Common arterial trunk associated with a homeodomain mutation of NKX2.6

Kirsten Heathcote1, Claire Braybrook2, Lulu Abushaban3, Michelle Guy1, Maher E. Khetyar1, Michael A. Patton1, Nicholas D. Carter1, Peter J. Scambler2,* and Petros Syrris1

1Department of Clinical Developmental Science, St George’s Hospital Medical School, London, SW17 0RE, UK, 2Molecular Medicine Unit, Institute of Child Health, London WC1N 1EH, UK and 3Department of Cardiology, The Chest Hospital, Kuwait City, 13041 Safat, Kuwait

Received October 19, 2004; Revised December 10, 2004; Accepted January 4, 2005

Persistent truncus arteriosus (PTA) is a failure of septation of the cardiac outflow tract (OFT) into the pulmonary artery and the aorta. A common arterial trunk (CAT) is often diagnosed as PTA in the absence of evidence of embryological mechanism. We have used autozygosity mapping of a large consanguineous family segregating CAT to map the causative locus to chromosome 8p21. An F151L mutation was identified in the homeodomain of NKX2.6, a transcription factor expressed in murine pharyngeal endoderm and embryonic OFT myocardium. Although expression of Nkx2.6 during murine embryogenesis is strongly suggestive of a role for this gene in heart development, mice homozygous for a targeted mutation of Nkx2.6 are normal. However, in these mice, it has been shown that Nkx2.5 expression expands into regions lacking Nkx2.6, suggesting functional complementation. As transcriptional targets of NKX2.6 are unknown, we investigated functional effects of the mutation in transcriptional and protein interaction assays using NKX2.5 as a surrogate. Introduction of F157L into human NKX2.5 substantially reduced its transcription activating function, its synergism with partners at the atrial natriuretic factor (ANF) and connexin-40 (Cx40) promoters and its specific DNA binding. We tested NKX2.5 target promoters for NKX2.6 activity. NKX2.6 was inactive at ANF but weakly activated transcription of a Cx40 promoter, whereas the F151L mutant lacked this activity. These findings indicate a previously unsuspected role for NKX2.6 in heart development, which should be re-evaluated in more sophisticated model systems.

INTRODUCTION

Common arterial trunk (CAT) is a rare congenital heart anomaly accounting for 1% of congenital heart defects (1). The neonate is well at birth but without surgery heart failure develops, and untreated infants rarely survive beyond 6 months of life. Surgical advances have led to much improved survival and quality of life in recent years (2), although even after surgery, CAT is classified as a severe lesion (3). The defect is attributed to incomplete septation (persistence) of the truncus arteriosus (PTA), which is the distal portion of the outflow tract (OFT) of the embryonic heart tube. Septation occurs when mesenchymal cell proliferation causes endocardial ridges to form within the OFT during the fifth week of human development, and cardiac neural crest cells migrate through the primordial pharynx and pharyngeal arches to populate these ridges during the sixth week; this invasion may initiate the fusion of these ridges to form a septum (4). This spiralling aorticopulmonary septum divides the OFT into the two great vessels, which arise from the heart. If this process is deficient, OFT defects occur. These congenital abnormalities may occur as isolated anatomical defects such as PTA, tetralogy of Fallot or double outlet right ventricle, or as part of defined syndromes such as deletion of 22q11 (DiGeorge and velocardiofacial syndromes) (5).

Several mouse mutations have been associated with PTA. Mutations in transcription factors (e.g. Pax3, cited2, AP2a, Pitx2) and signalling proteins (e.g. sema3C, BmprII, Alk2, Dvl2) highlight the importance of the neural crest contribution (6) and laterality (7) to OFT septation. However, recent work has drawn attention to the role of the pharyngeal endoderm and anterior (secondary) heart field in OFT morphogenesis (8).

