Functional dissection of sequence-specific NKX2-5 DNA binding domain mutations associated with human heart septation defects using a yeast-based system

Alberto Inga1, Stella Marie Reamon-Buettner2, Juergen Borlak2,‡ and Michael A. Resnick1,*†

1Chromosome Stability Section, Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, NIH, PO Box 12233, Research Triangle Park, NC 27709, USA and 2Department of Drug Research and Medical Biotechnology, Fraunhofer Institute of Toxicology and Experimental Medicine, Nikolai-Fuchs-Strasse 1, D-30625 Hannover, Germany

Received March 18, 2005; Revised May 10, 2005; Accepted May 20, 2005

Human heart development requires an orderly coordination of transcriptional programs, with the homeodomain protein NKX2-5 being one of the key transcription factors required for the differentiation of mesodermal progenitor cells. Indeed, lack of Nkx2-5 in mice arrests heart development prior to looping, resulting in embryonic lethality. There are 28 germline NKX2-5 mutations identified in humans that are associated with congenital heart disease, and we recently reported multiple somatic mutations in patients with complex cardiac malformations. To address the functional consequences of single and multiple mutations of NKX2-5, we developed a functional assay in the budding yeast Saccharomyces cerevisiae, which could determine transactivation capacity and specificity of expressed NKX2-5 alleles towards targeted response element (RE) sequences. We focused on mutants of the third helix, which provides DNA binding specificity, and characterized mutations that were highly associated with either ventricular (VSD) or atrioventricular (AVSD) septal defects. Individual mutants exhibited partial to complete loss of function and differences in transactivation capacity between the various REs. The mutants also exhibited gene dosage rather than dominant effects on transcription. Surprisingly, all AVSD patients (22/23) had a single K183E mutation in the DNA binding domain, which resulted in transcriptional inactivation. None of the VSD patients had this mutation; yet 14/29 had at least one mutation in the third helix leading to either inactivation or reduction of NKX2-5 transactivation. Therefore, mutations of somatic origin in the binding domains of NKX2-5 were associated specifically with AVSD or VSD and resulted in loss of protein function.

INTRODUCTION

The homeodomain (HD) protein Nkx2-5 is an evolutionarily conserved transcription factor required for the organogenesis of the heart (1) and the development of the cardiac conduction system (2). The human NKX2-5 gene maps to chromosome 5q34 and consists of two exons encoding a protein of 324 amino acids. The HD portion of the protein forms three alpha helices, and helix 3 is responsible for binding specificity. Other conserved regions of Nkx2-5 are the TN regions of the transactivation domain (TAD) and the NK2-specific domain (NK2-SD), with autoregulatory functions (3).

Several transcription factors cooperate in the regulation of heart development. Results from whole animals and cellular models suggest complex functional interplays and the necessity for proper gene dosage of these regulators (reviewed in 4–6). Deletion of both Nkx2-5 alleles in the mouse results in lethality due to impaired cardiac looping.

*To whom correspondence should be addressed. Email: resnick@niehs.nih.gov and Juergen.Borlak@item.fraunhofer.de
†Present address: Laboratory of Experimental Oncology B, Department of Translational Oncology, National Cancer Research Institute, IST, Largo R. Benzi X, 16132 Genoa, Italy.
‡These two authors contributed equally as Senior Authors.

Published by Oxford University Press 2005.
overgrowth, whereas hemizygosity can result in hypoplasia of the conduction system with variable penetrance, leading to a mild first-degree atrioventricular (AV) block (2,9). A ventricular myocyte-restricted knock-out mouse model further established a role for Nkx2-5 in the formation, maturation and maintenance of the conduction system (10). Although no structural heart defects were observed during embryonic development, all adult mice exhibited progressive cardiomyopathy with complete heart block and trabecular muscle overgrowth.

In humans, 28 different heterozygous germ line NKX2-5 mutations have been identified in patients suffering from congenital heart disease (CHD) (reviewed in 11). Although most are in sequences affecting the HD, there is no clear genotype–phenotype correlation. Similar to what is observed in the heterozygous Nkx2-5 mouse model, progressive AV conduction delay is the common phenotype, but a near complete penetrance of conduction abnormalities (12) and a diversity of cardiac malformations are observed. Cardiac malformations of patients with NKK2-5 germline mutations were mainly ASD with or without AV block, although there were reports of patient(s) with tetralogy of Fallot, ventricular septal defect (VSD), double-outlet right ventricle, interrupted aortic arch, truncus arteriosus, L-transposition of the great arteries, hypoplastic left heart syndrome and coarctation of the aorta.

The phenotypic discrepancies between CHD associated with NKX2-5 germline mutations in humans and in the haplo-insufficient Nkx2-5 mouse models have been ascribed to the dominant negative impact of mutant Nkx2-5 proteins towards wild-type Nkx2-5 or other cardiac transcription factors that act in cooperation and physically associate with Nkx2-5, such as Gata4 and Tbx5 (5). However, the spectrum of germline human NKX2-5 mutations is diverse in terms of mutation type, position of the affected amino acids and predicted impact of the mutations on protein–protein interactions (11,13,14). Indeed, there appears to be no common mutation identified in familial or sporadic cases of CHD. Although evidence of dominant negative properties of NKX2-5 proteins has been presented on the basis of transcription assays in model systems (11,14), the in vivo relevance of these observations remains to be established. In fact, the transcriptional targets of NKX2-5 that contribute to the heart chamber maturation and the development of the conduction system are largely unknown (5), and the results in animal models showed weak correspondence between alterations of gene expression in vivo and identification of direct target genes obtained using transfected cell lines, with over-expression of NKX2-5 alone or of other regulators of its transcriptional network (15).

