The Homeodomain Transcription Factor NK-4 Acts as either a Transcriptional Activator or Repressor and Interacts with the p300 Coactivator and the Groucho Corepressor*

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NK-4 (tinman) encodes an NK-2 class homeodomain transcription factor that is required for development of the Drosophila dorsal mesoderm, including heart. Genetic evidence suggests its important role in mesoderm subdivision, yet the properties of NK-4 as a transcriptional regulator and the mechanism of gene transcription by NK-4 are not completely understood. Here, we describe its properties as a transcription factor and its interaction with the p300 coactivator and the Groucho corepressor. We demonstrate that NK-4 can activate or repress target genes in cultured cells, depending on functional domains that are conserved between Drosophila melanogaster and Drosophila viridis NK-4 genes. Using GAL4-NK-4 fusion constructs, we have mapped a transcriptional activation domain (amino acids 1–110) and repression domains (amino acids 111–188 and the homeodomain) and found an inhibitory function for the homeodomain in transactivation by NK-4. Furthermore, we demonstrate that NK-4-dependent transactivation is augmented by the p300 coactivator and show that NK-4 physically interacts with p300 via the activation domain. In addition, cotransfection experiments indicate that the repressor activity of NK-4 is strongly enhanced by the Groucho corepressor. Using immunoprecipitation and in vitro pull-down assays, we show that NK-4 directly interacts with the Groucho corepressor, for which the homeodomain is required. Together, our results indicate that NK-4 can act as either a transcriptional activator or repressor and provide the first evidence of NK-4 interactions with the p300 coactivator and the Groucho corepressor.

Homeobox genes encode sequence-specific DNA-binding proteins that play important roles during development by regulating transcription in organisms as diverse as insects, nematodes, mammals, and plants (1, 2). These homeodomain transcription factors can act as transcriptional activators or repressors that require separate but sometimes overlapping functional domains including a DNA-binding domain for their transcriptional activities (3–5). In addition, it was recently demonstrated that homeoproteins interact with other transcription factors that lead to their functional specificity in vivo, utilizing a specific but conserved domain of the homeoproteins for these interactions (5–11). In general, sequence-specific DNA-binding proteins recruit a coactivator or corepressor for their transcriptional activation or repression of target genes (12, 13), yet the mechanism of activation or repression by homeoproteins is still poorly understood.

The NK-4 homeobox gene tinman (14–17), belongs to the NK-2 class that includes a large number of vertebrate homeobox genes (18–20). Analysis of tinman mutant embryos revealed that tinman function is required for development of the dorsal mesoderm including the heart during Drosophila embryogenesis (16, 17). Additionally, tinman activity is required for the formation of glia-like dorsal-medial cells (21), gonadal mesoderm (22–24), and a subset of somatic body wall muscles (16). In mammals, targeted disruptions of the mouse Nkx-2 genes (Nkx-2.1/TTF1, Nkx-2.2, and Nkx-2.5) revealed their functions in organogenesis including heart development (25–27). Likewise, it has recently been revealed that human NKX2–5 is involved in nonsyndromic, human congenital heart disease (28). Also, cross-phylum rescues of the NK-2 class of homeobox genes have recently been demonstrated (29, 30). For example, zebrafish nax 2.5 efficiently rescues a ceh-22 mutant when expressed in the pharyngeal muscle of Caenorhabditis elegans (29), and mouse Nkx2–5 rescued only visceral mesoderm when tested for its ability to rescue the tinman mutant phenotype of Drosophila (30). Thus, genetic evidence clearly indicates that NK-4 plays an important role during development and suggests that its function is well conserved in the animal species.

NK-4 is initially expressed in the presumptive mesoderm at the cellular blastoderm stage, continues to be expressed in all mesodermal cells during germ band elongation, and eventually becomes restricted to dorsal mesodermal cells including precursor cells of the heart (15). Recently, NK-4 has been shown to be regulated by the myogenic factor Twist during embryogenesis (31, 32). In addition, previous work has shown that inductive signals from dorsal ectodermal cells such as Dpp can induce tinman expression in the dorsal mesoderm (33–35). Similarly, chicken Nkx-2.5, a potential vertebrate homologue of the Drosophila NK-4 gene, was shown to be induced by BMP-2 and BMP-4 (36), suggesting that regulatory pathways for its induction are also conserved. We have previously demonstrated that NK-4 binds to a target sequence (5′-TCAAGTG-3′) (31, 37) that is related to the binding sites of other NK-2 class

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homeodomain proteins (20, 31, 38–41) and autoregulates its own gene in cultured cells (31). Additionally, it has recently been shown that NK-4 directly activates the D-mef2 gene in cardiac cells (37) and ventral muscle founder cells (42), making D-mef2 the first known in vivo target of NK-4 to be described. Cofactors of NK-4 are totally unknown. However, physical interaction of Nhx-2.5 with GATA-4 has been reported, suggesting that they may act as mutual cofactors (43–45).

