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Gene expression pattern

Transient cardiac expression of the *tinman*-family homeobox gene, *XNkx2-10*

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Abstract

In *Drosophila*, the *tinman* homeobox gene is absolutely required for heart development. In the vertebrates, a small family of *tinman*-related genes, the cardiac NK-2 genes, appear to play a similar role in the formation of the vertebrate heart. However, targeted gene ablation of one of these genes, *Nkx2-5*, results in defects in only the late stages of cardiac development suggesting the presence of a rescuing gene function early in development. Here, we report the characterization of a novel *tinman*-related gene, *XNkx2-10*, which is expressed during early heart development in *Xenopus*. Using in vitro assays, we show that *XNkx2-10* is capable of transactivating expression from promoters previously shown to be activated by other *tinman*-related genes, including *Nkx2-5*. Furthermore, *Xenopus* Nkx2-10 can synergize with the GATA-4 and SRF transcription factors to activate reporter gene expression.

Keywords: *Nkx*; *tinman*; Heart; Pharyngeal endoderm

1. Results and discussion

Somewhat unexpectedly, mice lacking Nkx2-5 function are able to form beating heart tissue and the majority of myocardial differentiation markers are expressed normally (Lyons et al., 1995). This result suggests that additional *tinman*-related genes expressed in the developing heart may partially substitute for Nkx2-*5* function in the knock-out mouse (Lyons et al., 1995). Using low stringency screening we have identified *XNkx2-10*, a new member of the vertebrate *tinman*-related gene family (Fig. 1A). The XNkx2-10 protein is 263 amino acids long, shorter than the *Xenopus* homologues of *XNkx2-3* (338 amino acids) and *XNkx2-5* (299 amino acids) and closer in length to the zebrafish *nkx2-7* coding region (267 amino acids). The XNkx2-10 homeodomain sequence differs from all previously described Tinman-related proteins (Fig. 1B). Specifically, the methionine and glutamine residues located at homeodomain positions 11 and 15, respectively, are not present in any other Tinman family proteins (Harvey, 1996). Indeed, the XNkx2-10 homeodomain differs at ten positions from chicken cNkx2-8, at nine positions from XNkx2-5, at six positions from XNkx2-3 and at five positions from zebrafish Nkx2-7. Outside of the homeodomain, XNkx2-10 contains both a TN domain and an NK-2 Specific Domain related to those in other Tinman-family proteins. The primary sequence therefore suggests that *XNkx2-10* is a novel member of the vertebrate *tinman* gene family.

By RNase protection assay (Fig. 1C) *XNkx2-10* is first transcribed in the early neurula stage embryo. This is later than the onset of *XNkx2-5* expression which occurs at the mid-gastrula stage in *Xenopus* (Tonissen et al., 1994). By whole-mount in situ hybridization, early expression of *XNkx2-10* closely resembles that of *XNkx2-3* and *XNkx2-5*. *XNkx2-10* transcripts are first visible at the late neurula stage in a position consistent with the location of precardiac tissue (Fig. 2A). By the early tailbud stage, cardiac precursor cells