NKX2-5 genetic heterogeneity and mosaicism in the diseased tissue could be a source of the phenotypic variability that is associated with human CHD, but is not recapitulated in animal models. This hypothesis is based on recent results from this (J. Borlak) laboratory of NKX2-5 mutations identified in diseased tissues from explanted formalin-fixed hearts of patients affected by complex cardiac malformations that included ASD, VSD and AVSD (16,17). Particularly interesting was the observation that non-synonymous multiple NKX2-5 mutations were identified in almost all diseased tissue samples. However, there were no mutations in unaffected heart sections of the same patients or lymphocytic DNA from unrelated individuals with CHD. Although mutations in the genes encoding the cardiac transcription factors TBX5 and GATA4 were also found in a fraction of these samples (18,19), there did not appear to be a general mutagenesis for other genes coding for cardiac transcription factors such as MEF2C (unpublished data). Indeed, MEF2C maps to chromosome 5 (5q14) close to NKX2-5 (5q34) but did not carry mutations in the sequences encoding its DNA binding domains in 68 hearts and 12 lymphocytic DNA samples of patients with CHD, as well as in 50 lymphocytic DNA samples of normal individuals. Thus, the results were indicative of NKX2-5 mutations of somatic origin, with multiple haplotypes and mosaicism generated through an unknown mechanism of localized hyper-mutagenesis. Although not nearly as extensive as for NKX2-5, multiple somatic mutations were also reported for the HPRT gene in kidney tubular epithelial cells (20). We have identified an additional 16 cases in which germline NKX2-5 mutations were detected in the affected heart tissues in conjunction with other mutations (17). Taken together, these results suggest that the wide phenotypic spectrum of congenital heart disease may be due, at least in part, to somatic mutations in transcription factor genes that control heart development in progenitor cells.

To address the functional significance of individual and multiple somatic NKX2-5 mutations in CHD, particularly VSD and AVSD, we created a functional assay for this homeobox transcription factor in a model organism, the budding yeast Saccharomyces cerevisiae. We focused mainly on newly observed somatic NKX2-5 gene mutations affecting amino acids in the third helix of the HD, a region that provides for specific binding to the DNA target response elements (NK-REs). Similar to a system that we had developed to examine the tumor suppressor p53 (21), the yeast-based assay measures transactivation capacity under conditions of tight, ‘rheostatable’ regulation of NKX2-5 expression and utilizes a set of reporter constructs under isogenic conditions. Different defined NK-REs derived from the promoters of known NKX2-5 target genes are placed upstream of the reporters to stimulate transcription in an otherwise constant chromatin environment. The tight regulation of expression of the NKX2-5 transcription factor also renders the functional assay sensitive to gene dosage, thus providing for analysis of the dominant negative potential of NKX2-5 mutants when heterozygous with wild-type NKX2-5.

The newly developed functional assay in yeast provides a robust tool for the in vivo assessment of the transactivation capacity of wild-type NKX2-5 and of single or multiple mutants towards a variety of REs. Nearly all AVSD patients had a single mutation which was found to be transcriptionally inactive. In contrast, none of the VSD patients had this mutation. However, there was always at least one mutation in this region that led to inactivation of NKX2-5 transactivation capacity, with some individual mutations exhibiting partial function. Loss-of-function NKX2-5 mutants did not exhibit dominance when co-expressed at equal levels with the wild-type allele, but instead resulted in a gene-dosage reduction in function.
RESULTS
NXX2-5 can act as a sequence-specific transcription factor in yeast

The model organism \textit{S. cerevisiae} has provided opportunities to examine the transactivation capacity of wild-type and mutant forms of various mammalian transcription factors, often in the absence of a yeast homolog (reviewed in 22). The success of this approach is due to the conservation of the basal transcription machinery and the consequent ability of mammalian transcription factors bound to a promoter to establish protein–protein interactions, resulting in stimulation of transcription. The genetic tractability of yeast along with a remarkable proficiency for targeted homologous recombination greatly facilitate the construction of gene reporter constructs and the use of expression vectors for functional assays (23,24). Yeast promoters can be easily modified and made uniquely responsive to a mammalian transcription factor by removing native upstream activating sequences and replacing them with REs for the chosen mammalian factor (22).

Our recent development of a system for \textit{in vivo} site-directed mutagenesis by oligonucleotides (25) has greatly improved the ability to modify yeast promoters for transcription studies (21,26). By applying this methodology to the analysis of the transactivation capacity of human p53 towards many different REs, we have also recently developed conditions that result in tight regulation of expression of the transcription factor along with rheostatable induction of the \textit{GAL1} promoter using variable levels of galactose in the culture medium (21). In a separate study demonstrating the utility of our approach, the combination of tight regulation of p53 levels and the transactivation analysis towards many REs greatly improved the analysis and classification of p53 mutations associated with cancer (26).

Drawing upon the experience with p53, we constructed expression vectors pTSG–NXX2-5 and pUSG–NXX2-5, which are low copy centromeric plasmids, to examine the human homoeodomain NXX2-5 protein in yeast, as described in Materials and Methods. In addition to the HD, which provides for DNA binding, two other domains are conserved in the NK2 class of homeoproteins to which NXX2-5 belongs. The specific domain (NXX2-SD) may mediate protein–protein interactions and appears to have an inhibitory function on sequence-specific transactivation in model systems (27). The TN domain contains a motif weakly conserved in other transcription factors and may contribute to transactivation (27). Thus, to establish a system based in yeast to address homoeodomain transcription factors, four different NXX2-5 constructs were tested. They included the wild-type full-length protein, a C-terminal truncation that lacked the NXX2-SD and might result in higher activity and also those same two constructs fused to an acidic TAD derived from p53 (Fig. 1A). Given the absence of a well-defined NXX2-5 activation domain and previous reports of weak activity in yeast of some classes of TADs (22), constructs with the p53 chimeric TAD were developed with the view that they could provide transactivation if sequence-specific binding were obtained.