The anterior heart field comprises a population of splanchnic mesoderm cells which contribute to OFT and right ventricular...
myocardium, as marked by the islet-1 LIM-homeodomain transcription factor (9). Tbx1 null mice develop PTA, and conditional mutagenesis has defined two distinct roles for Tbx1 in OFT development, neither of which directly involves the neural crest (10). Tbx1 regulates proliferation of anterior heart field cells destined for the OFT and is also required in Nkx2.5 expressing pharyngeal endodermal cells for septation to occur (10). One role of Tbx1 is to regulate fibroblast growth factor expression during embryogenesis (10), including cells in the OFT (11). Tissue-specific inactivation of Fgf8 using Tbx1-Cre results in OFT defects (12). Tbx1 is hemizygoosly deleted or heterozygously mutated in human patients with the 22q11 deletion syndrome (13). NKK2.5 has also been implicated in human congenital heart defects (14), including atrial septal defects, and it interacts at target promoters with two other transcription factors mutated in patients with cardiac septation defects, TBX5 (mutated in the Holt–Oram syndrome) (15) and GATA4 (16). Recently, it has been shown that Tbx2 is essential for both normal OFT development and patterning of the atrioventricular canal. However, Tbx2 does not appear to be required for an anterior heart field or neural crest contribution, implying mesenchymal cell growth or differentiation defects in the absence of this gene (17). Tbx2 is known to antagonize Tbx5 activity at the atrial natriuretic factor (ANF) promoter (18). Thus, it appears that Nkx, GATA and Tbx transcription factors interact to regulate one another’s activity and are key to normal cardiac morphogenesis.

Despite the rarity of large human kindreds segregating congenital heart defects, human genetic analyses have been important in elucidating some of these pathways. For instance, linkage and mutation analyses in just two families allowed the identification of GATA4 mutations in patients with cardiac septal defects (16). Here, we report linkage analysis of a previously described family comprising six individuals with CAT, where these patients are offspring of double first-cousin marriages. Candidate gene screening identified an amino acid substitution, F151L, at position 20 of the homeodomain of the transcription factor NKX2.6. Functional analysis of this mutation revealed diminished transcriptional activation, and the corresponding mutation of NKX2.5 reduced DNA binding and synergistic activation of target promoters in conjunction with GATA4 and TBX5. These findings indicate a previously unsuspected role for NKX2.6 in cardiac morphogenesis and the importance of the F151 residue for NKX transcription factor activity.

RESULTS

Genetic linkage analysis

The family under study (Fig. 1A) has a history of first-cousin marriages for generations (19). Six affected children have been born to this family, but three have died during surgery to repair the cardiac lesion, therefore it has not been possible to include them in this genetic study. Karyotype analysis excluded 22q11 microdeletion in the affected subjects, the common genetic cause of truncus arteriosus. Extensive phenotyping identified truncus arteriosus type 1 and no other visible abnormalities. As all affected individuals were the progeny of consanguineous unaffected parents, we initiated autozygosity mapping.

An initial screen of DNA from the three affected subjects using a 10 cM density set of microsatellite markers identified homozygosity common to all three samples at four locations. Further genotyping and haplotype analysis of the whole family excluded three of these loci, but consistent inheritance of markers identical by descent (IBD) was still observed on chromosome 8p21. The region IBD flanked marker D8S1771 and defined an interval of ~14 cM as deduced from the Marshfield genetic map (20), between heterozygous markers D8S1734 and D8S1711. Owing to software constraints, we had to analyze the pedigree as three separate nuclear families reducing the power of our analysis. Linkage analysis (HOMOZ) gave maxLOD of 2.9 between D8S1771 and D8S1809. We typed additional microsatellite markers and single nucleotide polymorphisms within our candidate interval, reducing the critical region of homozygosity to ~6 Mb between D8S1734 and D8S1218 (Fig. 1A).

