable to that noted in the heart (H) and anterior larynx (A, La) (see Figure 8). The number of Csx-staining cells has decreased by the newborn stage (c). As a negative control, preimmune serum is used for staining the 15.5-dpc embryo (d). We did not detect the tongue Ep nuclear staining where Nkx2.3 is expressed. Bar = 100 μm.
embryonic tongue did not show any detectable staining, whereas moderate background cytoplasmic staining was detected in the heart (Figure 6d).

Since tongue muscle is derived from occipital somite, we examined whether Csx is expressed in other cranial skeletal muscles. We readily detected the Csx-positive staining in extraocular muscles, pterygoid muscle, and occipital muscles (Figure 7A). To confirm the localization of Csx in these skeletal muscles, we performed double-immunostaining analysis with Csx Ab and MF20 anti–sarcomeric myosin heavy chain antibody using confocal microscopy. As shown in Figure 7B, Csx-expressing cells (Figure 7B-a) were colocalized with a subset of the MF20-positive skeletal muscle cells. When FITC-Csx Ab staining is merged with MF20 antibody rhodamine signals, they changed into yellowish green (c), indicating that Csx localized to the cytoplasm. Csx mRNA expression is detected by RT-PCR. We used the tail RNA (lane 1) as a negative control and the neonatal heart RNA (lane 6) as a positive control. Bar=100 μm.

Figure 7. Csx-positive staining (arrows) in cranial skeletal muscle. A, In the transverse section of the 15.5-dpc embryo at the eye level, Csx-positive signals are detected in extraocular muscles (EOM) (a and a’), pterygoid muscles (Ptery) (a), and occipital muscle layers (Occi) (b, b’, and b”). B, Serial section was double-immunostained with Csx Ab (a) and MF20 antibody (b) and then examined under the confocal microscopy. Csx-expressing cells were colocalized with a subset of the MF20-positive skeletal muscle cells. When FITC-Csx Ab staining is merged with MF20 antibody rhodamine signals, they changed into yellowish green (c), indicating that Csx localized to the cytoplasm. C, In the tissues containing Occi (lane 2), back muscles (lane 3), tongue (lane 4), and liver (lane 5) from 15.5-dpc embryo, Csx mRNA expression is detected by RT-PCR. We used the tail RNA (lane 1) as a negative control and the neonatal heart RNA (lane 6) as a positive control. Bar=100 μm.

Csx Expression in Anterior Larynx

At 11.5 dpc, Csx-positive cells were localized at the anterior larynx (Figure 8a and 8b) ventral to the larynx (asterisks in Figure 8). At 15.5 dpc, strong Csx expression was still observed (Figure 8c and 8d), although it was restricted between the epithelium of the larynx (arrow upper right of asterisk in Figure 8c and 8d) and the layer of sternohyoid muscle (SH in Figure 8c and 8c’). These Csx-positive cells were compact and roundish, suggesting that they are mesenchymal cells. In the transverse section (Figure 8e and 8e’), Csx-expressing cells (double asterisks in Figure 8e) were localized anterior to the lumen of the larynx (asterisk in Figure 8e), adjacent to the chondrified lateral thyroid cartilage (LTC in Figure 8e’). The thyroid glands localized laterally between the larynx and esophagus at this stage were negative for Csx (Figure 8e). To confirm the localization of thyroid primordia, we performed in situ hybridization using the Csx homeobox cRNA probe, which cross-hybridized with several NK-2 family genes, including TTF-1/Nkx2.1, a tissue-restricted marker for embryonic thyroid. In addition to the strong Csx hybridization signals in the anterior larynx (double asterisks in Figure 8f), bilaterally localized thyroid glands were visualized between the larynx and esophagus (arrowheads in Figure 8f). Laryngeal mucosa and several laterally localized tissues in the anterior larynx were also detected with this homeobox probe. Furthermore, we detected other hybridizing signals in the frontal part of the same section, such as the primordium of the follicle vibrissa (Figure 8g), which may correspond to Nkx2.3 mRNA signals. However, we did not detect Csx Ab staining in these areas (Figure 8h). Thus, Csx Ab does not seem to cross-react with at least TTF-1/Nkx2.1 and Nkx2.3 in situ. Additionally, in 10.5-, 12.5-, 13.5-, and 15.5-dpc embryos, we could not detect Csx Ab staining in the hypothalamus (where TTF-1/Nkx2.1 mRNA is
expressed) or in the forebrain (where Nkx2.2 mRNA is expressed) (data not shown).

Csx Expression in Spleen and Liver
In Figure 9a, inferior to the stomach, a ribbon-like spleen was clearly stained with Csx Ab at 12.5 dpc. In this section, almost all cells in the spleen express Csx (Figure 9a'). At this stage of embryo, the spleen has just formed as a mesenchymal aggregate, originating from the celomic mesothelium within the dorsal mesentery of the stomach. In the neonatal spleen (Figure 9b), Csx-positive cells were sparsely distributed because of the blood cells situated in a network of mesenchymal cells. By 2 weeks after birth, only a few cells around the pulp expressed Csx (Figure 9c). No signals were detected in the neonatal spleen with the preimmune sera (data not shown).