For the initial functional analyses of NXX2-5 towards an RE, we chose the distal NK-RE derived from the atrial natriuretic factor (ANF) promoter that has been used in transactivation assays in mammalian cells (28). The distal ANF-RE comprises two closely spaced inverted monomer-binding sites for NXX2-5 and can bind a dimer of NXX2-5 proteins, according to \textit{in vitro} results (28). However, both monomer-binding sites contain a mismatch from the NXX2-5 consensus sequence (27) and their activity could be weak, especially when isolated from their natural promoter. As adjacent REs have shown cooperative interactions, and previous reports of weak activity in yeast (27), we also introduced and examined transactivation at different numbers of the distal NK-RE.

As shown in Figure 1B and C, wild-type NXX2-5 can greatly stimulate transcription in yeast. This activity required a wild-type NXX2-5 HD and the presence of an NKX-RE in the promoter. This is the first report of a human HD protein functioning in transcription in yeast. Transcription also required the acidic TAD provided in the chimERIC construct. The C-terminal truncation that lacks the NXX2-SD exhibited up to 3-fold higher activity when compared with the full-length construct (Fig. 1C), suggesting the conservation in the yeast system of the inhibitory effect seen in mammalian cells (29). At high expression, SD-truncated NXX2-5 showed low levels of transactivation even in the absence of the exogenous activation domain, whereas the chimeric construct is at least 50 times more active.

Our results show that the HD-containing protein NXX2-5 can act as a sequence-specific transcription factor in yeast but it is intrinsically very weak. This observation suggests that it would be possible to use the system to screen for additional transcription factors or cofactors that can physically associate and cooperate with NXX2-5 function, as in the case of the cardiac transcription factors \textit{GATA4} and \textit{TBX5} in human cells (30,31). As our aim was to study the impact of HD NXX2-5 mutations on sequence-specific transactivation, in the subsequent analyses, we took advantage of the robust activity of the chimeric full-length NXX2-5 with an exogenous acidic TAD and also examined transactivation at several different REs.

Impact of RE sequence variation on NXX2-5 transactivation capacity

We explored the effect that variation in NK-RE sequence and structure can have on NXX2-5 transactivation capacity. Besides allowing comparison of results between an \textit{in vivo} functional assay and an \textit{in vitro} DNA binding measurements with naked DNA (29), this analysis was expected to generate a set of reporter strains for the subsequent analysis of NXX2-5 mutations.

The distal ANF-RE, which provides for a dimer-binding site, showed relatively weak activity. Two copies of this RE could mediate only low levels of transactivation as evidenced...
Figure 1. (A) Diagram of NKX2-5 expression vectors and reporter yeast strains. Full-length NKX2-5, the NK2-SD deletion (Δ-SD) and the chimeric cDNA constructs with the acidic p53 TAD were cloned downstream of the inducible GAL1 promoter in the yeast expression vector backbone pTSG. The vector also contains the CYC1-derived transcriptional terminator, centromere (CEN) and origin of replication (ARS) for stable low-copy transmission in yeast and the TRP1 selection marker (URA3 in the otherwise identical pUSG backbone). Upon transformation of yeast cells, NKX2-5 expression can be induced by varying the amount of galactose inducer in the culture medium. The yeast strains contain a modified ADE2 locus on chromosome XV, with a minimal CYC1-derived promoter upstream of either the ADE2 or the firefly luciferase cDNAs. NKX2-5 response elements (NK-RE) are introduced upstream of the minimal promoter resulting in NKX2-5-dependent reporter genes. (B) Example of visual (red/white) assay. Yeast reporter strains transformed with the indicated expression vectors were streaked on a master glucose plate containing a high amount of adenine to allow equal growth of all transformants and prevent the accumulation of red pigment. The master plate is then replica plated to a series of tester plates that contain low amounts of adenine to allow visual identification of ADE2 transcribing clones based on colony color. The tester plates contain variable amount of galactose inducer to achieve modulation of NKX2-5 expression. At low galactose concentration, all clones are red because induced NKX2-5 protein levels are insufficient to stimulate transactivation. At higher galactose concentration, expression of the chimeric wild-type NKX2-5 (TAD::NKX) led to transactivation, as evidenced by the appearance of pink (0.004%) and white (0.064%) colonies. The p53-derived acidic TAD is required for this activity. The K192E mutation in the third alpha helix of the NKX2-5 HD results in reduced transactivation (pink color is visible only at 0.064%). The empty vector and a vector expressing a mutant p53 allele do not show transactivation from the NK-RE reporter. (C) Example of quantitative luciferase assay. The average light units per microgram of protein extracts induced by the variable expression (0.008 and 0.12% galactose corresponding to low and moderate expression) of the indicated constructs in a NKX2-5 yeast reporter strain is presented. The fold induction relative to the p53 mutant (p53-R175H) control is indicated, as well as the standard deviation of three biological replicates.
by the appearance of pink colonies in the *ADE2*-based assay at the high
 NKX2-5 expression obtained with 0.5% galactose (data not shown). Increasing the number of ANF-REs resulted in cooperative interaction. Seven copies of the RE (i.e. seven adjacent NKX2-5 dimer-binding sites) were nearly 30 times more active (pink colonies at 0.004% galactose, Fig. 1B).