Mutation screening of positional candidate genes

Genes within the 6 Mb interval not previously implicated in human disease and with reported expression in cardiac tissues or with putative roles in heart development were considered positional candidates. Genes including FZD3, FBXO16, BNIP3L, TNFRSF family members and NKK3A were individually sequenced in members of the pedigree with CAT, and no mutations were found when compared with normal DNA sequence. We also sequenced all novel genes in the region predicted by the Ensembl server (21) including the predicted human homologue of Nkx2.6. Nkx2.6 encodes a homeobox transcription factor expressed in murine cardiogenesis (22,23) and as such is a good candidate gene when considering cardiac OFT development.

Sequence analysis of Nkx2.6 detected a c.451T>C nucleotide transition in exon 2 of the gene. All three affected individuals were homozygous for C451, parents of affected children were heterozygous (Fig. 1B and C), and the sequence variant was absent in 100 Kuwaiti, 100 Arab and 250 Caucasian control DNA samples. This novel Nkx2.6 mutation is predicted to substitute phenylalanine for leucine at position 151 in the predicted protein sequence (F151L) and position 20 of the homeodomain (when considering homeodomains, this residue would be referred as F20). The Nk-2 subgroup of the homeodomain family comprises six vertebrate homologues (Nkx2.3, 2.5, 2.6, 2.7, 2.8, 2.9) on the basis of their close structural similarity to tinman, a transcription factor essential for the development of heart-like dorsal vessel in Drosophila (24). The F151 residue is conserved in Nk-2 proteins (23) from at least 10 different species (Fig. 2).

F151L missense mutation impairs transcriptional activity of Nkx2.6 and Nkx2.5

In the absence of known downstream genes or a binding site recognition sequence for Nkx2.6, we used Nkx2.5 as a surrogate in biochemical studies to assess functional consequences of the F151L homeodomain substitution. Nkx2.5 was chosen firstly because of the strong conservation with
NKX2.6, especially throughout the homeodomain (Fig. 2), and secondly because biochemical pathways utilizing NKX2.5 are relatively well characterized. Thirdly, there are in vivo data suggesting Nkx2.5 may compensate for the lack of Nkx2.6 during embryogenesis (see Discussion) and therefore indicating that some DNA binding and transcriptional regulatory activities will be shared.

Transcriptional activity of wild-type NKX2.6 (NKX2.6WT) and wild-type NKX2.5 (NKX2.5WT) was compared with the mutant proteins (NKX2.6 F151L and NKX2.5F157L); F20 of the homeodomain was mutated in each case. Transcriptional activation was assessed by transient transfection assays using a 2.6 kb ANF promoter or 1.1 kb Cx40 promoter upstream of a luciferase reporter (ANF-luc and Cx40-luc).

Over-expression of NKX2.5WT led to activation of both ANF and Cx40 promoters, consistent with previous studies. Addition of wild-type NKX2.6WT had no effect on this activation. However, when equivalent amounts of protein were expressed, NKX2.5F157L displayed less transcriptional activation of both ANF-luc and Cx40-luc when compared with NKX2.5WT (Figs. 3A and 4A). Co-expression of NKX2.5WT and either TBX5 or GATA4 resulted in co-operative activation at the ANF promoter as previous studies have shown (15,25), whereas synergistic transcriptional activation was reduced with co-expression of NKX2.5F157L and TBX5 and lost with co-transfection of NKX2.5F157L and GATA4 (Fig. 3A). NKX2.6WT did not activate the ANF or Cx40 promoter in U2OS cells, but weakly activated the Cx40-luc in H9c2 cardiomyocytes. However, this transcriptional activity was lost with co-transfection of NKX2.6F151L.