We also detected Csx-positive cells sparsely localized in the liver caudal to the heart in a 12.5-dpc embryo (Figure 10A-a and 10A-b) and in a 15.5-dpc embryo (Figure 10A-c). This staining pattern was not observed with the preimmune sera (data not shown). Csx protein was not detectable in the adult liver (data not shown).

Csx Expression in Stomach
At 11.5 dpc, Csx-positive cells surrounded the mucosal layer of the distal part of stomach (Figures 10B-a and 10B-a'). However, the body of the stomach adjacent to this Csx-positive area (ST in Figure 10B-a) was negative for Csx. At 13.5 dpc (Figure 10B-b), the number of Csx-positive cells was decreased and localized at the inferior part of the distal stomach. These cells might be mesenchymal cells within the dorsal mesentery. Csx seems to be transiently expressed only during the fetal stage of stomach formation, since we could not detect Csx protein expression in the neonatal stomach (data not shown).
Discussion

Csx/Nkx 2.5 or its homologous genes are one of the earliest markers of the cardiac primordium in Drosophila, zebrafish, Xenopus, chick, mouse, and human. The conservation of Csx/Nkx2.5 among these diverse species strongly suggests that Csx/Nkx2.5 plays a fundamental role in heart development in all species and may play other roles in extracardiac tissues. The present study is the first to examine Csx protein expression at the single-cell level in all major organs from 7.8 dpc to the adult stage. All previous studies were performed at the mRNA level.

Among homeobox genes, mRNA expression does not always correspond to protein expression because of posttranscriptional regulations. In fact, we were unable to detect Csx protein in thyroid and in NIH 3T3 cells, whereas Csx/Nkx2.5 mRNA is expressed in these cells (Reference 9 and authors’ unpublished data). On the other hand, we can clearly detect cardiac expression of nuclear-localized Csx protein in all stages of heart development: from the cardiac primordium to the adult heart.

Because of the embryonic lethality observed in Nkx2.5/Csx-targeted mice, the role of Csx/Nkx2.5 in the later stages of cardiac development has not been defined. However, it is possible that this transcription factor may be necessary for maintaining differentiation of cardiac myocytes. Csx protein is not detectable in the valvular tissues (derivatives of the endocardial cushion), the endocardium, the epicardium (a derivative of mesothelial cells derived from the splanchnopleuric mesoderm), or the coronary vasculature (the same origin as the epicardium). In addition, using primary cultured cardiac cells, we demonstrated that Csx/Nkx2.5 is expressed only in the cardiac myocytes and not in the nonmyocytes. In this respect, Csx differs from other cardiac-restricted transcription factors. In addition to their expression in cardiomyocytes, GATA-4 protein is also expressed in the endocardium and endocardial cushion tissues, and MEF2 proteins are expressed in vascular smooth muscle cells. Several lines of evidence suggest that the endoderm plays an important role in early heart specification. Secreted factors, such as bone morphogenic proteins, induce precardiac mesoderm differentiation. In this early phase of cardiac development, the adjacent endoderm also expresses Csx. Thus, it is possible that Csx expression is regulated by these secreted factors in both endoderm and mesoderm.

We also noticed that during the fetal stages of development, Csx protein is readily detected in the spleen, distal stomach, and tongue, which is similar to the Nkx2.5 mRNA expression pattern described by Lints et al. In our previous study, we did not detect significant extracardiac expression of Csx mRNA, probably because of the lower signal-to-noise ratio of the oligonucleotide probe used for in situ hybridization studies. In contrast to the mRNA study by Lints et al., we did not detect Csx protein expression in the thyroid but detected Csx protein in the anterior larynx, liver, and a subset of cranial skeletal muscles. Detection of Csx proteins in these tissues is either due to a higher sensitivity and better spatial resolution of the antibody staining compared with mRNA in situ hybridization or due to posttranslational regulation of the protein expression in these tissues.

The Csx Abs that we raised seem specific for Csx/Nkx2.5 in situ, as available evidence suggests that it does not...
cross-react with murine TTF-1/Nkx2.1, Nkx2.2, Nkx2.3, and Nkx2.6/Tix9 in tissue sections. However, Csx pAb weakly cross-reacted with Nkx2.2 when overexpressed in COS cells. Therefore, it is possible that some of the Csx protein signals that we detected might come from other, as-yet-unidentified, NK-2 family proteins if they were expressed at very high levels. It is not known whether there are murine homologues of zebra fish nksx2.7 and chick Csx2.8, 11

