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The Different Cardiac Expression of the Type 2 Iodothyronine Deiodinase Gene between Human and Rat Is Related to the Differential Response of the *dio*2 Genes to Nkx-2.5 and GATA-4 Transcription Factors

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By producing T_3 from T_4, type 2 iodothyronine deiodinase (D2) catalyzes the first step in the cascade underlying the effect exerted by thyroid hormone. Type 2 iodothyronine deiodinase mRNA is expressed at high levels in human heart but is barely detectable in the corresponding rodent tissue. Although the heart is a major target of thyroid hormone, the role of cardiac D2 and the factors that regulate its expression are unknown.

Here we report that the human *Dio2* promoter is very sensitive to the cardiac transcription factors Nkx-2.5 and GATA-4. Nkx-2.5 transactivates a 6.5-kb human (h)*Dio2*-chloramphenicol acetyltransferase construct, with maximal induction reached with a 633-bp proximal promoter region. Interestingly, despite 73% identity with the corresponding human region, the rat *Dio2* promoter is much less responsive to Nkx-2.5 induction. Using EMSA, we found that two sites in the human promoter (C and D) specifically bind Nkx-2.5. In coexpression studies, GATA-4 alone was a poor inducer of the h*Dio2* promoter; however in synergy with Nkx-2.5, it activated D2 reporter gene expression in the human, but not the rat promoter. Functional analysis showed that both C and D sites are required for the complete Nkx-2.5 response and for the Nkx-2.5/GATA-4 synergistic effect. In neonatal rat primary myocardiocytes, most of the h*Dio2*-chloramphenicol acetyltransferase activity was suppressed by mutation of the Nkx-2.5 binding sites. Finally, a mutant Nkx-2.5 protein (N188K), which causes, in heterozygosity, congenital heart diseases, did not transactivate the *Dio2* promoter and interfered with its activity in cardiomyocytes, possibly by titrating endogenous Nkx-2.5 protein away from the promoter.

In conclusion, this study shows that Nkx-2.5 and GATA-4 play prime roles in *Dio2* gene regulation in the human heart and suggests that it is their synergistic action in humans that causes the differential expression of the cardiac *Dio2* gene between humans and rats. (*Molecular Endocrinology* 17: 1508–1521, 2003)

T_4, THE MAIN PRODUCT of the thyroid gland, must be converted into T_3 to exert its function in the cell. Type 2 iodothyronine deiodinase (D2) is an obligate outer-ring selenodeiodinase that catalyzes the conversion of T_4 to T_3 and of rT_3 to 3,3′-T_2 (for a review, see Ref. 1). This is the first step in the sequence of events underlying the action of thyroid hormone and serves to regulate the intracellular T_3 concentration in tissues that express D2. Type 2 iodothyronine deiodinase is critical for tissues such as rat brain, where it produces more than 75% of nuclear T_3 (2). Northern blot analysis showed that D2 expression in humans is more extensive than previously supposed (3). Human D2 mRNA has been found in thyroid, brain, spinal cord, skeletal muscle, placenta, and, albeit at low levels, also in kidney and pancreas (1). Although D2 has been highly conserved during evolution and has been found in all vertebrate species examined so far, its tissue distribution varies from species to species. The differences in D2 expression suggest that it exerts a species-specific function. In this regard, D2 mRNA is highly expressed in human heart (3) but apparently not in rat heart (4). The definition of human and rat *Dio2* promoters led to the identification of DNA regions that are critical for D2 expression, *e.g.* a functional cAMP responsive element (5) and two binding sites for the thyroid transcription factor-1 (TTF-1/Nkx-2.1) that are required for D2 expression in the thyroid (6).

The heart is a major target of thyroid hormone and one of the most sensitive organs to variations in

Abbreviations: ANF, Atrial natriuretic factor; CAT, chloramphenicol acetyltransferase; C/EBPα, CCAAT enhancer-binding protein-α; CMV, cytomegalovirus; DTT, dithiothreitol; Nkx-2.5-wt, wild-type Nkx-2.5; TTF, thyroid transcription factor.

plasma thyroid hormone levels (7). T_3 can increase myocardial inotropy and heart rate and can dilate peripheral arteries to reduce afterload (8). At a molecular level, at least three cardiac genes are known to respond transcriptionally to T_3, namely, α-myosin heavy chains (9, 10), the hyperpolarization-activated cyclin nucleotide-gated channel 2 (11), and sarcoplasmic reticulum calcium ATPase (12). The expression of D2 in human heart and its absence from rodent heart is one of the most intriguing differences in mammalian deiodinase physiology and indicates that in this regard, the rodent is not a faithful model of the human situation. The role of cardiac D2 and the transcriptional factors that regulate cardiac D2 gene expression are not known.

Nkx-2.5 and GATA-4 are among the main regulators of tissue-specific transcription in the heart (13). Nkx-2.5 is a homeobox-containing gene originally identified as a potential vertebrate homolog of the *Drosophila* gene *tinman* (14). It belongs to the NK2 class of homeobox proteins, which are characterized by a tyrosine residue at amino acid 54 of the homeodomain and a conserved 23-amino acid NK2-specific domain (15). Nkx-2.5 is expressed in the heart (14, 16) and heart progenitor cells in the adult and in the very early developmental stage when the two-heart primordials are symmetrically situated in the anterior lateral mesoderm (17). Mice lacking Nkx-2.5 die around embryonic d 11 (E11) due to the abnormal looping morphogenesis of the primary heart tube (18, 19). Overexpression of wild-type Nkx-2.5 in *Xenopus* and Zebrafish models increases the number of cardiac myocytes, resulting in cardiac enlargement (20, 21), whereas cardiac expression of a dominant negative Nkx-2.5 mutant in *Xenopus* embryos reduces heart size and, in the most severe cases, prevents the formation of the heart (22, 23). Recently, heterozygous mutations of human Nkx-2.5 were identified in patients affected by a variety of congenital heart diseases including progressive atrioventricular conduction delays (AV block), ventricular septal defect, tetralogy of Fallot, and tricuspid valve abnormalities (24, 25). However, only few downstream targets of the Nkx-2.5 protein have been identified, and the molecular mechanisms by which Nkx-2.5 mutations cause heart abnormalities are unknown. The atrial natriuretic peptide (26), A1 adenosine receptor (27), ventricular myosin light chain 2, and the cardiac ankyrin-repeat protein (CARP) (28) are among the *in vivo* downstream targets of Nkx-2.5. Nkx-2.5 protein binds with high affinity to the sequence 5′-TNNAGTG-3′, which is different from the typical 5′-TAAT-3′ core found in most homeodomain factor DNA-binding sites (15).

GATA-4 plays a key role in regulating heart-specific gene expression. During embryogenesis GATA-4 is expressed in the precardiac mesoderm at E7.5 and in the endocardial and myocardial layers of the heart tube (29). It alters transcription of target genes by binding to the WGATAR consensus sequence through its DNA-binding domain, which consists of two adjacent zinc fingers of the C2/C2 family. It regulates such cardiac structural genes as the α-myosin heavy chain, troponin-C, atrial natriuretic factor, and brain natriuretic peptide (30). Mice with GATA-4-targeted disruption develop apparently normal cardiomyocytes, but they die early in embryogenesis due to defects in the morphogenetic movements that are required for the formation of the linear cardiac tube (31, 32). Nkx-2.5 and GATA-4 specifically cooperate in activating atrial natriuretic factor (ANF) and other cardiac promoters and can physically interact both *in vitro* and *in vivo* (33–35).

Nothing is known about the potential role of any of the heart-specific transcription factors in deiodinase gene expression. In this study, we explored the molecular basis of human cardiac *Dio2* expression by analyzing the capability of Nkx-2.5 and GATA-4 to activate the *Dio2* promoter. We found that Nkx-2.5 is a potent inducer of the *Dio2* promoter and that Nkx-2.5/GATA-4 synergism is responsible for the high D2 mRNA levels found in the human, but not in the rat, heart. We also provide evidence that overexpression of a putative dominant negative Nkx-2.5 protein (N188K), which is frequently the cause of human genetic cardiac defects (36), down-regulates human (h)*Dio2* promoter activity in rat neonatal primary cardiomyocytes, in part by titrating endogenous Nkx-2.5 away from the promoter region.

RESULTS

D2 mRNA Expression in Human and Rat Hearts

We analyzed human (atrium and ventricle) and rat ventricle for D2 mRNA expression by using RT-PCR analysis with D2-specific primers (D2-1s and D2-2r, which recognize a D2 cDNA region that is 100% identical in rat and human). A 590-nucleotide band corresponding to D2 appeared in human ventricle cDNA and, at a lower intensity, in the atrium (Fig. 1A). The densitometric analysis of the D2 band, observed in the rat ventricle, revealed a signal about 3-fold less intense as compared with the human sample. Semiquantitative glyceraldehyde-3-phosphate dehydrogenase amplification is included as a control for equivalence of the cDNAs (Fig. 1B).