F157L missense mutation reduces DNA binding activity of NKX2.5

Random oligonucleotide selection experiments have determined binding site sequences with highest affinity for several Nk-2 proteins including NKX2.5, and in all cases, the binding site conforms to a consensus 5’-T(C/T)AAGTGG-3’ (26). Electromobility shift assay (EMSA) of in vitro translated NKX2.5WT protein efficiently retarded gel migration of radiolabelled probe containing two NKX and one TBX binding sites (NKX-BS) as previously described (27), but NKX2.6WT did not. However, an equivalent amount of NKX2.5F157L protein displayed less binding affinity for NKX-BS probe (Fig. 5A) than NKX2.5WT. Upon co-incubation of equal amounts of wild-type and mutant proteins, the presence of NKX2.5F157L did not affect the DNA binding affinity of NKX2.5WT for NKX-BS, but the DNA binding affinity of NKX2.5WT for NKX-BS was reduced in EMSA with 2-fold excess of NKX2.5F157L (Fig. 5B).
probe includes a TBX binding site, so we compared the DNA binding affinities of NKX2.5WT and NKX2.5F157L in the presence of TBX5. An increase in the amount of shifted NKX-BS probe was detected when NKX2.5WT was incubated with TBX5, but the amount of shifted NKX-BS probe was reduced when TBX5 was incubated with NKX2.5F157L (Fig. 5B). Synthesis of equal quantities of in vitro translated protein from FLAG-NKX2.5WT and FLAG-NKX2.5F157L expression plasmids was confirmed by radiolabelling with [35S]methionine and immunoblotting.

**DISCUSSION**

We analyzed a family with multiple double first-cousin marriages and six individuals with CAT as described previously (19). Clinical investigation identified the cardiac defect as PTA type 1, the situation in which the right and left pulmonary arteries arise from the truncus arteriosus. Autozygosity screening revealed a T>C nucleotide transition leading to an L-substitution in the homeodomain of the protein.

Several considerations indicate F151L is likely to be a pathogenic mutation. The F151 residue in α-helix I of the homeodomain is conserved in Nkx-2 proteins from at least 10 different species (23). F151 is not a predicted direct DNA contact residue, most of which occur in α-helix III (28), but it is involved in α-helices. X-ray crystallography on Drosophila engrailed protein (29) and subsequent threading analysis for Pitx2 (30) have identified F151 as one of the several hydrophobic residues predicted to stabilize intramolecular interactions between α-helices.

To date, two human genetic disorders are caused by mutation of F20 (the F151 equivalent residue) in homeodomain proteins. Most notably, an identical substitution of F20L has been reported in SHOX (Short HOMEObox containing gene) in Leri Weill dyschondrosteosis, a short stature syndrome (31). In addition, F20S in the homeodomain of PROP-1 causes combined pituitary hormone deficiency (32). These considerations, combined with the fact that the F151 homeodomain substitution which segregated with CAT in the pedigree described here was not observed in 900 control chromosomes, are strongly supportive of the notion that F151L is not a rare polymorphism and is indeed causative in our family. We have, however, failed to detect NKX2.6 mutations in 10 other sporadic cases of PTA type 1, indicating heterogeneity.

NKX2.6 is expressed in the caudal pharyngeal arches (22) and is also expressed in the sinus venosa and in the dorsal pericardium. Most notably, at E9.5, NKX2.6 is expressed in the OFT itself (33). Expression is not observed after E10.5. As discussed earlier, cells in the pharyngeal arches and, self-evidently, cells within the OFT are important for OFT septation.

Mutation of NKX2.5 in mouse by gene targeting results in embryonic lethality; although a linear heart tube forms, it fails to undergo looping morphogenesis (34). In contrast, targeted disruption of NKX2.6 results in normal mouse embryos with no obvious heart or pharynx abnormalities (35). Notably, NKX2.5 mRNA expression expands to the pharyngeal pouch endoderm and has been proposed to be indicative of functional compensation for loss of NKX2.6 in the pharyngeal pouches of these embryos (35). NKX2.6 is also expressed in branchial arch mesectoderm and ectoderm (22), tissues with a known role in cardiac morphogenesis (36). Furthermore, overlapping functions for NKX2.5 and NKX2.6 have been revealed in double knockout mouse embryos, which lack pharyngeal pouches because of reduced proliferation and survival of pharyngeal endodermal cells (37). Given the high degree of homology between Nkx2.5 and Nkx2.6 over the homeodomain (90% identity) and overlapping pharyngeal expression patterns, these data make it likely that Nkx2.5 can act at a subset of Nkx2.6 target genes, although there may be less activity in the reciprocal direction.