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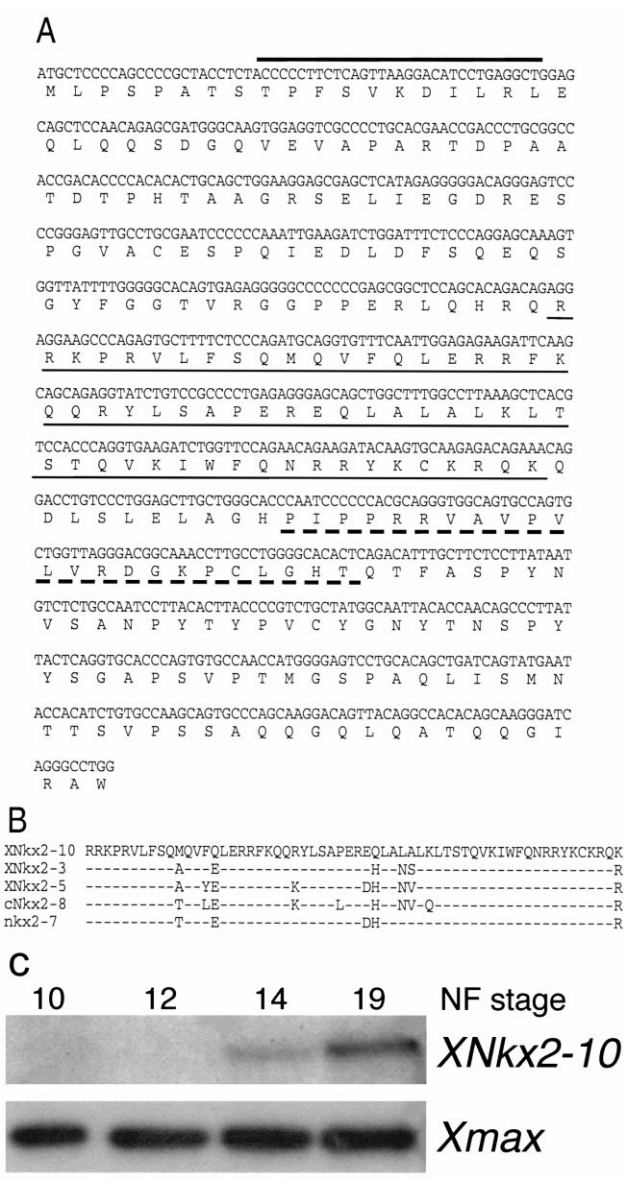

Fig. 1. (A) DNA sequence of the *XNkx2-10* gene. The coding region of *XNkx2-10* and the derived protein sequence are shown. Portions of this sequence were previously reported in Newman and Krieg (1998), where the gene was called Nkx2-9. The conserved TN, homeo- and NK-2 Specific domains are highlighted by overlines, underlines and dash underlines respectively. The GenBank accession number is AF127224. (B) Comparison the homeodomain sequences of cardiac NK-2 genes from frog, chick and zebrafish (Tonissen et al., 1994; Evans et al., 1995; Lee et al., 1996; Boettger et al., 1997; Brand et al., 1997; Reecy et al., 1997). (C) RNase protection assay to determine the onset of *XNkx2-10* gene expression. RNA samples from early (stage 10.5) and late (stage 12) gastrula and early (stage 14) and late (stage 19) neurula were assayed. The *XNkx2-10* probe consisted of the final 310 nt of the coding region. The ubiquitous *Xmax* gene (Tonissen and Krieg, 1994) is used as a loading control.

and also the overlying anterior pharyngeal endoderm are *XNkx2-10* positive (Fig. 2B,C,F,G). At stage 28, myocardial precursor cells initiate the expression of cardiac differentiation markers (Chambers et al., 1994; Drysdale et al., 1994). It is at this stage, that *XNkx2-10* expression begins to decrease in the forming myocardium (Fig. 2D,H). In contrast, both *XNkx2-3* and *XNkx2-5* transcripts remain abundant in the developing heart throughout embryonic development (Fig. 2E,K) (Tonissen et al., 1994; Evans et al., 1995; Cleaver et al., 1996). Whereas cardiac expression of *XNkx2-10* decreases in the mid-tailbud stage embryo, transcripts remain extremely abundant in the pharyngeal endoderm (Fig. 2D,E). *XNkx2-10* expression decreases in the pharyngeal endoderm and becomes undetectable by the late tadpole stage (stage 42). PCR analysis fails to detect Nkx2-10 expression in any adult tissue tested (heart, spleen, kidney, liver, lung, pancreas and skeletal muscle).