Transactivation of the h*Dio2* Promoter by the Homeodomain Factor Nkx-2.5

We hypothesized that the much higher D2 mRNA level in human heart compared with rat heart is due to differences in the response to a heart-specific transcription factor of the human *vs.* the rat *Dio2* gene. In a transient transfection assay of HeLa cells, a 6.5-kb h*Dio2*-CAT promoter construct was tested with or without two essential cardiac-specific transcription factors, Nkx-2.5 and GATA-4. We analyzed Nkx-2.5 because its consensus binding site is virtually identical

with the cognate transcription factor TTF-1 (38) that regulates D2 expression in human thyroid. GATA-4 was selected because computer-assisted analysis of the human and rat promoter regions for cardiac transcription factors revealed several GATA-4 consensus-binding sites within the proximal upstream regulatory region (Fig. 2).

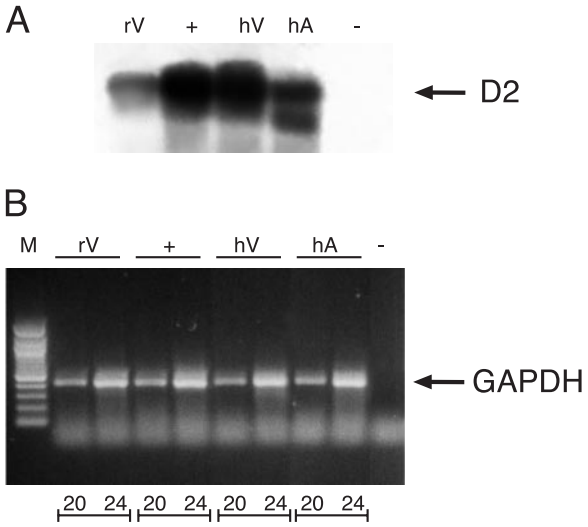

Fig. 1. D2 mRNA Is Expressed in Human and Rat Heart

D2 mRNA expression in human and rat heart was examined with semiquantitative RT-PCR. Total RNA was isolated from rat ventricle (rV), and human left atrium (hA) and left ventricle (hV). Human thyroid (+) cDNA served as a positive control for D2 amplifications. A, D2 amplifications (30 cycles) were detected after Southern blot analysis using a human D2 cDNA-labeled fragment as probe. B, Glyceraldehyde-3-phosphate dehydrogenase amplification products (20 and 24 cycles), used as an internal control, were viewed by ethidium bromide staining and photographed under UV light. The figure refers to one RT-PCR, which was repeated twice with the same tissues, with comparable results. (M): 100-bp DNA ladder. A and B, The (−) lane equates with no cDNA.

Nkx-2.5 transactivated the 6.5-kb h*Dio2* chloramphenicol acetyltransferase (CAT) reporter (Fig. 3A) to an extent similar to TTF-1; activity was maximal (13-fold) with 100 ng/dish of the transfected plasmid (Fig. 3B). GATA-4, the other heart-specific transcription factor tested, did not significantly increase h*Dio2*-CAT activity over basal level (Fig. 3A). To assess whether Nkx-2.5 is involved in the expression of D2 in adult thyroid, we analyzed Nkx-2.5 mRNA expression in adult thyroid by both Northern blot (using 5 μg of human thyroid Poly A+) and RT-PCR. No Nkx-2.5 expression was detected in human adult thyroid (data not shown), which confirms the cardiac specificity of Nkx-2.5 in D2 regulation.

To identify the *cis*-regulatory elements in the h*Dio2* promoter that mediate the transcriptional response to Nkx-2.5, we analyzed a broad series of h*Dio2*-promoter 5′-truncated constructs. As shown in Fig. 4A, deletion constructs from its 5′-end revealed that, within the 6.5-kb 5′-flanking DNA examined, the −633-bp DNA fragment reproducibly induced maximal CAT activity (32-fold over basal level). The −633-bp DNA fragment contains the C and D sites that are required for TTF-1 regulation in the thyroid gland (6). An additional 5′-deletion, which eliminates the C site, caused a lower Nkx-2.5 response (17- *vs.* 32-fold), which was further decreased (9- *vs.* 17-fold) when base pairs from −240 to −83 nt, including the D site, were deleted (h*Dio2*-83 bp). Interestingly, deletion of the region between −83 and −60 bp almost abolished the Nkx-2.5 response (h*Dio2*-60 bp), although that region did not bind Nkx-2.5 by EMSA (data not shown). Also the rat *Dio2* promoter constructs responded transcriptionally to Nkx-2.5, although to a much lesser extent with respect to the corresponding human promoter region (Fig. 4B). The −658-bp rat construct (r*Dio2*-3) was induced only 9-fold over the basal level by cotransfected Nkx-2.5 (Fig. 4B), a response that is less than 30% of the corresponding human promoter (Fig. 4A).

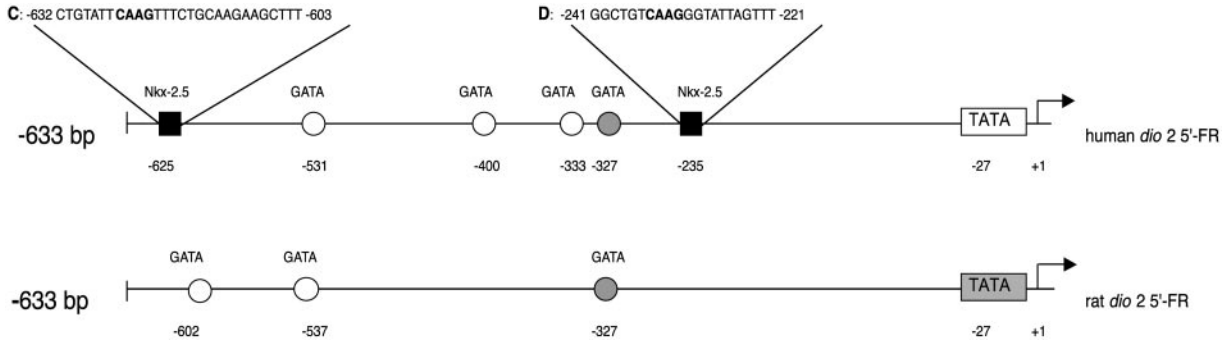

Fig. 2. Schematic Structural Organization and Comparison of the Proximal Human and Rat *Dio2* Promoters

Localization of the Nkx-2.5 and GATA-4 transcription factor binding sites in the human and rat proximal promoters are indicated. Putative binding sites deduced by computer-assisted analyses of the indicated region are indicated by *open circles*, and their locations relative to transcription start site are indicated. The only GATA-4 binding site conserved between human and rat sequences is indicated by a *shaded circle*. The C and the D sites and corresponding oligonucleotides are indicated.

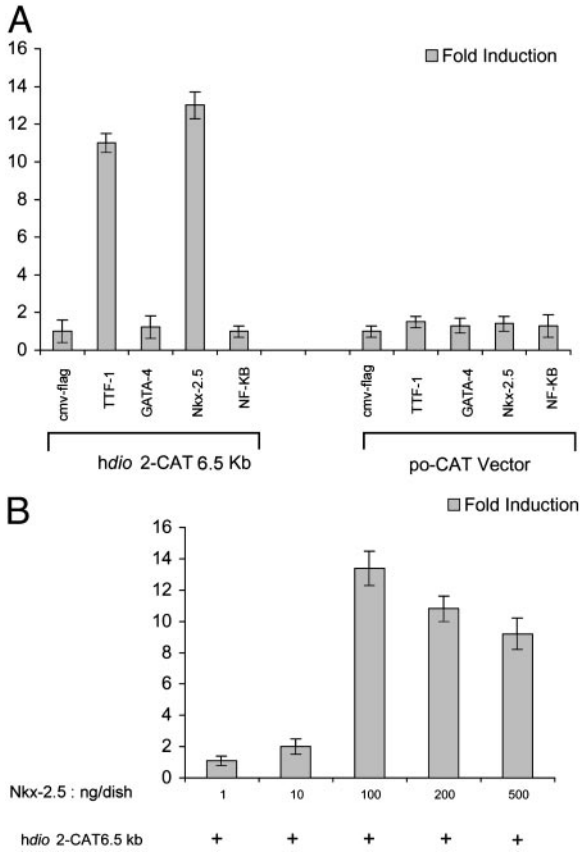

Fig. 3. Nkx-2.5 Transactivates the h*Dio2* Proximal Promoter in a Dose-Dependent Fashion

A, HeLa cells were transiently transfected with CAT reporter construct (3 μg/35-mm dish) containing the 6.5-kb *Dio2* 5′-flanking region (or the po2-CAT empty vector) together with CMV-Flag (0.2 μg/35-mm dish, set as 1 in the experiment) or the plasmids encoding the indicated transcription factors (0.2 μg/35-mm dish). B, The 6.5-kb-h*Dio2*-CAT promoter construct (3 μg/35-mm dish) was cotransfected with the indicated amounts of the Nkx-2.5 expression plasmid. Results are shown as the mean ± SD of the CAT/Luc ratios of at least three separate experiments done in duplicate.