Each monomer-binding site in the distal ANF-RE has one mismatch from the NK-RE consensus TYAAGTG (27) (Table 1). To explore the impact of single nucleotide changes on the NK-RE on NKX2-5 functionality, we tested six sequence variants that are present in proposed NKX2-5 target promoters. On the basis of the number of distal ANF-REs required for transactivation, each sequence motif was examined by cloning four adjacent monomer-binding sites into the *ADE2* reporter construct in direct and in inverse orientation. None of the sequences was capable of mediating NKX2-5 transactivation in direct orientation (Table 1). Instead, stimulation of transactivation was detected with two sequence motifs placed in inverse orientation, thus forming two palindromic dimer-binding sites. The endothelin converting enzyme (ECE) consensus sequence was 14 times stronger than the distal ANF-RE of the same structure. There was no transactivation with nucleotide changes at the following position of the consensus sequence: first (T>G, pANF), sixth (T>A, dANF-b or T>G, pDIO2) and seventh (G>T, dDIO2). The C>G mismatch (dANF-a) at the second position retained ~30% function. A combination of a weak and an inactive sequence motif explains the very low activity (~7% of the consensus) of the distal ANF-a+b RE. (Note: GTG is an E-box half-site, frequently contacted by bHLH TFs. Most likely the thymine is contacted by the NKX2-5 HD.)

Although based on a relatively small sampling of NK-RE sequence variants, our results suggest that the REs that are active in our *in vivo* functional assay are only a subset of the sequences that can bind the NKX2-5 protein *in vitro* (29). The observation that transactivation by NKX2-5 in yeast requires palindromic REs is consistent with the higher NKX2-5 binding affinity for dimer NK-REs measured by gel shift assays and the capacity of NKX2-5 to form homodimers (28). Palindromic NK-REs are present in the promoters of some NKX2-5 target genes (28); however, they are not a common feature of all proposed targets and the palindromic requirement for NKX2-5-dependent transactivation *in vivo* remains to be established. In the context of a complex promoter, where NKX2-5 mediates transcription in conjunction

### Table 1. Relative NKX2-5 transactivation capacity towards various response elements (visual *ADE2*-based assay)

<table>
<thead>
<tr>
<th>RE name</th>
<th>RE DNA sequence</th>
<th>RE structure</th>
<th>Relative transactivation capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>dANF-a+b</td>
<td>TgAAGTGCtCCTGA</td>
<td>4 monomers</td>
<td>N/T 1 28</td>
</tr>
<tr>
<td>ECE</td>
<td>TCAAGT7G</td>
<td>inactive</td>
<td>14</td>
</tr>
<tr>
<td>dANF-a</td>
<td>TgAAGT7G</td>
<td>inactive</td>
<td>4</td>
</tr>
<tr>
<td>dANF-b</td>
<td>TCAAGGAG</td>
<td>inactive</td>
<td>inactive</td>
</tr>
<tr>
<td>pANF</td>
<td>gCAAGTG</td>
<td>inactive</td>
<td>inactive</td>
</tr>
<tr>
<td>DIO2.1</td>
<td>TCAAGG7G</td>
<td>inactive</td>
<td>inactive</td>
</tr>
<tr>
<td>DIO2.1</td>
<td>TCAAG7TL</td>
<td>inactive</td>
<td>inactive</td>
</tr>
</tbody>
</table>

Figure 2. Functional impact of NKX2-5 HD mutations. Using the chimeric constructs with the exogenous acidic activation domain, the transactivation of wild-type and mutant NKX2-5 was determined at moderate GAL1 induction (0.12% galactose), using the ECE and distal ANF-a+b RE. The average light units and the standard deviations of three replicates are shown. The single mutant K183E and the double mutant K152T, K194R were associated with AVSD and VSD, respectively (Table 2).

#### Functional consequences of novel somatic NKX2-5 mutations associated with cardiac malformations

Although not identical in structure to the NK-REs found in the human promoters, palindromic ECE and ANF-REs provide opportunities with the yeast system to analyze the functional consequences of NKX2-5 DNA binding mutations. On the basis of our previous studies of mutant forms of the p53 transcription factor, where the impact of mutations on transactivation capacity differed between RE sequences, we chose to measure transactivation by NKX2-5 mutant proteins using various RE sequences and number of dimer-binding sites.

For this study, we focused on NKX2-5 gene mutations of both somatic and germline origin that were detected in VSD and AVSD heart malformations. The mutations resulted in amino acid changes at the third helix of the HD which mediates specific interactions with the major groove of the DNA at the NK-RE site. As molecular analysis of the NKX2-5 gene from diseased tissues revealed the presence of multiple mutants and different haplotypes (17), we were also interested in determining the functional consequences of individual and multiple mutations within a given haplotype. This information might provide clues to possible selection processes that give rise to the disease. Six different haplotypes from three VSD patients were analyzed (Fig. 2 and Table 2). We also investigated the mutant K183E (corresponding to amino acid 46 of the HD) that was highly associated with AVSD (observed in 22 out of 23 patients) and the stop codon mutant Q187X (corresponding to amino acid 50 of the HD). Except for the T178M mutation, which has also been found in the germline (32,33), none of the other HD mutants given in Table 2 have been analyzed in a functional assay. Further, we analyzed
A219V which is located in the NK2-SD and is also a germline mutation (34).