We have carried out preliminary experiments to determine the transcription regulating properties of Nkx2-10. First, we tested for transactivating ability by co-transfecting variable amounts of a plasmid containing *XNkx2-10* sequences, together with the 3× (A20)-TATA-LUC construct (Chen and Schwartz, 1996), into the CV-1 cultured cell line. In parallel experiments, *XNkx2-10* sequences were co-transfected with the minimal cardiac actin promoter construction. As shown in Fig. 3A, low levels of XNkx2-10 produce little effect, but higher levels cause a significant increase in reporter gene activity over background. In the case of 3× (A20)-TATA-LUC a 14-fold increase is observed and for Ca SRE1-TATA-LUC the stimulation is 7-fold. The ability of XNkx2-10 to co-operate with other transcription factors was also tested. As shown in Fig. 3B, transfection of 150 ng of *XNkx2-10* plasmid alone, results in a less than 2-fold increase in Ca SRE1-TATA-LUC reporter gene activity, and transfection with the MADS class transcription factor, SRF, results in a 6-fold increase in luciferase activity (Fig. 3B). However, co-transfection of both XNkx2-10 and SRF leads to a 17-fold activation of luciferase activity. Similarly, expression of GATA4 alone results in only a slight increase in luciferase activity, while co-transfection of both GATA4 and XNkx2-10 leads to a 4-fold increase (Fig. 3B). These studies indicate that XNkx2-10 functions as a weak transcriptional activator and this activity is synergized by co-operation with other cardiac-expressed transcription factors. Overall, Nkx2-10 is a weaker activator than Nkx2-5, but approximately equivalent to Nkx2-8 when tested in identical assays. For comparison, Nkx2-5 co-transfection with GATA-4 increases Ca SRE1-TATA-LUC reporter activity approximately 15-fold while Nkx2-8 plus GATA-4 results in an approximately 2-fold increase (Sepulveda et al., 1998).

We have also tested the effects of *XNkx2-10* expression in the *Xenopus* embryo. Although previous studies indicate that over-expression of either *Nkx2-3* or *Nkx2-5* in the *Xenopus* embryo results in enlarged hearts (Chen et al., 1996; Cleaver et al., 1996), we have been unable to replicate this effect using Nkx2-10. Although one must be cautious with the interpretation of negative results, these experiments suggest that the biological activities of Nkx2-5 and Nkx2-10 are not identical.