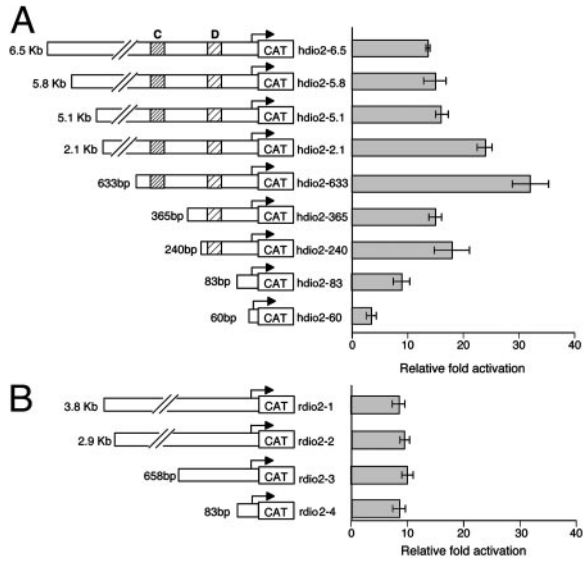

Fig. 4. Definition of the Regions within Human and Rat *Dio2* Promoter Constructs that Respond Transcriptionally to Cotransfected mNkx-2.5

HeLa cells were transiently cotransfected with various human (A) or rat (B) *Dio2* promoter constructs, (3 μg/35-mm dish, *left panels*), and constant amounts of Nkx-2.5 plasmid or empty vector (0.1 μg/35-mm dish) (see *Materials and Methods*). Data are shown as the mean ± SD of the CAT/Luc ratios of at least four separate experiments done in duplicate.

EMSA Analysis of the Interaction of Nkx-2.5 with the h*Dio2* Promoter

To correlate the functional response of the putative Nkx-2.5 binding sites in the h*Dio2* promoter to DNA binding regions, we performed EMSA using nuclear extracts from Nkx-2.5-transfected Bosc-23 cells with either the D or the C corresponding probes (see Fig. 2). We used Bosc-23 cells (a clone derived from HEK-293, see *Materials and Methods*) to obtain a large amount of transfected Nkx-2.5 protein. As shown in Fig. 5A, addition of the nuclear extracts of Nkx-2.5-transfected cells to the radiolabeled D oligonucleotide produces a single-shifted band. A weaker band, probably corresponding to endogenous Nkx-2.5, appeared in nontransfected cells. Northern blot analysis with Bosc-23 cell PolyA+ confirmed the presence of a novel Nkx-2.5 mRNA (data not shown). Nkx-2.5 binding to D is specific because it was competed by the nonradiolabeled probe in a dose-dependent manner and was specifically displaced by the cold NKE-2 oligonucleotide (Fig. 5A), which contains the high-affinity Nkx-2.5 binding site from the proximal ANF promoter (35). Because the transfected Nkx-2.5 protein contains a flag epitope at its NH_2 terminus (see *Materials and Methods*), we were able to supershift the Nkx-2.5/D-probe complex using an anti-flag antibody (Fig. 5B). The C oligonucleotide, corresponding to the 5′-putative Nkx-2.5 binding sites in the −633 h*Dio*-promoter, also specifically binds Nkx-2.5 (Fig. 5A). The EMSA pattern was complex and contained three major bands, all of which were significantly competed by an excess of nonradiolabeled self-oligonucleotide. However, only the lower band of the doublet (*open arrow*) was efficiently displaced by cold NKE-2, and its corresponding Nkx-2.5/C-probe complex formation was shifted by the anti-flag antibody (Fig. 5, A and B). Because Nkx-2.5 binds to several proteins as transcriptional partners (39–42), the multiple bands observed in this study may correspond to the formation of a ternary complex with accessory proteins in the context of the C oligonucleotide. The relative binding affinities obtained from the densitometric analysis of the EMSA data (Fig. 5A) suggest that D binds Nkx-2.5 with a higher affinity when compared with C.

To assess whether the D site binds Nkx-2.5 in human heart, we examined nuclear extracts from human

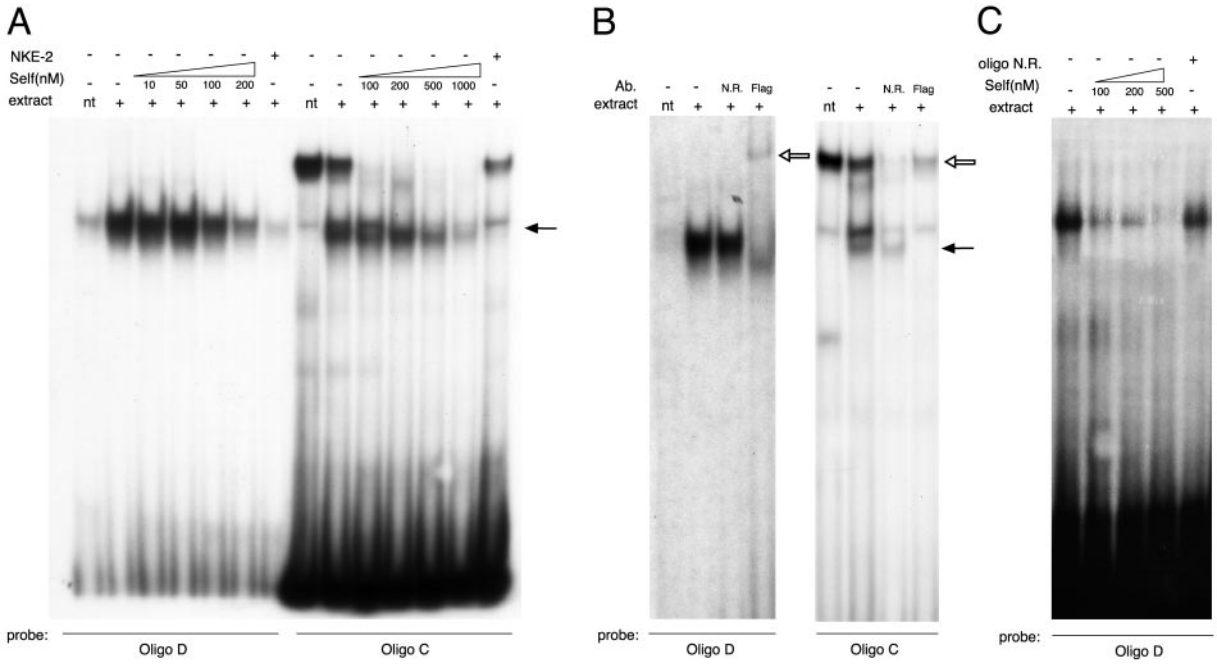

Fig. 5. Semiquantitative EMSA Analysis for the Interaction of the Mouse Nkx-2.5 Protein to the D and C Sites of h*Dio2* 5′-FR

A, EMSA analysis with oligonucleotides D and C. Nuclear extracts (2 μg) from Bosc-23-transfected cells with (+) or without (nt) Nkx-2.5 expression plasmid were added to radiolabeled oligonucleotides D and C as indicated. The "self" row indicates the unlabeled probe at the concentrations used for competition. The *solid arrow* indicates the lower band of the doublet that was specifically displaced by the rat ANF NKE-2 oligonucleotide (*last lane*). Autoradiogram exposure time was 16 h. B, Supershift analysis of the mouse Nkx-2.5 binding to the D and C sites. Nuclear extracts without (n.t.) or with (+) transfected mNkx-2.5 were preincubated with an anti-Flag (Flag) or unrelated (N.R.) antibody for 30 min before EMSA analysis. The *open arrow* indicates the bands supershifted by the anti-Flag antibody. C, Interaction of cardiac protein with the D oligonucleotide. Endogenous Nkx-2.5 in human ventricular extract binds the D probe. Nuclear extracts from human ventricle were assayed for the capacity to form complexes with the D oligonucleotide. Unlabeled competitor oligonucleotide and unrelated oligonucleotide (N.R.) were used at the indicated nanomolar concentrations.

left ventricle by EMSA. As shown in Fig. 5C, the D oligonucleotide formed a specifically shifted band with heart nuclear proteins; the binding was competed in a dose-dependent manner by an excess of cold D but not by an unrelated oligonucleotide. The band disappeared in a supershift experiment with an anti-Nkx-2.5 antibody (data not shown).

Both C and D Sites Are Required for a Complete Functional Response of h*Dio2* to Nkx-2.5 and for the Expression of h*Dio2* in Rat Neonatal Cardiomyocytes

To assess the functional contribution of sites C and D to the transactivation of h*Dio2* promoter by Nkx-2.5, we disrupted both sites in the context of the −633-bp construct and evaluated the functional response to Nkx-2.5. Mutation of the D site only partially reduced the transactivation response to Nkx-2.5 (Fig. 6), and a similar, although slightly more potent, reduction was observed by deletion of the C site. When both C and D sites were mutated within the −633 promoter, the h*Dio2* response was drastically reduced (Fig. 6). The double mutant construct h*Dio*-633 (2 m), in which the CAAG core motif for both C and D sites is mutated to CGTG, resulted in only a 9-fold induction by Nkx-2.5, which is comparable to that observed with the rat *Dio2*-3 constructs (see Figs. 4 and 6). This finding is consistent with the absence of C and D sites in both constructs.