The single and multiple mutants were constructed in the pTSG-TAD::NKX2-5 vector backbone (see Materials and Methods) and the functional analysis was conducted using the quantitative luciferase reporter at moderate levels of NKX2-5 allele expression (0.12% galactose). The normalized average light units for four mutants and the controls are presented in Figure 2. The frequently appearing AVSD-associated K183E amino acid change resulted in complete loss of transactivation, whereas the double mutant K192R and A219V, either together or individually, had only a modest decrease in transactivation for ANF (14d), but the combined mutations have a strong impact when measured against the ECE-REs. In addition, some mutations exhibited some degree of functional alteration, particularly when tested with the strain containing the most sensitive reporter (two NKX dimer-binding sites of the ECE consensus sequence ECE 2d). A mild effect was seen with the frequent mutation A219V that affects an amino acid in the NK2-SD regulatory domain and is not directly involved in sequence-specific DNA binding. The negative phenotype of the triple mutant haplotypes from patient D03 appears to be due to the T178M mutation, an amino acid change that causes a loss-of-function. This germline mutation, also found in familial CHD patients, was shown to have reduced in vitro DNA binding resulting in loss of transactivation in human cells (14).

It is important to note that the impact of various mutations and combination of mutations can be dependent on the particular RE used to address functionality. This observation is illustrated in Figure 3, where the relative transactivation capacities of NKX2-5 mutations exhibiting partial function are presented in the form of a line graph to emphasize the different patterns of response with the various REs. The overall pattern of activity was similar for the ECE (2d)- and ECE (4d)-REs, whereas responses were more variable and dependent on specific NKX2-5 mutations with the ANF-REs, particularly with the high activity ANF (14d), which is generally less sensitive to NKX2-5 functional mutants. For example, K192R and A219V, either together or individually, have only a modest decrease in transactivation for ANF (14d), but the combined mutations have a strong impact when measured against the ECE-REs. In addition, some mutants (e.g. K192T, A219V) led to changes in spectrum of activation, not simply a general reduction, with ANF (14d)

### Table 2. Dissecting the transactivation capacity of complex NKX2-5 clinical mutants using response elements with different strength in a luciferase reporter assay

<table>
<thead>
<tr>
<th>Patient</th>
<th>Allele/haplotype</th>
<th>Disease</th>
<th>Response elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO3</td>
<td>T178M, K192R, A219V</td>
<td>VSD</td>
<td>100 ± 15.3</td>
</tr>
<tr>
<td></td>
<td>T178M, K194R, A219V</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K192T, A219V</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178V, K192T, A219V</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td>DO3</td>
<td>T178M, K192R</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K219V</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K194R</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K192T</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td>E03</td>
<td>T178M, K192R</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K194R</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K219V</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M, K192T</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td>K192R, A219V</td>
<td>27 ± 11.1</td>
<td>29.8 ± 6.5</td>
<td>47.7 ± 11.8</td>
</tr>
<tr>
<td>K194R, A219V</td>
<td>38.4 ± 6.9</td>
<td>44.9 ± 6.3</td>
<td>57.5 ± 16.5</td>
</tr>
<tr>
<td>K192T, A219V</td>
<td>21.3 ± 2.6</td>
<td>25.4 ± 2.1</td>
<td>26.7 ± 5.9</td>
</tr>
<tr>
<td>E04</td>
<td>K192R, K194R</td>
<td>VSD</td>
<td>31 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>K192T, K194R</td>
<td>VSD</td>
<td>11.4 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Also germline</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>T178M</td>
<td>VSD</td>
<td>0</td>
</tr>
<tr>
<td>K192R</td>
<td>56.2 ± 2.3</td>
<td>68.3 ± 13.8</td>
<td>61.6 ± 14.8</td>
</tr>
<tr>
<td>K192T</td>
<td>28.6 ± 7.4</td>
<td>31.3 ± 7.5</td>
<td>ND</td>
</tr>
<tr>
<td>K194R</td>
<td>68.8 ± 10.6</td>
<td>ND</td>
<td>48.4 ± 8.3</td>
</tr>
<tr>
<td>A219V</td>
<td>65.0 ± 5.9</td>
<td>63.1 ± 16.1</td>
<td>60.1 ± 4.3</td>
</tr>
<tr>
<td>FO4</td>
<td>K183E</td>
<td>AVSD</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Q187X</td>
<td>VSD</td>
<td>0</td>
</tr>
</tbody>
</table>

The average transactivation capacities relative to wild-type NKX2-5 are summarized in Table 2. Disease-associated haplotypes are indicated by the patient identifier in the first column and the disease name in the third column. Overall, disease-associated somatic NKX2-5 mutations were non-functional or retained little activity as haplotypes, generally below the effect obtained with hemizygosity (discussed subsequently). Almost all the individual mutations exhibit some degree of functional alteration, particularly when tested with the strain containing the most sensitive reporter (two NKX dimer-binding sites of the ECE consensus sequence ECE 2d). A mild effect was seen with the frequent mutation A219V that affects an amino acid in the NK2-SD regulatory domain and is not directly involved in sequence-specific DNA binding. The negative phenotype of the triple mutant haplotypes from patient D03 appears to be due to the T178M mutation, an amino acid change that causes a loss-of-function. This germline mutation, also found in familial CHD patients, was shown to have reduced in vitro DNA binding resulting in loss of transactivation in human cells (14).
being particularly affected. The pattern of transactivation responses also shows how different amino acid changes at the same position can lead to significant differences in transactivation, as found for K192R versus K192T. Overall, these results are reminiscent of those previously reported for mutations in the tumor suppressor p53 and suggest that mutations in the sequence binding domain can have markedly different effects, depending on the RE being transactivated and the specific mutation. This is particularly important for genes such as \textit{NKX2-5} that can regulate many genes from various REs. As for the case of p53, we suggest that \textit{NKX2-5} may also be a ‘master gene of diversity’ (26).