Using ANF and Cx40 reporters, we demonstrate that a homeodomain F151L substitution impairs transcriptional activation by NKX2.6 at the Cx40 promoter and reduces transcriptional activation function of NKX2.5 at both Cx40 and ANF promoters. Furthermore, synergistic transcriptional activation is reduced between NKX2.5F157L and both cofactors GATA4 and TBX5 at the ANF promoter. EMSA showed that NKX2.5F157L retains some DNA binding activity, but at reduced levels compared with NKX2.5WT. Together, our results suggest that reduced transcriptional activation function of NKX2.5F157L is a consequence of both impaired DNA binding and interaction with cofactor proteins.

A model for synergistic activation of ANF by Nkx2.5 and GATA4 has been proposed (38), and it describes how
hydrophobic interactions between C- and N-terminal domains of NKX2.5 protein could prevent access to target gene binding site recognition sequences. In its inactivated state, NKX2.5 has a low affinity for its target binding sequence, but interaction with cofactors, such as GATA4 or TBX5, could remove steric hindrance effects, thereby increasing specific DNA binding affinity of NKX2.5. Consistent with this model, we observed an increase in shifted NKX-BS probe when NKX2.5<sup>WT</sup> was incubated in the presence of TBX5 which was reduced with NKX2.5<sup>F157L</sup> and TBX5. These results indicate that the DNA binding affinity of NKX2.5<sup>WT</sup> for NKX-BS probe is enhanced by the presence of cofactor TBX5 and that the F157 residue is important for this interaction. To summarize, using NKX2.5 as a surrogate in our functional analyses, we have shown that the F157 residue (homeodomain F20) is important for co-operative protein interactions involved in transcription of downstream genes and is likely to have a role in specific DNA binding.

The genetic and biochemical data presented here implicate NKX2.6 in the process of OFT septation. This role was not evident from the Nkx2.6 null mouse model, even though Nkx2.6 is expressed in tissues known to play a vital role in the septation process. This discrepancy might be explained by a species difference in the requirement for Nkx2.6 or because this mouse mutant is highly unlikely to produce any Nkx2.6 protein. In patients homozygous for NKX2.6<sup>T451C</sup>, we predict that stable protein would be produced and capable of residual but largely non-functional DNA binding and protein interactions. The presence of NKX2.6<sup>T451L</sup> protein could lead to a diminished ability of NKX2.5 to compensate for the loss of function aspects of the NKX2.6 mutation. This hypothesis could be tested once a bona fide target promoter shared between both transcription factors has been identified. Alternatively, the mutant protein could exert a dominant negative effect, but we feel this is unlikely given the recessive nature of the condition. The identification of NKX2.6 targets, especially those shared with NKX2.5, will be important for further understanding of OFT septation, and for testing whether NKX2.6, like NKX2.5, forms complexes with TBX and GATA family proteins. As Tbx1 null mice have abnormal development of derivatives of the pharyngeal endoderm and PTA (39) and Tbx2 null mice have OFT defects (17), these would be logical candidates to test for epistatic and/or biochemical interactions.

**MATERIALS AND METHODS**

**CAT family and controls**

Informed consent was obtained from participating individuals, and the study was approved by the Ministry of Health in Kuwait. Blood samples were collected, and DNA was...
extracted using standard methods. The same procedure was followed for control samples.