To assess the relevance of endogenous Nkx-2.5 in *Dio2* expression in the heart, we transfected primary neonatal rat cardiomyocytes with either human or rat *Dio2* promoter constructs. As shown in Fig. 7, several h*Dio2* promoter deletion constructs significantly induced CAT expression in primary cardiomyocytes. The rat promoter (−3.8 kb) was much less efficient in driving CAT expression in rat cardiomyocytes (Fig. 7), a finding consistent with the weaker D2 expression in the rat heart. Neither the rat-dio2#2 construct nor the rat-dio2#3 shorter promoter construct differs significantly from the −3.8-kb promoter when transfected in primary cardiomyocytes (data not shown). With h*Dio2*-633 (2 m) in which both Nkx-2.5 sites were mutated, CAT levels were significantly lower than with h*Dio2*-633, thereby confirming that Nkx-2.5 binding sites C

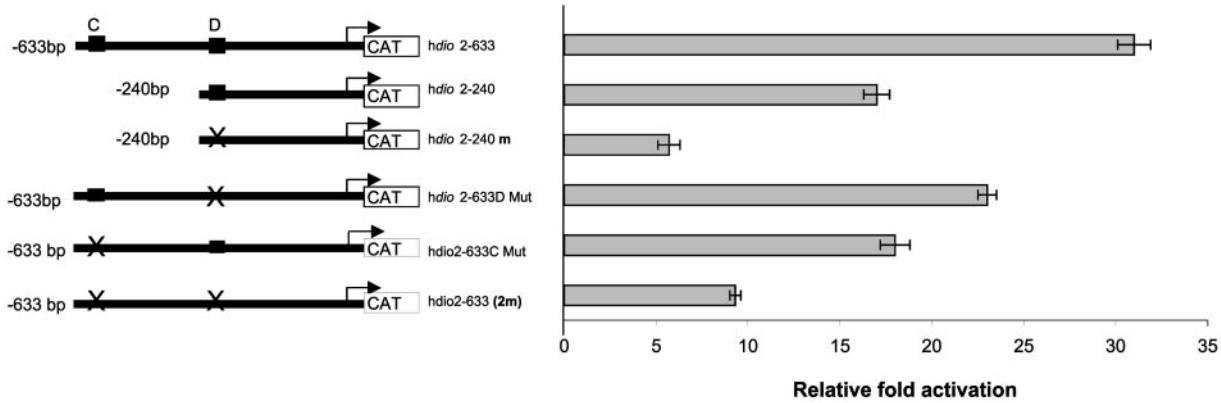

Fig. 6. Effect of Mutations in the C and D Sites of the h*Dio2* Gene on the Functional Response to Transiently Expressed Nkx-2.5 in HeLa Cells

The C and D sites were mutated individually or together by site-directed mutagenesis in the context of the −633-bp or −310-bp h*Dio2* promoter constructs. Data are shown as the mean ± SD of the CAT/Luc ratios of four separate experiments done in duplicate.

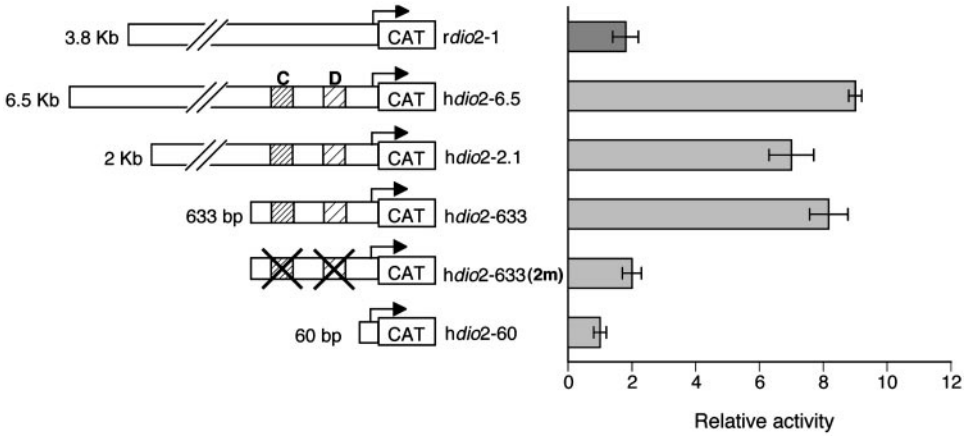

Fig. 7. The C and D Nkx-2.5 Binding Sites Contribute to h*Dio2* Promoter Activity in Neonatal Cardiomyocytes

Transfections were carried out in neonatal primary cardiomyocytes from 1-d-old rats as described in *Materials and Methods*. The results are expressed relative to the activity of the h*Dio2*-60-bp minimal promoter, taken as 1. Data are shown as the means ± SD of three independent experiments based on the CAT/Luc ratios.

and D play critical roles in the cardiac expression of *Dio2*.

Nkx-2.5 and GATA-4 Synergistically Transactivate the h*Dio2* Promoter through the Nkx-2.5 Binding Sites

Because the h*Dio2*−633 promoter construct contains multiple putative GATA-4 binding sites (see Fig. 2), and although GATA-4 is unable by itself to transactivate h*Dio2*-promoter (see Fig. 3), we examined the possibility that Nkx-2.5 and GATA-4 could functionally interact at the level of the h*Dio2* promoter. When the h*Dio2*-633-bp 5′-flanking region of the *Dio2* gene was cotransfected with the Nkx-2.5 plasmid in limiting conditions (5 ng of transfected Nkx-2.5/dish), transactivation of the h*Dio2* promoter was modest (Fig. 8); similarly, promoter activity did not significantly increase with the GATA-4 protein. Interestingly, transfection of Nkx-2.5 and GATA-4 together induced a potent transactivation of h*Dio2*-633 bp (~21-fold), which was much stronger when compared with the single effects (or their sum) of the two transcription factors (Fig. 8). These results indicate that Nkx-2.5 and GATA-4 synergistically transactivate the h*Dio2* promoter, suggesting that both factors contribute to *Dio2* gene regulation in human heart. A previous report on the ANF promoter indicates that the synergy between Nkx-2.5 and GATA-4 depends on the integrity of the Nkx-2.5 binding sites (33). We investigated whether this was the case also with the rat *Dio2* promoter and found there was no cooperative effect with the rat construct, which lacks both the D and C Nkx-2.5 binding sites (Fig. 8). This suggested that the C and D sites are required for Nkx-2.5 and GATA-4 to act in synergy. We next intro-

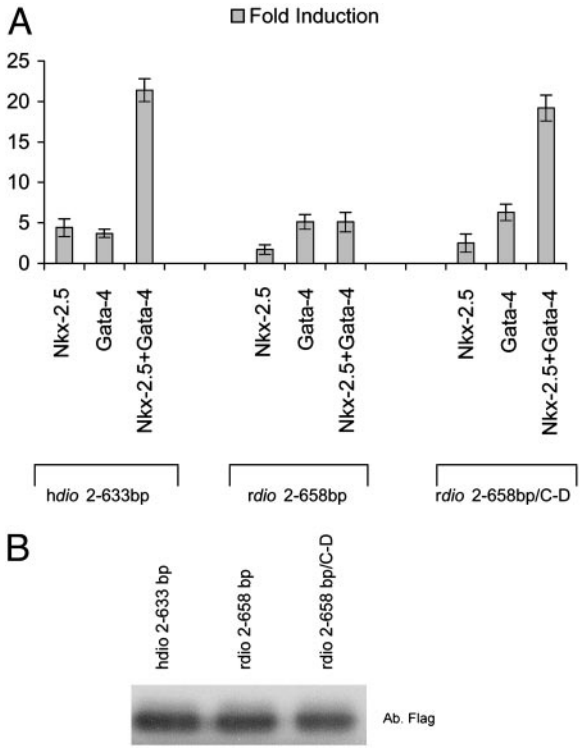

Fig. 8. Nkx-2.5 and GATA-4 Synergistically Transactivate the Human, But Not Rat, *Dio2* Promoter

A, HeLa cells were cotransfected with the −633-bp human or −658-bp rat *Dio2* promoter constructs and the expression plasmid of Nkx-2.5 (5 ng/plate) and/or GATA-4 (50 ng). Nkx-2.5 and GATA-4 synergistically transactivated the human reporter construct, but not the corresponding rat construct unless it contained the human C and D sites (r*Dio2*-658 bp/C-D). The data (CAT/Luc ratios) represent the mean of three independent experiments done in duplicate and *error bars* correspond to SD. B, To examine relative Nkx-2.5 expression levels produced with different cotransfected plasmids, Western blot analysis was performed with cell extracts derived from a parallel experiment. Nuclear extracts prepared as described in *Materials and Methods* were loaded on an SDS-PAGE gel at 20 μg of protein per lane and tested with the indicated antibody.

duced the human D and C sites into the rat *Dio2* promoter (rdio 2-658 bp/C-D) and assayed the artificially created promoter for synergy. Under these conditions there was a synergistic effect between Nkx-2.5 and GATA-4 as opposed to the wild-type rat *Dio2* (Fig. 8). When we used the single mutant of the hdio2-633-bp construct, either the hdio2-633-Cmut or hdio2-633-Dmut, we still observed cooperation between Nkx-2.5 and GATA-4 (15- and 18-fold, respectively), although their intensity was slightly reduced as compared with the wild-type promoter containing both Nkx-2.5 binding sites (data not shown). These data demonstrate that the synergism between Nkx-2.5 and GATA-4 is sequence specific and that reintroduction of an Nkx-2.5 binding site is necessary and sufficient for such an effect.