Addressing dominance of wild-type and mutant \textit{NKX2-5}

The yeast-based functional assay can be easily adapted to the analysis of dominance of mutant transcription factor alleles when heterozygous with the wild-type. The tight regulation of protein expression obtained with the inducible \textit{GAL1} promoter combined with the availability of reporters with different responsiveness also provides the opportunity to develop functional assays that are sensitive to gene dosage. We constructed vector pUSG-TAD::NKX2-5 that expresses wild-type NKX2-5 fused to the acidic TAD and contains the \textit{URA3} selectable marker (see Materials and Methods). This plasmid can then be selected along with the original pTSG-TAD::NKX2-5 vectors (\textit{TRP1} marker) that contain either wild-type NKX2-5 or mutants. We chose reporter strain with the ECE (2d)-RE because, overall, it showed the highest discrimination between NKX2-5 mutants.

The results of the functional assay using moderate co-expression of two NKX2-5 alleles are presented in Table 3. The induction of the luciferase reporter was clearly sensitive to the copy number of wild-type NKX2-5. As expected, expression from the two vectors was nearly identical as they only differ in the selection marker. Selection of both clones resulted in nearly double the amount of transactivation when the two wild-type NKX2-5 proteins were expressed. There was no apparent dominance by the mutants because co-expression of loss-of-function mutants (from pTSG-TAD vectors) with wild-type NKX2-5 (from pUSG-TAD::NKX2-5) was functionally equivalent to the expression of the wild-type allele only, and a partial function allele showed an additive effect with the wild-type. The lack of dominance for these particular VSD-associated haplotypes may also result from an inability of the mutant proteins to form heterodimers with the wild-type protein (28).

**DISCUSSION**

\textit{Nkx2-5} is a transcription factor that is required during cardiac development, and mutations in the human \textit{NKX2-5} gene have been associated with CHD. Mutations of the \textit{NKX2-5} gene are typically identified in lymphocytic DNAs and most are familial. Among a total of 28 reported \textit{NKX2-5} germline mutations, 10 are located in the HD. Of these, eight are missense (R142C, L171P, T178M, Q187H, N188K, R190H, Y191C) and two are nonsense (Q149X, Q170X) mutations. Five are located in the third helix (Q187H, N188K, R189G, R190H, Y191C), two are in the second helix (Q170X, L171P), one is in the first helix (Q149X), one is in the loop between the second and third helix (T178M) and the remaining mutation is in the NLS region (R142C). The appearance of \textit{NKX2-5} mutations in the lymphocytic DNA of patients with non-familial heart defects is uncommon. Among 608 patients, only 18 (3\%) revealed an \textit{NKX2-5} mutation in their lymphocytic DNA, with 12 distinct mutations (35). Interestingly, 16 of these 18 patients with an \textit{NKX2-5} mutation had no family history of CHD. Similarly, in a recent population-based study, 227 infants with CHD were examined for the presence
of five established germline NKX2-5 mutations, but none were detected (36). Taken together, there is little correlation between NKX2-5 genotypes and CHD. On the basis of the recent studies discussed subsequently, these results are consistent with sporadic septal defects arising from multiple mutations during early development.

Previous functional analysis of the eight missense HD mutations showed localization of all mutant proteins to the nuclei, but some exhibited anomalous nuclear distribution (e.g. T178M) (11). All mutants had exhibited markedly decreased DNA binding and reduced transcriptional activation, whereas interaction with transcriptional partners varied. We have undertaken functional studies on mutations affecting the HD of NKX2-5. Unlike the situation for single mutations being identified in lymphocytic DNA, the NKX2-5 gene from diseased cardiac tissues of patients with complex cardiac malformations typically contained multiple mutations (17). Neither have the reasons for the multiple mutations been established nor has the role of the individual mutations in the appearance of the disease been directly addressed. It remains to be determined whether heart disease associated with the single germinal mutations is due only to the single mutations or, alternatively, whether additional mutations arise in the heart that amplify the effect of the germinal mutations. Further, mutations in other transcription factor genes including GATA4, TBX5, FOG2 and CFC1 have been associated with CHD in humans, and there is emerging evidence from mutational studies for additional transcription factors to have a role in CHD (37). Recently, three individuals with common arterial trunk (CAT) were found to be carrying a homozygous c.451T>C (F151L) mutation in the HD of NKX2-6 (38). Similar to other germinal mutations in cardiac-specific transcription factor genes, F151L was familial and not detected in 10 sporadic cases of CAT. Therefore, studies in which there is only an examination of lymphocytic DNA may not reveal the molecular basis of CHD.

The assay we developed, which is the first to examine HD transcription factors in yeast, provides a convenient and powerful system for the functional characterization of single and multiple mutations to elucidate mechanisms of CHD. Noteworthy among the mutations examined with the yeast system is K183E, which was detected in 22 of 23 patients exhibiting AVSD, in seven of 16 with ASD, but was not detected among 29 VSD patients. The single K183E mutation led to complete loss of transactivation activity. In constrast to AVSD, where K183E was the only mutation in the third helix and specific for AVSD, there were four mutations in patients with VSD (Q187X, K192R, K192T, K194R). Two patients had combined K192R and K194R, which were located in the same allele. In VSDs, 14/29 had at least one mutation in the third helix, which led to inactivation or reduction of transactivation.