**Linkage analysis and recombination mapping**

Microsatellite analysis excluded the DiGeorge region on chromosome 22q11 (data not shown). The genome-wide screen used the ABI Linkage Mapping Set Version 2.0 (Applied Biosystems), according to protocols supplied by the manufacturer. PCR amplification and separation of amplicons on an ABI PRISM 377 DNA sequencer were performed using standard protocols. GENESCAN and GENOTYPER software (Applied Biosystems) were used to identify alleles. Areas of homozygosity were investigated with unlabelled microsatellite markers analyzed by PCR and polyacrylamide gel electrophoresis. These additional markers were identified on the Marshfield genetic map and the Ensembl physical map. Linkage analysis was performed using MAPMAKER/HOMOZ software assuming autosomal recessive inheritance, 100% penetrance and trait-allele frequency of 0.0001. One-hundred control chromosomes were typed to establish allele frequencies of relevant markers in the Kuwaiti population.

**Candidate gene identification and sequence analysis**

Positional candidate genes were identified using information from Project Ensembl (21) and GenBank (40). All exons encoding the open reading frame of candidate genes were amplified by PCR using intronic primers. PCR products were sequenced using BigDye terminators v3 (Applied Biosystems) and analyzed by ABI PRISM 377 DNA sequencer using standard protocols. All three individuals with a CAT phenotype, parents and an unrelated control DNA sample were screened for sequence variants.

**Mutation analysis**

We analyzed NKX2.6 for the 451T>C sequence variant in all family members who had provided DNA samples and at least 900 control chromosomes from people of mixed ethnic origins including 200 Kuwaiti, 200 Arab and 500 Caucasian chromosomes. Mutation analysis was performed using either direct sequencing or restriction fragment length polymorphism (RFLP) analysis of PCR products as the 451T>C mutation creates a BsaHI site (affected: 210 bp + 178 bp fragments; unaffected: 388 bp fragment). Reference sequence for NKX2.6 was taken from Ensembl, gene number ENSG000000180053. Sequences of all primers and PCR conditions used in this study are available on request. For RFLP-PCR analysis, 10 μl of PCR product was incubated with 0.5 U BsaHI restriction enzyme, according to standard protocols and digested samples resolved on 2% agarose gels.
Recombinant plasmids and site-directed mutagenesis

For expression constructs, cassettes flanked by EcoRI and XhoI restriction sites were generated by PCR using NKX2.6-specific primers (5'-ATGCTGCTGAGCCCCGT-3' and 5'-CCAGGCCCTGACACCCTGCA-3') and NKX2.5-specific primers (5'-ATGTTCCCCAGCCCTGCTCTCA-3' and 5'-AGGCTCGGATACCAATGCA-3') incorporated and cloned into pcDNA3-N-FLAG vector (Invitrogen). F157L or F151L missense mutations were introduced in FLAG-NKX2.5 and FLAG-NKX2.6 plasmids, respectively, by in vitro mutagenesis (Altered Sites II System, Promega, UK) and verified by sequencing. pGVB-ANF-luc, HA-NKX2.5 and FLAG-TBX5 were provided by Hiroshi Akasawa and Issei Kumuro (Chiba University Graduate School of Medicine, Japan). N-Myc-GATA4 was provided by Deepak Srivastava (University of Texas, Southwestern Medical Center, USA) and pGL3-Cx40-luc was donated by Birgit Teunissen and Marti Bierhui-zen (University Medical Center, Utrecht, The Netherlands).

Reporter gene constructs and transient transfection assays

U2OS (osteosarcoma) or H9c2 (rat cardiomyoblast) cell lines were routinely maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Subconfluent cells in six-well plates were transfected using Effectene transfection reagent (Qiagen), with 100 ng reporter construct (either ANF-luc or Cx40-luc) and 100 ng β-gal-CMV as an internal control for transfection variability. The following FLAG-epitope tagged expression constructs were transfected either alone or in combination: FLAG-NKX2.5WT, FLAG-NKX2.5F157L, FLAG-TBX5, GATA4-Myc, FLAG-NKX2.6WT and FLAG-NKX2.6F151L. Total plasmid amount per well was adjusted to 400 ng using empty pcDNA3 plasmid DNA. U2OS cells were harvested 48 h after transfection using 200 μl lysis buffer (H9c2 cells 24 h after transfection), and then assayed for luciferase activity using a luminometer (Turner TD-20/20) and β-galactosidase activity. Differences in transfection efficiency were corrected relative to expression levels of β-galactosidase. At least three independent experiments were performed in triplicate. Equal expression levels of protein using FLAG-NKX2.5WT and FLAG-NKX2.5F157L expression constructs were confirmed by immunoblotting, using anti-FLAG monoclonal antibody (α-FLAG Ab) (Sigma).