The differences in induction were not due to discrepancies in Nkx-2.5 expression as demonstrated by the immunoblotting experiment shown in Fig. 8B.

A N188K Nkx-2.5 Mutant Does Not Transactivate the h*Dio2* Promoter and Reduces Endogenous Nkx-2.5 Activity

Many Nkx-2.5 nonsense and frame-shift mutations have been reported in patients with congenital heart disease (24, 25). Among these, an N188K missense mutation on a single allele affecting the Nkx-2.5 homeodomain region causes congenital cardiac malformations. To investigate the putative effect of this mutation on D2 expression, we tested the capacity of the Nkx-2.5-N188K mutant to transactivate the h*Dio2*-promoter. Interestingly, the Nkx-2.5 mutant was unable to transactivate the h*Dio2*-633-CAT (Fig. 9A), indicating that a functional Nkx-2.5 homeodomain region is necessary for *Dio2* promoter transactivation. EMSA showed that Nkx-2.5-N188K does not bind the D oligonucleotide (Fig. 9B). Neither the loss of the transcriptional effect nor the DNA binding properties of the Nkx-2.5 mutant was caused by effects exerted by the mutation on protein expression or stability because Western blots showed comparable levels of transiently expressed wild-type and mutant Nkx-2.5 protein (Fig. 9C).

Because Nkx-2.5 can dimerize to exert its functional activity (36), we tested whether N188K could affect the action of wild-type Nkx-2.5 on *Dio2* gene expression. Neonatal rat cardiomyocytes were cotransfected with h*Dio2*-633 bp and either wild-type Nkx-2.5 (Nkx-2.5-wt) or the Nkx-2.5 N188K mutant. The transfected Nkx-2.5-wt increased *Dio2* CAT activity by almost 2-fold over basal level, whereas transfection with the Nkx-2.5-N188K-encoding plasmid reduced CAT activity to less than 40% of the control (Fig. 10A). To gain insights into the apparently dominant negative effect exerted by N188K, we performed EMSA after preincubating increasing ratios of N188K-transfected nuclear extract with wild-type Nkx-2.5 for 20 min before adding the D probe. Nkx-2.5-N188K interfered with the shifted band, reducing Nkx-2.5-wt binding to the D probe in a dose-dependent fashion (Fig. 10B). To better characterize the effect exerted by N188K, we performed a functional titration assay in HeLa cells by cotransfecting increasing amounts of N188K *vs.* Nkx-2.5 wild type and measuring the functional effect on the hdio2-633-bp reporter construct. At a 1:1 molar ratio of N188K/Nkx-2.5, induction by Nkx-2.5 was almost completely abrogated on the h*dio2*-633-bp promoter (Fig. 10C) and on the rat dio2 constructs (rdio2#1, rdio2#3, data not shown). These data suggest that the N188K mutant might exert its dominant-negative effect *in vivo* in heterozygosity, in part by titrating Nkx-2.5-wt away from the h*Dio2* target DNA. Although we introduced the N188K mutation into the mouse Nkx-2.5 protein, the 100% identity of the human and mouse homeodomain regions suggests that

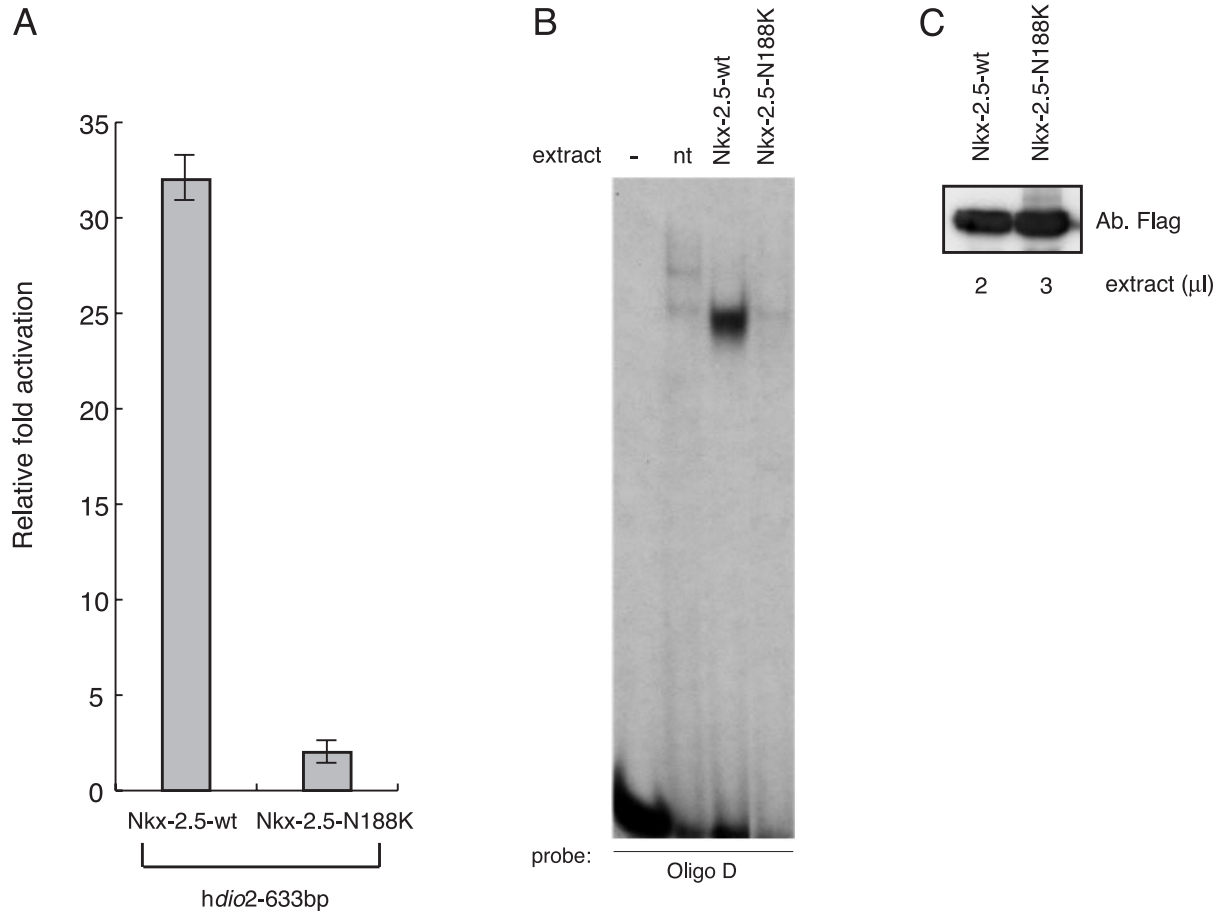

Fig. 9. Nkx-2.5-N188K Neither Transactivates the h*Dio2* Promoter nor Binds h*Dio2* DNA

A, HeLa cells were cotransfected with Nkx-2.5-wt expression plasmid or Nkx-2.5-N188K mutant and the h*Dio2*-633 construct. Data are shown as the mean ± SD of the CAT/Luc ratios of four separate experiments. B, The D oligonucleotide binds strongly to wild-type Nkx-2.5, but not to the N188K mutant. Comparison of the binding of Nkx-2.5-wt and Nkx-2.5-N188M to D oligonucleotide by EMSA analysis. Equal amounts of Nkx proteins were used for each lane. C, To examine relative expression levels, Western blot analysis was performed with cells transfected in parallel with Nkx-2.5-wt and Nkx-2.5-N188K plasmid. Nuclear extracts prepared as described in *Materials and Methods* were loaded on an sodium dodecyl sulfate-polyacrylamide gel at 10 μg of protein per lane and tested with the indicated antibody. The amount of extract used for each lane is also indicated.

the mouse mutant is a faithful model of regulation at the human promoter. Preliminary data obtained in our laboratory with the cloned wild-type and mutant human Nkx-2.5 proteins support this hypothesis.