Dissection of the triple mutant haplotypes revealed that the individual K192R, K192T and K194R reduced transactivation to a level that ranged from 30 to ~80% of wild-type; some pair wise combinations of mutations had a much more dramatic effect (i.e. K192T, K194R). Also, there were examples where changes in transactivation differed greatly between REs, as found for ECE (2d) versus ANF (14d) with the K192R, A219V mutant protein. Although the transition from these results with a model system to clinical manifestations must be done with reservation, these observations with various single and multiple mutants and with different REs suggest that the various mutants might give rise to a variety of cardiac phenotypes.

In this regard, it is interesting that T178M and A219V are common mutations in three haplotypes from the same patient that also contain a third distinct mutation, suggesting that they originated prior to the third mutation (Table 1 and patient D03 in Table 2). T178M is a loss-of-function mutant in our assay, as well as in mammalian cell-based studies (13,14). A219V showed instead a mild reduction in function for all REs examined. T178 is located in the loop between the second and third helix of the HD, whereas A219 is located in the NK2-SD domain. Both T178M and A219V changes have been identified as germline CHD-associated mutations (33,34). Interestingly, there is preliminary evidence for mutations within NK2-SD contributing to CHD (http://cardiogenomics.med.harvard.edu/groups/proj1/pages/nk2-sd_home.html). On the basis of our molecular identification of mutations in disease-associated tissues (17) along with functional analyses of mutations, we propose that a germinal A219V mutation is a risk factor that when combined with other somatic mutations can increase the likelihood of CHD.

As the double mutant T178M/A219V is loss-of-function, a disease selection hypothesis would imply that the subsequent mutation confers additional negative impact on NKX2-5 functions. Given the mosaic nature of the diseased tissues, the presence of functional NKX2-5 proteins in individual cells cannot be excluded (17). Hence, the third mutations in the haplotypes might confer a dominant negative phenotype to the already transactivation defective mutant protein when engaged in dimers with functional NKX2-5 proteins. However, we were unable to detect dominance from the triple mutant protein in the yeast-based assay (Table 3). Alternatively, the multiple mutations may disrupt functions of NKX2-5 separate from the sequence-specific DNA binding. Along this line, it was shown that the NKX2-5 region between amino acids 182 and 199 is responsible for the physical interaction with the cardiac transcription factor GATA4 and that the K194I mutants abolished the interaction.
in vitro (28). Physical interaction between GATA4 and Nkx2-5 can result in transcriptional synergy (31,39–41). Recently, three germline GATA4 gene mutations have been associated with CHD (30,42): missense G296S, immediately after the C-terminal finger; a frameshift E359del and another frameshift mutation leading to a premature stop codon at amino acid 403. In addition, 12 somatic GATA4 mutations affecting the zinc fingers were detected in our patient cohort (19). Specifically, mutant C292R, which affects a zinc coordinating cysteine in the C-terminal zinc finger, was detected in 19 of 29 patients with VSD, suggesting that targeting the functional synergy between GATA4 and Nkx2-5 may result in septation defects. Taken together, these results suggest that disrupting the interaction between Nkx2-5 and GATA4 can contribute to the clinical manifestations of CHD.

In conclusion, the yeast-based functional assay provides opportunities to examine the consequences of single and multiple mutations in the master regulatory Nkx2-5 protein that is essential in normal heart development. Overall, the functional dissection of individual mutations contained in different haplotypes from the same patient suggested the selection in the disease tissues of Nkx2-5 variants impaired both in sequence-specific DNA binding and in physical interaction with other cardiac transcription factors. The approach that we have taken can be adapted to the direct cloning of separated Nkx2-5 alleles from genomic DNA isolated from heart tissue, especially because the gene comprises only two large exons. Similar approaches have been developed for the detection of p53 mutations in tumor tissue (43,44). This would facilitate the identification of mutations with reduced functionality in mixed samples. The system could also be extended to the analysis of other transcription factors involved in heart development whose function is altered in CHD, such as GATA4 and TBX5, and their functional interplay with Nkx2-5 in the activation of the transcriptional network of heart development.

MATERIALS AND METHODS

Cardiac malformations, genomic DNA isolation and detection of Nkx2-5 mutations

The characterization of malformed hearts, genomic DNA isolation and mutation detection in Nkx2-5 gene have been described previously (16). Briefly, we investigated 68 formalin-fixed hearts collected from 1954 to 1982 from patients with complex cardiac malformations. These malformed hearts were obtained from the Institute of Anatomy, University of Leipzig, Germany. Hearts were grouped according to their septal defects: 29 ventricular (VSD), 16 atrial (ASD) and 23 atrioventricular (AVSD). Most patients died at early infancy and were affected by multiple cardiac malformations.

Genomic DNA was isolated with NucleoSpin Tissue Kit (Macherey-Nagel, Düren Germany). Nkx2-5 fragments were amplified by PCR reaction and directly sequenced with specific primers. Mutations were confirmed by PCR–RFLP assay, when possible, or by cloning of the PCR fragments into TOPO TA Cloning Kit for re-sequencing (Invitrogen, Karlsruhe, Germany).