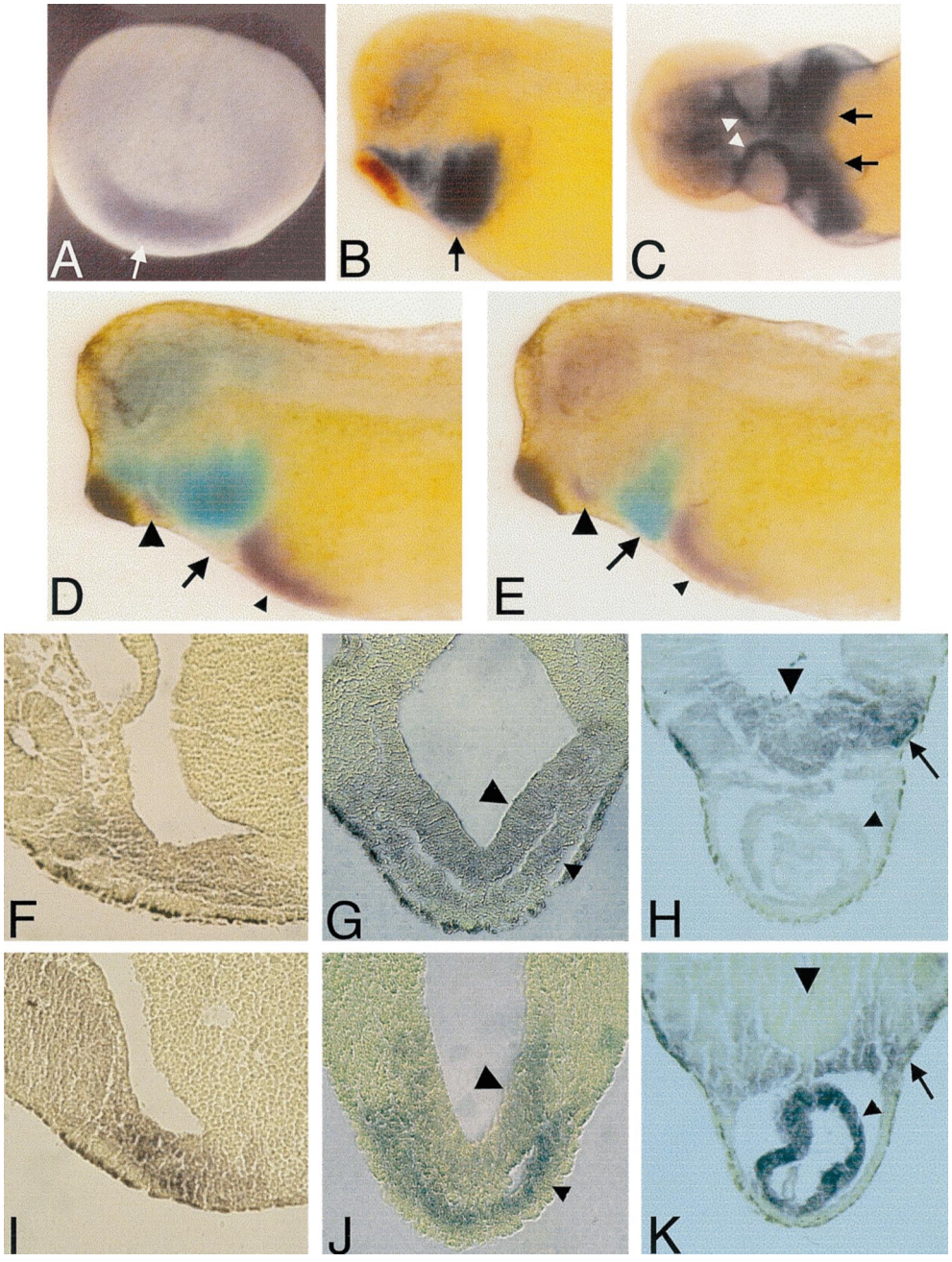

Fig. 2. Expression pattern of *XNkx2-10* by wholemount in situ hybridization. (A) Anterior view of a late neurula stage embryo showing *XNkx2-10* expression in endodermal and mesodermal cells (arrow) adjacent to the neurual tube. (B) Lateral view of an early tailbud embryo showing *XNkx2-10* transcripts in the pharyngeal endoderm and cardiac primordia (arrow). (C) Ventral view of the embryo in (B) showing both cardiac (black arrows) and pharyngeal (white arrowheads) staining. (D) Mid tailbud embryo (stage 32) stained for both *XHex* (purple), which marks the developing liver (Newman et al., 1997) and *XNkx2-10* (blue). The heart (arrow), directly anterior to the liver (small arrowhead) is negative for *XNkx2-10* at this stage. Note the extensive pharyngeal staining, extending anteriorly past the forming thyroid (large arrowhead). (E) Mid tailbud embryo (stage 32) stained for both *XHex* (purple) and *XNkx2-5* (blue). Compare with (D) and note the strong cardiac staining (arrow). (F–H) Sections of embryos stained for *XNkx2-10*. (I–K) Sections of embryos stained for *XNkx2-5*. (F and I) Parasagittal section through an early tailbud stage (stage 22) embryo showing expression of both *XNkx2-10* and *XNkx2-5* in the endoderm and the mesoderm. (G and J) Cross-section through a tailbud stage embryo (stage 26) showing transcripts in both the pharyngeal endoderm (large arrowhead) and cardiac mesoderm (small arrowhead). (H and K) Cross-section through a late tailbud stage embryo. Note the lack of *XNkx2-10* expression in the folded heart (small arrowhead) and the lack of *XNkx2-5* expression in the pharyngeal endoderm (large arrowhead). Arrows highlight expression of both *XNkx2-5* and *XNkx2-10* in the mesoderm immediately dorsal to the heart. In situ analysis was performed as described by Harland (1991).