DISCUSSION

Type 2 iodothyronine deiodinase is a critical component of the homeostatic mechanism regulating tissue T_3 concentration. It is a selenoenzyme of short half-life, which rapidly responds to T_3 demand through a direct regulation by T_3 and T_4 concentrations at both transcriptional and posttranscriptional levels, respectively (1). The expression of D2 in the myocardium raises the possibility that, in humans, this organ can respond not only to changes in plasma T_3, but also to changes in T_4, which is the main thyroid product and also the optimal D2 substrate. This would account for the association between increased heart rate and slight increases in circulating T_4 frequently seen in patients with subclinical hyperthyroidism or under treatment with L-T_4 (8). The evolutionary advantage of such a D2-mediated mechanism would be enormous in situations of reduced T_4 production, *e.g*. iodine deficiency or mild hypothyroidism. In this context, the higher expression of cardiac D2 in humans as compared with rodents confers a more efficient homeostatic mechanism by which to protect the heart from low T_3 concentrations.

The aim of this study was to determine the molecular mechanisms governing D2 expression in human and rat myocardium. Hitherto, it was debated whether or not the rat heart contained D2 (43, 44). Using RT-PCR, we provide the first demonstration of a D2 mRNA transcript in the rat heart. Extending the analysis to

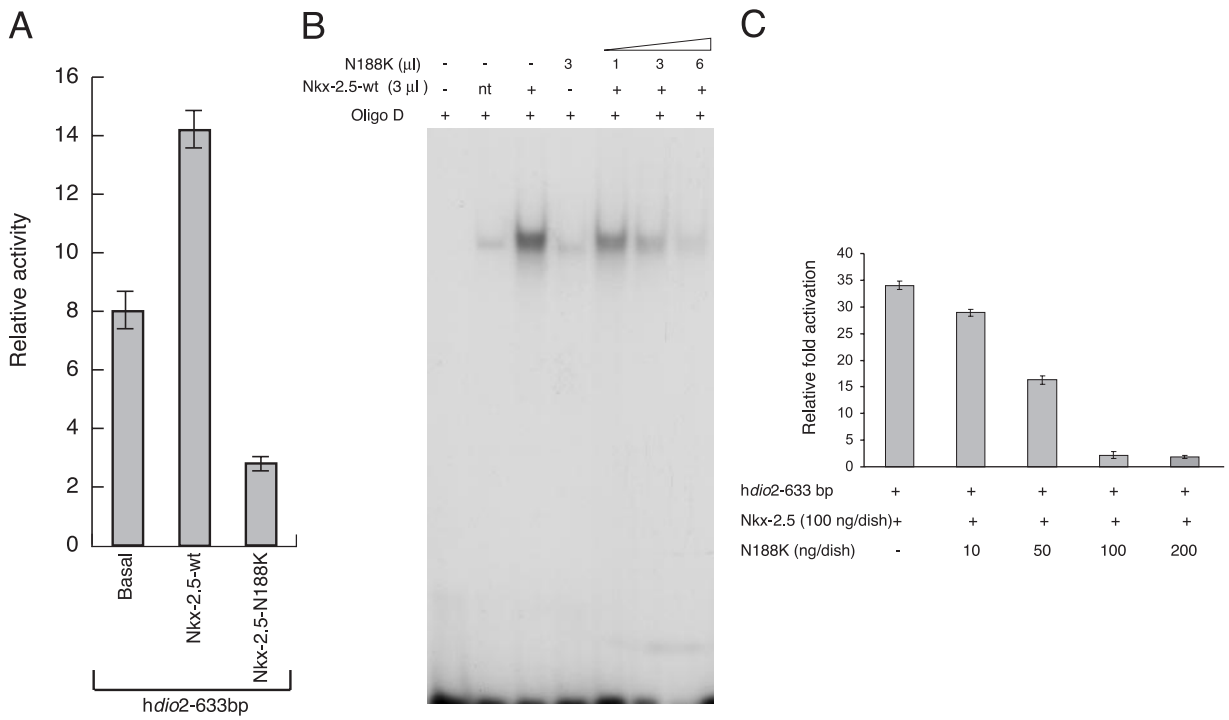

Fig. 10. Nkx-2.5-N188K Reduces Endogenous Nkx-2.5 Action by Interfering with the Binding of Nkx-2.5-wt from the D Oligonucleotide

A, Neonatal rat cardiomyocytes were transfected with the h*Dio2*-633-bp promoter and the indicated plasmids. The results (CAT/Luc ratio) are expressed relative to the activity of the h*Dio2*-60-bp minimal promoter, taken as 1. Data are means ± SD of three independent experiments. B, To assess whether the Nkx-2.5-N188K mutant affected the DNA binding activity of Nkx-2.5-wt, nuclear extract from Nkx-2.5-wt transfected cells were mixed with increasing amounts of nuclear extracts from Nkx-2.5-N188K before binding to the radiolabeled D probe. EMSA analysis was performed with the preincubated mixed nuclear extracts as indicated. The amounts of nuclear extracts used, which were previously analyzed by Western blot for Nkx levels (see Fig. 9C), are indicated. C, HeLa cells were transiently cotransfected with h*dio2*-633-bp reporter, constant amounts of Nkx-2.5 plasmid, and increasing amounts of Nkx-2.5-N188K mutant. Data are shown as the mean ± SD of the CAT/Luc ratios of at least three separate experiments done in duplicate.

human left atrium and ventricle, we show that, in the material we examined, D2 mRNA levels are higher in the ventricle than in the atrium. Whether the higher D2 mRNA in the ventricle when compared with the corresponding atrium is the typical expression pattern remains to be established. Interestingly, the atrium expressed a lower D2 band that was not found in the ventricle or in human thyroid. In this regard, alternative splicing variants of human D2 mRNA have been identified (45). Moreover, Gereben *et al*. (46) reported a 77-nt deletion chicken D2 variant (compatible with the extra band we observed in the human atrium), which corresponds to a D2 enzyme without functional activity. Studies are required to clarify the role of different D2 transcripts in the deiodinase physiology of the human heart.

The human *Dio2* promoter responds transcriptionally to TTF-1, a thyroid-specific transcription factor that belongs to the NK homeobox gene family (47). In contrast to the highly specific biological functions of individual homeobox genes, *in vitro* DNA binding studies demonstrated that most protein-containing homeodomains bind to similar short consensus sequences (48). In these cases, *in vivo* specificity is achieved by multiple mechanisms, such as tissue specificity and spatio-temporal patterns, interaction with other factors (49), translational regulation of homeobox gene expression (50), subcellular localization (51), and phosphorylation state (52). Furthermore, small differences in DNA binding affinities to target sites due to sequences flanking and outside the core motif play an important role *in vivo* in the action of homeodomain proteins and in their binding to target DNA. Given the foregoing, our approach was to determine whether Nkx-2.5 is involved in cardiac D2 expression and, if so, whether it could account for the different level of D2 expression in human and rat myocardium.

The present study indicates that D2 expression in the human heart is positively controlled by Nkx-2.5 via two DNA binding sites. In HeLa cells, also the rat D2 promoter responds transcriptionally to Nkx-2.5, although to a much lesser extent.

Furthermore, we observed that the human promoter hdio2-83-bp construct (which is 97% identical with the corresponding rat promoter), although deprived of the C and D binding sites, partially responded transcrip-

tionally to cotransfected Nkx-2.5 in HeLa cells. This effect was lost with a further 23-nt deletion (hdio-2-60, Fig. 4A). EMSA showed no Nkx-2.5 binding to that 23-bp DNA region (data not shown), indicating that this DNA sequence, although involved in Nkx-2.5 responsiveness, does not directly bind Nkx-2.5. Computer-assisted prediction analysis of the putative transcription factors able to bind that region indicated that, within the 23 nt (between −83 and −60 bp), there is a consensus binding site for CCAAT enhancer-binding protein-α (C/EBPα). It has recently been shown that C/EBPα can, synergistically with Nkx2.1, promote transcription of the Clara cell secretory protein (53). It is conceivable that in HeLa cells, within the human and rat minimal 83-bp promoter region, there is an Nkx-2.5-induced transcriptional effect mediated by binding to other transcription factors such as C/EBPα.