Antibodies, SDS–PAGE and immunoblotting

FLAG-epitope tagged NKX2.5WT, NKX2.5F157L, NKX2.6WT and NKX2.6F151L expression plasmids were transfected into U2OS cells as described earlier. Protein expression was evaluated by western blotting using α-FLAG Ab 48 h after transfection (Sigma). Protein samples were prepared by lysis of cells in RIPA sample buffer (150 mM NaCl, 1% NP-40, 50 mM Tris, pH 7.5, 0.5% DOC, 0.1% SDS), separated on 12% SDS–PAGE gels and electroblotted onto ECL nitrocellulose (Amersham, UK). Following blocking in 5% Marvel/1 x TBS/0.1% Tween-20 (blocking buffer) for 2 h at room temperature or overnight at 4°C. Primary antibody α-FLAG Ab was diluted 1:1500 in blocking buffer and hybridized to
the blots for 1 h at room temperature. Subsequently, anti-mouse polyclonal antibody conjugated to peroxidase (Amer- sham) was used at 1:5000 for 1 h at room temperature. Bands were visualized using enhanced chemiluminescence (Amer sham).

Electromobility shift assays

EMSAs were used to assess DNA binding properties of NXX2.5F157L compared with NXX2.5WT. The NXX-BS probe used for EMSA contained two NXX-binding sites (TNAAGTG) and an overlapping TBX5 binding site (TCA- CACCT) (−264 to −227 bp in the ANF proximal promoter (27)). Oligo 5′-TCACACCTTGAGTGGGCGCTCTTT- GAGGCAATC-3′ was annealed to 5′-GATTGGCTTCAAGG- GAGGCCCACTTAAAGGTTGA-3′ and labelled with [32P]dATP using Klenow polymerase. Wild-type and mutant proteins were transcribed and translated using the TnT-coupled reticulolysate system (Promega). Part (2.5 μl) of the reaction was incubated at room temperature for 20 min in 20 μl binding buffer (20 mM HEPES, pH 7.9, 60 mM KCl, 1 mM MgCl2, 0.5 mM DTT and 10% glycerol) with 2 μg of polydl-dC and 1 μl of labelled oligo. Protein/ DNA complexes were separated on a 6% polyacrylamide gel in Tris–borate buffer at 4°C. Gels were dried and exposed to film. Supershift EMSAs were carried out by pre-incubating NXX2.5WT or NXX2.5F157L with 2 μl of α-FLAG Ab for 30 min on ice prior to addition of labelled oligo. Equal transcription/translation efficiency of NXX2.5WT and NXX2.5F157L. FLAG-tagged fusion proteins was confirmed by transcription/translation of proteins in the presence of [35S]-labelled methionine, with subsequent SDS–PAGE analy- sis and immunoblotting using α-FLAG Ab.

ACKNOWLEDGEMENTS

We would like to thank the family for participating in this study. Plasmids were kindly provided by Hiroshi Akasawa, Issei Kumuro, Deepak Srivastava, Birgit Teunissen and Marti Bierhuizen. We thank Koen Devriendt, Maria Cristina Digilio and Katrina Prescott who provided DNA samples from sporadic cases of CAT. K.H. thanks Mark Adams for assistance with preparation of line art for figures. P.S. is funded by British Heart Foundation; MK and MG are funded by Birth Defects Foundation (UK).

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