Cloning of wild-type Nkx2-5 into yeast expression vectors

Total RNA was isolated from normal heart tissue with RNAeasy Kit and RT–PCR was carried out with Omniscript RT Kit, according to the manufacturer’s procedure (Qiagen, Hilden, Germany). The coding region of Nkx2-5 was amplified using the same PCR conditions described for formalin-fixed samples (16) and PCR primers with incorporated Bam HI and Xho I sites. PCR fragments were digested with Bam HI and Xho I and cloned into the pTSG and the pTSG-TAD vectors using standard procedure. pTSG is a centromeric yeast expression vector based on the inducible GAL1 promoter (Fig. 1A) (21). pTSG-TAD is identical to pTSG but it contains the TAD derived from the human transcription factor p53 downstream GAL1 and allows the construction of A-terminal fusions with full-length Nkx2-5. Cloning of the Nkx2-5-Δ-SD was performed using a similar approach. DNA from the resulting plasmids pTSG-Nkx2-5, pTSG-TAD::Nkx2-5, pTSG-Nkx2-5-Δ-SD and pTSG-TAD::Nkx2-5-Δ-SD were isolated using Qiaprep Spin Miniprep Kit (Qiagen) and inserts were analyzed by restriction digestion and confirmed by DNA sequencing. To allow simultaneous selection of two Nkx2-5 expression vectors for the dominance assay, plasmid pUsG-TAD::Nkx2-5 was constructed by replacing the TRP1 selection marker in pTSG-TAD::Nkx2-5 with URA3 using standard cloning procedures.

Cloning of wild-type Nkx2-5 into yeast expression vector

Total RNA was isolated from normal heart tissue with RNAeasy Kit and RT–PCR was carried out with Omniscript RT Kit, according to the manufacturer’s procedure (Qiagen). The coding region of Nkx2-5 was amplified using the same PCR conditions described for formalin-fixed samples (16) and PCR primers with incorporated Bam HI and Xho I sites. PCR fragments were digested with Bam HI and Xho I and cloned into the pTSG and the pTSG-TAD vectors using standard procedure. pTSG is a centromeric yeast expression vector based on the inducible GAL1 promoter (Fig. 1A) (21). pTSG-TAD is identical to pTSG but it contains the TAD derived from the human transcription factor p53 downstream GAL1 and allows the construction of A-terminal fusions with full-length Nkx2-5. Cloning of the Nkx2-5-Δ-SD construct, with deletion of the C-terminal domain starting from amino acid 208, was performed using a similar approach. DNA from the resulting plasmids pTSG-Nkx2-5, pTSG-TAD::Nkx2-5, pTSG-Nkx2-5-Δ-SD and pTSG-TAD::Nkx2-5-Δ-SD were isolated using Qiaprep Spin Miniprep Kit (Qiagen) and inserts were analyzed by restriction digestion and confirmed by DNA sequencing. To allow simultaneous selection of two Nkx2-5 expression vectors for the dominance assay, plasmid pUsG-TAD::Nkx2-5 was constructed by replacing the TRP1 selection marker in pTSG-TAD::Nkx2-5 with URA3 using standard cloning procedures.
Preparation of yeast expression vectors with different NKX2-5 mutations

Multiple NKX2-5 mutations (e.g. triplets) were obtained from TOPO-TA cloned PCR fragments as part of the verification procedure for NKX2-5 variants from fixed heart samples (previously mentioned). Multiple mutations were brought into the wild-type NKX2-5 plasmids by standard digestion (KpnI/BsrI) and ligation protocols. Sequential dissections of three NKX2-5 mutants to obtain double and single mutations were carried out with the GeneTailor™ Site-Directed (SD) Mutagenesis System (Invitrogen). To facilitate amplification, NKX2-5 multiple mutants cloned in the small pBluescript plasmid (Stratagene, 3 kb) were used as templates for GeneTailor SD-mutagenesis. Q-solution (Qiagen) was also added during amplification step. Clones containing only the desired NKX2-5 mutation(s) were identified by DNA sequencing.

Phenotypic and quantitative yeast-based NKX2-5 functional assays

The construction of NKX2-5 reporter strains was performed using the master reporter strains yAFM-ICORE (ADE2 color reporter) and yLFM-ICORE (luciferase reporter), as previously described (26). Briefly, the ICORE cassette contains the counter-selectable KLYRA3, the reporter KanMX4 and the I-SceI endonuclease gene under control of the GAL1 promoter and its unique 18 nt recognition site. Induction of I-SceI on galactose leads to the generation of a single DNA double-strand break at the ICORE site, resulting in a dramatic increase in homologous DNA targeting by oligonucleotides along with coincident loss of the ICORE (25). The cassette is integrated upstream of a minimal cyl1 promoter which was placed to regulate the expression of the reporters at the ADE2 locus on chromosome XV. To generate NKX2-5 responsive strains, the ICORE was replaced with oligonucleotides containing the desired NK-REs, according to the delitto-perfetto protocol (25). NK-REs were derived from the promoters of proposed and validated NKX2-5 targets. The sequence and the structure of the NK-REs examined in this study are reported in Table 1. The ANF-, ECE- and DIO2-REs are derived from the promoters of the ANF, ECE and isoform 1b and the iodothyronine deiodinase genes, respectively. Details on strain construction are available upon request. The ADE2-based visual transactivation assay was performed on plates with low adenine (5 mg/l) to allow color detection (21). Transactivation of ADE2 results in a change of the colony color from red to pink or white. Differences in transactivation capacities with the different REs are estimated based on the amount of NKX2-5 required for transactivation, which is dependent on the amount of galactose inducer, and is based on the approach we developed to analyze p53 transactivation capacity (21). Luciferase assays were performed using protein extracts obtained from the mechanical lysis of yeast cells grown in liquid cultures, as previously described (26). The averages for induced light units and the standard deviations of at least three biological replicates are presented in Figures 1C and 2 and reported in Tables 2 and 3.

ACKNOWLEDGEMENTS

We thank Annika Roskowetz and Andreas Hiemisch for technical support, and we appreciate the financial support to J.B. (grant no. 25A.5-76251-99-3/00) of the Lower Saxony Ministry of Science and Culture, Germany.

Conflict of Interest statement. None declared.

REFERENCES