However, the difference in Nkx-2.5 induction observed in HeLa cells between the human and rat *Dio2* 5′-flanking regions (14-fold and 9-fold, respectively) could not account for the remarkable difference in D2 mRNA expression between the two species. Previous reports have shown that Nkx-2.5 can cooperate with GATA-4 to activate transcription of the ANF promoter (33–35). Moreover, Nkx-2.5 and GATA-4 directly interact *in vivo* and *in vitro* via the Nkx-2.5 homeodomain and the GATA-4 zinc-finger domain (34, 54). The presence of several putative GATA-4 binding sites in both the human and the rat *Dio2* proximal promoter regions (see Fig. 2) prompted us to examine the effect of GATA-4 on *Dio2* promoter activity. Although GATA-4 alone induced no significant increase in the human and rat *Dio2* promoter activities, cotransfection experiments revealed that Nkx-2.5 and GATA-4 positively cooperate to stimulate the human, but not the rat, *Dio2* promoter. The absence of a cooperative effect of GATA-4 on the rat *Dio2* promoter is due to the absence of Nkx-2.5 binding sites; in fact, introduction of the human C and D sites into the rat promoter context was sufficient to reestablish this synergism. These results suggest that the presence or absence of synergy between Nkx-2.5 and GATA-4, rather than the transcriptional effects of either factor alone, underlies the different D2 expression in human and rat heart. This finding is reinforced by evidence that Nkx-2.5/GATA-4 transcriptional cooperation depends on the promoter context; cooperation is positive when the promoter contains Nkx-2.5 binding sites irrespective of GATA-4 sites, and negative when the promoter contains only GATA-4 sites (35). Consistent with this notion, there is a positive cooperation between Nkx-2.5 and GATA-4 in the human promoter, which, unlike the rat promoter, possesses two potent Nkx-2.5 binding sites. Also the mouse heart has very low D2 activity and mRNA level (Larsen, R., personal communication), which is consistent with the absence of the C and D sites in the mouse *Dio2* gene sequence (55). What is the physiological relevance of *Dio2* gene regulation by Nkx-2.5? In a transgenic mice model overexpressing human Nkx-2.5, mRNA levels of such cardiac genes as atrial natriuretic peptide, brain natriuretic peptide, cardiac ankyrin-repeat protein, and sarco (endo) plasmic reticulum Ca^{2+}-ATPase-2 (Serc2), which are downstream targets of Nkx-2.5, are up-regulated and, conversely, the expression of these genes is perturbed in mice that do not express Nkx-2.5 (19). These data point to a direct correlation between Nkx-2.5 level and the expression of its target genes. Furthermore, Nkx-2.5 itself is differentially regulated by important myocardial stimulators, *e.g.* isoproterenol, phenylephrine, and pressure overload, at least during early phases of the latter (48). It is tempting to speculate that such conditions may impact directly on D2 levels. Thus, Nkx-2.5 functional deficiencies, may correspond to a reduction in cardiac D2, and a consequent reduction in local T_3 production. Moreover, in an animal model of reduced Nkx-2.5 activity, cardiomyocyte differentiation is inhibited, whereas Nkx-2.5 overexpression promotes cardiomyocyte differentiation (56). Because increased thyroid hormone concentrations are generally associated with differentiation as opposed to a proliferative state, when T_3 levels are low, Nkx-2.5 could increase D2-catalyzed T_4 to T_3 conversion as part of its effects to accelerate differentiation. In this regard, it would be of interest to assess whether the *in vivo* models of altered Nkx-2.5 state display corresponding variations in cardiac D2, although the much lower cardiac D2 in rodents makes them a poor model for the human heart.

Several heterozygous mutations have been identified in patients with congenital heart disease (24, 25). Among them, an Asn 188 Lys missense mutation within the homeodomain region markedly reduces the binding of the Nkx-2.5 protein to the ANF promoter region and the ability to transactivate the ANF promoter (36). Here we have analyzed the capability of the Nkx-2.5-N188K mutant to transactivate human *Dio2* promoter and bind the D site. When cotransfected with the *Dio2* promoter CAT reporter gene, Nkx-2.5 N188K did not bind to the D site nor did it activate the *Dio2* promoter. Furthermore, in rat cardiomyocytes, Nkx-2.5-N188K protein reduced h*Dio2* promoter activity to 40% of the basal activity.

To explore possible mechanisms whereby the N188K mutant could interfere with the endogenous Nkx-2.5 protein, we incubated the N188K mutant protein with nuclear extracts containing Nkx-2.5-wt and found that it inhibited binding of Nkx-2.5-wt to the DNA in a dose-dependent manner. Accordingly, functional analysis in HeLa cells showed that N188K is able, in a dose-dependent assay, to inhibit wild type-induced transcription. These data suggest that at least part of the functional inhibition observed *in vivo* is due to blockade of DNA binding of Nkx-2.5-wt to DNA. Given the complex protein-protein interactions affecting the *in vivo* action of homeobox transcription factors, the possibility of sequestering associated protein cannot be excluded.

An appropriate thyroid hormone level is critically important for the coordination of developmental pro-

cesses in all vertebrate species. An examples of this is the critical role played by locally produced T_3 in amphibian metamorphosis (57) and in cochlear maturation and the onset of auditory function in rodents (58). In both cases, tissue- and time-specific overexpression of D2 is required for an optimal intracellular T_3 concentration. In this context, we postulate that the human cardiac malformations due to Nkx-2.5 mutations may be due, in part, to intracellular hypothyroidism consequent to reduced cardiac D2 activity. It will be of interest to determine whether or not this is the case in human cardiac developmental malformations and to investigate in greater detail the functional relevance of reduced cardiac D2 levels.

In conclusion, our study provides novel insights into a potential interaction between thyroid status and homeobox-dependent cardiomyocyte differentiation. *Dio2* is one of the few genes the cardiac expression of which differs greatly between humans and rodents. The identification of the type 2 deiodinase gene as a downstream target of Nkx-2.5, together with initial insights into the mechanisms governing *Dio2* expression in the myocardium, might help us to understand the mechanisms by which Nkx-2.5 regulates development and the differentiation state of cardiac cells.

MATERIALS AND METHODS

Eukaryotic CAT Expression Vectors and Constructs

A mouse Nkx-2.5 pBluescript (SK) vector (kindly provided by Dr. G. Condorelli) was used as DNA template in a PCR with oligonucleotides Nk1s and Nk3r (Table 1). The 960-nt PCR product, corresponding to the mouse Nkx-2.5 cDNA coding region, was subcloned into pFLAG-CMV-2 vector (Sigma, St. Louis, MO); To generate the Nkx-2.5-N188K mutant, we used recombinant PCR with two sets of oligos (M188s and M188r, and Nk1s and Nk3r) to introduce the N188K mutation into the Nkx-2.5. Briefly, the two PCR products [cytomegalovirus (CMV)-Flag/M188r and M188 s/Nk3r] were combined by PCR using CMV-flag and Nk3r as outside oligonucleotides, and the final PCR product was reinserted into the pFLAG-CMV-2 vector (Sigma). mGATA-4-expressing plasmid was kindly provided by Dr. G. Condorelli; nuclear factor-κB p65 transcriptionally active subunit was kindly provided by Dr. A. Leonardi.

The h*Dio2*-CAT contructs containing the 6.5-kb hdio2 5′-fragment (h*Dio2*-6.5) and some of its 5′-truncation products (−633 and its mutated forms, 633 Cmut, 633D mut, 633 2 m) have been previously described (6) as well as the rat promoter-CAT constructs (nos.1, 3, and 4). The hdio2-60-bp construct was generated by PCR with oligo hD2-60 s and CATr. The resulting fragment, which contained the −60-nt minimal promoter region, was subcloned in the po-CAT vector. The hdio-2 240 bp and the corresponding hdio-2 240 m version construct was generated by PCR with oligo hD2-240 s or hD2-240 m as sense oligonucleotides and CATr as antisense nucleotide (Table 1). The resulting fragment, which contained the −240-nt wt promoter and its corresponding D mutated version (CAAG to CGTG), was subcloned into po-CAT vector. The h*dio* −365-bp plasmid was obtained by *Sac*I/*Bam*HI deletion of the h*dio*2-633. The rat promoter-CAT construct rdio2-CAT no. 2 was generated by digestion of the rdio2-CAT no. 1 with *Eco*RI-*Xba*I. To generate the rdio2-658-bp/C-D construct, the binding site core motif CAAG present in the human promoter was introduced into the rat promoter gene at the positions that corresponded to the C and D human sequences. Briefly, the two PCR products (Crat-s/Drat-r and Drat-s/F-r) were combined by PCR using Crat-s and F-r as outside oligonucleotides, and the final PCR product, containing the mutated sites, digested with *Sac-Bgl*II, was reinserted into r*dio*-2#2 vector in the identical position. All the oligonucleotides used are shown in Table 1 and all the plasmids produced were sequenced for sequence control.

DNA Transfection and CAT and Luc Expression Assays

The reporter CAT and Luc plasmids were cotransfected into HeLa cells with the calcium phosphate precipitation method. For each 60-mm dish, 3 μg po-CAT2-reporter vector were cotransfected with 0.1 μg Nkx-2.5 expressing vector or 0.4 μg GATA-4 expressing vector and 0.3 μg Rous sarcoma virus-Luc vector as internal control in HeLa or COS7 cells at 60% confluency. The synergistic effect of Nkx-2.5 and GATA-4 was tested in cotransfection assays performed with limiting amounts of transcriptional factors Nkx-2.5 and

Table 1. Oligonucleotides Used to Generate Plasmids and for EMSA

Oligonucleotide	Sequence (5′-3′)	Orientation
CMV-flag	ACCATGGACTACAAAGACGATGACG	Sense
M188s	ATCTGGTTCCAG**AAA**CGGCGCTACAAG	Sense
M188r	CTTGTAGCGCCGTTTCTGGAACCAGAT	Antisense
NK1s	CGGAATTCCTTCCCCAGCCCTGCGCTCACACCCAC	Sense
NK3r	GCTCTAGAGTTAGAGTCTGGTCCTGCCGCTGTC	Antisense
C-rat-s	AAGCTTTAGTAAAACCCAAATTAACAATTGTATT**CAAG**GTTTGGGA	Sense
D-rat-s	GAGACTTGT**CAAG**GGTAACTTTC	Sense
hD2-240s	TGAAGGCTGT**CAAG**GGTATTAG	Sense
hD2-240(M)s	TGAAGGCTGT**CGTG**GGTATTAG	Sense
hD2-60s	CACTTCTCTATTGCAGCAATTAGC	Sense
CAT-r	CTCACCGTCTTTCATTGCCATACGG	Antisense
D-rat-r	GAAAGTTACCCTTGACAAGTCTC	Antisense
F-r	GCCTAATCTTGGTAAAGATCTTGACGTCATTGAG	Antisense
D	GGCTGTCAAGGGTATTAGTTT	Sense
C	CTGTATTCAAGTTTCTGCAAGAAGCTTT	Sense
NKE-2	CCTTTGAAGTGGGGGCCTCTTGAGGCAA	Sense

The mutations in the wild-type constructs are *underlined*.