2. Experimental procedures

Approximately 10^6 plaques of a *Xenopus* stage 42 tadpole cDNA library were screened at low stringency with the coding region of the chicken *cNkx2-8* gene. All filters were washed in 5× SSC/0.1% SDS at 37°C. RNase

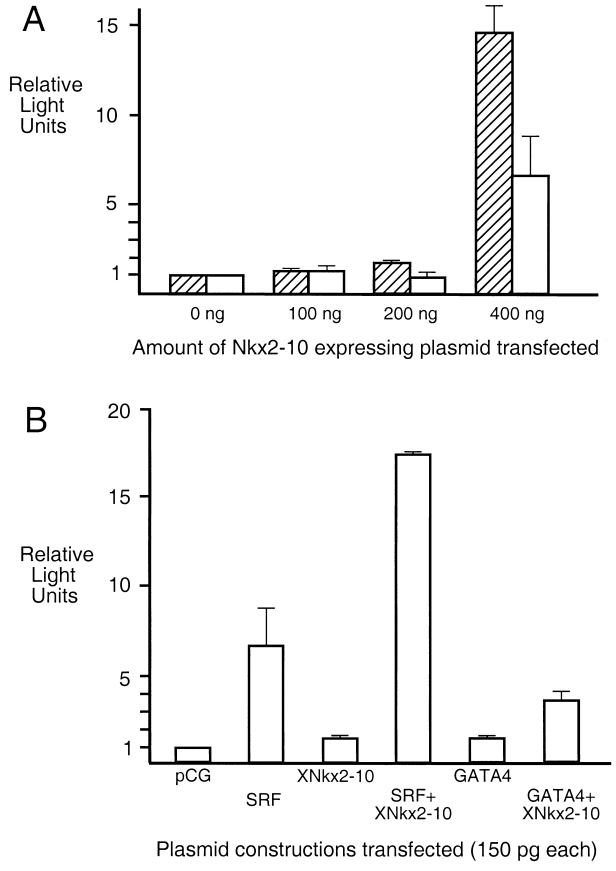

Fig. 3. Transcription regulation properties of XNkx2-10. (A) Relative light units after transfection of cultured cells with varying amounts of *XNkx2-10* plasmid. The reporter construction was either 3× (A20)-TATA-LUC (solid bars) or Ca SRE1-TATA-LUC (open bars). Activation is assessed relative to the control experiment using pCG vector alone (indicated as 0 ng). The mean and standard deviation of two independent experiments are presented. (B) Relative activation of transcription of the Ca SRE1-TATA-LUC reporter construction, following transfection with combinations of plasmids. In all cases, 150 ng of DNA encoding the transcription factor was transfected into the cultured CV1 cells. The mean and standard deviation of two independent experiments are presented.

protection analysis was performed as described by Krieg and Melton (1987) using ten embryo equivalents of total RNA.

For transcription studies, CV-1 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. Proliferating CV-1 fibroblasts were transfected using Lipofectamine (Gibco), with a total of 2.5 μg of DNA, consisting of 3× (A20)-TATA-LUC or Ca SRE1-TATA-LUC (1 μg; Chen and Schwartz, 1995; Chen et al., 1996) in the presence or absence of pCS2-XNkx2-10 and/or pCGN-SRF (150 ng, gift of Dr. Ron Prywes). All transfections were balanced for a constant amount of pCGN control vector. After 6 h at 37°C, 3 ml of DMEM containing 3% horse serum and 15 μg/ml of insulin was added to the transfected cells, which were then incubated for an additional 60 h. Cells were washed with phosphate buffered saline and lysed with 400 μl of Reporter Lysis Buffer (Promega). Lysates were analyzed for luciferase activity as described previously (Reecy et al., 1997).

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