One of the unexpected findings of the present study is that we detected Csx protein signals in a subset of cranial skeletal muscles, such as extraocular muscle, pterygoid muscle, and occipital muscle. Interestingly, the staining was localized not in the nucleus but rather in the cytosol (Figure 7B). Although functional significance of this observation remains to be determined, to our knowledge, this is the first example of differential localization of a homeoprotein in the vertebrate. The cytoplasmic localization might be one of the regulatory mechanisms of Csx transcriptional activity, or it might play a role in the cytosol, like the Drosophila homeobox protein bicoid. 22,43 Also, Csx Ab staining seems not uniform even in the same muscle bundle (Figure 7B). Thus, Csx antibody might be detecting a distinct subset of cells that may express a different set of genes. Interestingly, in the extraocular muscles and jaw muscles, expression of the cardiac α-myosin heavy chain gene has been reported at both the mRNA and protein levels.44,45

Shiojima et al36 reported an alternatively spliced form of Csx that would encode a carboxyl-terminally truncated protein without the homeodomain. This alternatively spliced product is reported to be transcriptionally active in a transient transfection assay. Our initial report on the murine Csx cDNA had an amino terminus8 different from that of Nkx2.5 cDNA reported by Lints et al. 9 The subsequent analysis of some 40 additional Csx cDNA clones has indicated that the different 5′ end in our original clone was most likely due to a recombination artifact, and there was no evidence for alternatively spliced mRNA that would change the Csx protein coding region (K. Lang, D. Turbay, and authors’ unpublished data). Thus, in the mouse, different tissues seem to express the same protein, and a posttranslational mechanism is likely to be operative for the differential Csx protein localization.

We found that Csx protein is strongly expressed at 15.5 dpc in the anterior larynx adjacent to the chondrified thyroid cartilage.ucci et al46 have reported that in humans, a single median mesenchymal tissue forms all structures along the midline of the thyroid cartilage, including intermediate lamina of the thyroid cartilage, Broyles’ ligament, insertion fibers of vocal muscles, and connective tissues. Chondrification of intermediate lamina of thyroid cartilage proceeds between 11 and 20 weeks in human embryos until the cartilage becomes continuous with the two lateral thyroid cartilages (LTC in Figure 8), which have already chondrified by 7 to 8 weeks.46 If a similar process applies to the formation of the mouse anterior larynx, the tissue strongly positive for Csx in Figure 8 is likely to be a mesenchymal band that will form the intermediate lamina of thyroid cartilage, ligament, connective tissues, and fibers. Interestingly, in contrast to other pharyngeal arch cartilages that develop from the neural crest of the midbrain and hindbrain regions, the cartilages of arch 4 and 6 (thyroid cartilage and cricoid cartilage) develop from the lateral plate mesoderm.31 We could not verify Csx protein expression in the thyroid primordium, where mRNA was reported to be expressed,5 since the thyroid primordium is localized in the anterior larynx and is difficult to separate from the mesenchymal band at 11.5 dpc. However, at 15.5 dpc, Csx protein is not expressed in the laterally localized thyroid glands. It is possible that Csx mRNA expression in the thyroid is transient at the early stage of embryo or that Csx mRNA is not translated in thyroid glands.

To date, the function of Csx/Nkx2.5 in the extracardiac tissues has not been determined. In the Nkx2.5-targeted mice, death occurred before these tissues had been fully formed.4 Thus, it is impossible to study the function of Csx in these tissues using the conventional gene-targeting approach. Csx seems to be expressed in the early developmental stages of the mesodermal derivatives, except for the early endoderm expression adjacent to the precardiac mesoderm. It is possible that Csx might determine the subdivision of cell fate, cell growth, cell movement, or the localization of the already specified cells in these tissues. It is also possible that the role Csx plays may be different among different tissues. It will be interesting to study the Csx/Nkx2.5 function in these extracardiac tissues by rescuing the cardiac lethality of the Csx/Nkx2.5-targeted mouse.

In summary, we have described the developmental pattern of Csx protein expression using immunohistological technique. Csx protein is present in nucleus of the myocardial cells throughout the cardiac development and transiently in several other tissues or a subset of cells in a given tissue. In contrast, Csx protein is localized to the cytoplasm of the developing cranial skeletal muscle. Further studies are necessary to determine the functions of Csx/Nkx2.5 in cardiac and noncardiac tissues.

Acknowledgments

This study was supported in part by a National Institutes of Health grant (Dr Izumo) and a Research Fellowship from the American Heart Association, Michigan Affiliate, Inc (Drs Kasahara and Tanaka). Dr Schinke is supported by Deutsche Forschungs Gemeinschaft. We thank M.W. Russell for a critical review of the manuscripts and E. Nabel and D. Fox for their help in tissue section and monoclonal antibody production.

References

Csx/Nkx2.5 Protein Expression


Cardiac and Extracardiac Expression of Csx/Nkx2.5 Homeodomain Protein
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Circ Res. 1998;82:936-946
doi: 10.1161/01.RES.82.9.936

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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