GATA-4 (10 ng and 100 ng, respectively) and 3 μg po-CAT2-based vector. For the uninduced control, the pFLAG-CMV-2 empty vector was cotransfected in the same quantity of Nkx-2.5 or GATA-4-expressing vector. CAT activities were measured 48 h after transfection, and differences in transfection efficiency were corrected relative to the luciferase activity level. Each construct was studied in duplicate in at least three separate transfections; data (CAT/Luc ratios) are shown as mean ± SD.

Rat cardiomyocytes cultured in 12-multiwell plates, with F12 containing 10% horse serum, were cotransfected with a total of 3.5 μg of po-CAT2-reporter vector, 0.5 μg Rous sarcoma virus-Luc as internal control, and 0.3 μg Nkx-2.5-expressing vector mixed using LipofectaMINE (Life Technologies, Paisley, Scotland, UK) for 12 h in 1.5 ml Optimem (Life Technologies), after which Optimem was substituted with medium supplemented with 10% horse serum. Cardiomyocytes were harvested 24 h after transfection, and CAT and Luc activities were determined using standard procedures. Experimental data are presented as the mean of three independent duplicate transfection assays normalized by Luc activity.

Bosc-23 cells (ATCC, clone number CRL-11270), grown in 100-mm dishes at 70% confluency, were transiently transfected with LipofectaMINE (Life Technologies, Inc., Gaithersburg, MD). Nkx-2.5-wt (10 μg) or Nkx-2.5-N188K vector was mixed with 1.6 ml Optimem and 40 μl LipofectaMINE for 45 min and then added to the cells in 6 ml total Optimem. After 48 h, cells were washed and harvested by scraping in 2 ml PBS 1× (pH 7.4). After centrifugation at 500× *g*, pellets were frozen at −80 C until required for the preparation of other nuclear extracts.

Semiquantitative EMSA and Supershift Assays

Double-stranded oligonucleotides corresponding to the putative Nkx-2.5 binding sequence were used as probes for EMSA. The oligonucleotide sequences (one strand) were: oligonucleotide C (from −632 to −603) 5′-CTGTATTCAAGTTTCTGCAAGAAGCTTT-3′, and oligonucleotide D (from −241 to −221) 5′-GGCTGTCAAGGGTATTAGTTT-3′. As a positive control, we used the high-affinity Nkx-2.5 binding site oligonucleotide NKE-2 described for the rat ANF promoter. Antisense oligonucleotides were labeled with a T_4 polynucleotide kinase (New England Biolabs, Beverly, MA) reaction with radioactive γ-^{32}P-ATP. Double-stranded oligonucleotides were purified by passing through NICK columns containing Sephadex G-50 DNA Grade (Pharmacia Biotech, Piscataway, NJ).

Nuclear extracts of transfected BOSC-23 cells were prepared as follows: cells grown on 100-mm plates were washed with PBS 1× buffer, harvested by scraping and centrifuged at 500 × *g* for 20 min at −4 C; the pellet was frozen at −80 C. The cells were resuspended in low-salt buffer [10 mM HEPES, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.1 mM EGTA (pH 7); 0.5 mM dithiothreitol (DTT); pepstatin, 4 mg/ml; 2 mM benzamidine; aprotinin, 20 mg/ml; 10 mM leupeptin; and 1 mM phenylmethylsulfonylfluoride] and centrifuged for 3 min at 4 C at 300 × *g*; the pellets were resuspended in low-salt buffer and passed through a 25-gauge needle. Nuclei were pelleted by centrifugation at 500 × *g* for 3 min and resuspended in 100 μl of extraction buffer (10 mM HEPES, pH 7.9; 0.4 M NaCl; 1.5 mM $MgCl_2$; 0.1 mM EGTA, pH 7; glycerol 5%; 0.5 mM DTT; pepstatin, 4 mg/ml; 2 mM benzamidine; aprotinin, 20 mg/ml; 10 mM leupeptin; and 1 mM phenylmethylsulfonylfluoride) and incubated for 30 min at 4 C. After a centrifugation at 15,000 × *g* for 20 min, the supernatant was used as a nuclear extract for EMSA. For antibody interference assays, proteins were incubated with cold competitors or antisera (Ab-Flag: M2, Sigma Chemical Co.; Ab-Nkx−2.5: N1889, Santa Cruz Biotechnology, Inc.) 15 min before addition of the probe.

Human heart nuclear extracts were prepared from frozen tissues using standard procedures (37). The tissue was homogenized in the presence of low-salt buffer and the nuclear extracts were prepared as previously described. EMSAs were performed in 30-μl reaction mixtures at room temperature at a final concentration of 20 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM DTT, 10% glycerol, 1 mg/ml BSA, 1 mg/ml poly-(dIdC). A typical assay contained 10 μg of nuclear extracts and 10 fmol of the probe. For EMSA competition assays, proteins were incubated at room temperature with cold competitors for 15 min before addition of the probe.

Culture and Transfection of Primary Neonatal Rat Cardiomyocytes

Primary cultures of neonatal rat ventricular cardiac myocytes were prepared from 1-d-old Wistar rats (Charles River Laboratories, Wilmington, MA). Briefly, cardiac myocytes were dispersed from the ventricles by digestion with collagenase type IV (Sigma), 0.1% trypsin (Life Technologies), and 15 μg/ml DNase I (Sigma). Cells were applied on a discontinuous Percoll gradient (1.060/1.086 g/ml) prepared in Ads buffer [116 mM NaCl, 20 mM HEPES, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl, 0.8 mM $MgSO_4$ (pH 7.35)] and centrifuged at 3000 rpm for 30 min.

Cells were grown in the cardiac myocyte culture medium containing DMEM/Ham's F-12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite (Life Technologies), 2 g/liter BSA (fraction V), 3 mmol/liter pyruvic acid, 15 mmol/liter HEPES, 100 μmol/liter ascorbic acid, 100 μg/ml ampicillin, 5 μg/ml linoleic acid, and 100 μmol/liter 5-bromo-2′-deoxyuridine (Sigma). We obtained cell cultures in which more than 95% of the cells were myocytes as assessed by immunofluorescence staining with a monoclonal antibody against sarcomeric myosin (MF20). Culture media were changed to serum free at 24 h.

RT-PCR Assays

mRNA was extracted from all the tissues (see above) using the Trizol (Life Technologies) reagent. mRNA (1 μg) was used for the reverse transcription (RT) with random hexamers (SuperScript Kit, Life Technologies). The RT products were used for PCR with D2-specific oligonucleotides. To exclude the PCR products amplified from the genomic DNA, D2 primers (D2-1 s: CTCTATGACTCGGTCATTCTGCTC and D2-2r: TAAGTCATGTTGGAGTTATTGTCC) were designed to span one intron. After electrophoresis, PCR products were transferred onto the nylon membrane and hybridized with the γ-^{32}P dCTP random-labeled rat D2 DNA probe. Rat ventricle, obtained from a male Sprague Dawley rat (120 g), was dissected and immediately frozen in liquid N_2. Human thyroid was used as positive control. Human heart samples were from a 60-yr-old patient undergoing a heart transplantation at the Cardio-Surgery Division, Second University of Naples. Samples were collected soon after surgery and immediately snap frozen in liquid nitrogen to preserve mRNA. All human tissues were obtained under protocols approved by the Institutional Review Board of the University of Naples "Federico II." The RT-PCR bands were quantified by densitometric analysis (using a PhosphorImager, GS525, Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blot Analysis

Nuclear extracts of transfected Bosc-23 cells, prepared as described above, were boiled in Laemmli buffer and resolved by 12% SDS-PAGE. The gel was blotted on Immobilon P (Millipore Corp., Bedford, MA) for 12 h at a constant current of 150 mA. Immunodetection of Nkx-2.5 was performed by using a monoclonal anti-FLAG antibody (M2, Sigma) diluted 1:3000 in Tris-buffered saline containing 0.5% nonfat milk (Bio-Rad Laboratories, Inc., Richmond, CA), and the filter was

treated with a 1:3000 dilution of goat antimouse IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL).

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