Despite the growing body of evidence that supports an important role for NK-4 during embryogenesis, the properties of NK-4 as a transcriptional regulator and the mechanism of gene transcription by NK-4 are still poorly understood. In this study, we characterize the functional domains of NK-4 that are responsible for its transcriptional activity. Our analysis reveals that NK-4 acts as either a transcriptional activator or repressor that utilizes separate functional domains for its transcriptional activity. Furthermore, we provide the first evidence of interaction of NK-4 with the p300 coactivator and the Groucho corepressor.

MATERIALS AND METHODS

GAL4 Fusion Constructs—For the generation of GAL4-NK-4 fusion constructs (Fig. 2A), corresponding coding regions for NK-4 were amplified by PCR (10 cycles of 1 min at 95 °C, 1 min at 55 °C, 2 min at 72 °C) with specific primers, and amplified DNA fragments were codigested with EcoRI and XbaI (in the case of A4 and A5 constructs, EcoRI digestion). These DNA fragments were subcloned into the EcoRI/XbaI sites (in the case of constructs A4 and A5, EcoRI site) of the pSG424 expression vector (46). Open reading frames of each construct were confirmed either by sequencing with specific primers or by expression of fusion genes in E. coli (Fig. 5; see below) or by expression of GFP-NK-4 fusion genes in CV-1 cells. Specific primer sets used for PCR amplifications are as follows: primer A1, primer 20 and primer 502; A13, primer 20 and primer 69; A4, primer 20 and primer 70; A5, primer 20 and primer 25; A6, primer 20 and primer 501; A7, primer 20 and primer 502; A8, primer 20 and primer 500; A10, primer 519 and primer 501; A14, primer 9513 and primer 502; A15, primer 915 and primer 501; A12, primer 912 and primer 502; A18, primer 915 and primer 502; A19, primer 925 and primer 502; A2, primer 422 and primer 502; A23, primer 913 and primer 502; A24, primer 953 and primer 502; A31, primer 913 and primer 9538; A32, primer 915 and primer 9535; A33, primer 915 and primer 423, A34, primer 915 and primer 9537; A35, primer 915 and primer 9538.

The sequences of specific primers (restriction site is underlined) are as follows: primer 20, 5′-TGGGAAATCTGCAGGGGACCATCATG-3′; primer 25, 5′-CTAGAATTCATCTGCAGGGGACCATGTT-3′; primer 26, 5′-CAATCATGAAAGCTTTGGCGCCATAT-3′; primer 27, 5′-GGGATATTCTGGGGCGAGCTGAGTTAC-3′; primer 33, 5′-GGGATATTCTGCAGGGACCATCATG-3′; primer 42, 5′-GGGATATTCTGGGGCGAGCTGAGTTAC-3′; primer 43, 5′-GGGATATTCTGGGGCGAGCTGAGTTAC-3′; primer 500, 5′-CAATCATGAAAGCTTTGGCGCCATAT-3′; primer 501, 5′-CAATCATGAAAGCTTTGGCGCCATAT-3′.

For the NKA6 construct (Fig. 3A), DNA fragments containing the activation domain (aa 411–490) of VP-16 were amplified by PCR with specific primers (primer VPAD1 (5′-GATGGTACGTCGACAGTGAGGC-3′) and primer VPAD2 (5′-GATGGTACGTCGACAGTGAGGC-3′)) and subcloned into the EcoRI site of pSG424. The NK4HD construct that contained the NK-4 homeodomain (aa 297–364), DNA fragments were amplified by PCR with specific primers (primer NK4–53 (5′-GATGGATTCACAAGGCCTCGGGATGAGGCCAAG-3′) and primer 9538) and subcloned into the EcoRI/XbaI sites of the pSG424 vector. For the cloning of the NK-1 homeodomain (aa 531–628), DNA fragments were amplified by PCR with specific primers (primer NK1–53 (5′-CAGAGGTATCCGTAAATGCGGCGGAGCTTCG-3′) and primer NK1–55 (5′-CGAGGTATCCGTAAATGCGGCGGAGCTTCG-3′)) and subcloned into the EcoRI/XbaI sites of the pSG424 vector to generate the NK4-NK1HD plasmid. For constructions of series of the GAL4-NK-4 fusion expression vectors used in Fig. 3, plasmids containing the activation domain of either NK-4 (NKA6 or NK46Dm) or VP16 (SG424VP16) were digested with EcoRI, and DNA fragments were eluted from the gel and subcloned into the EcoRI site of either the GAL4-NK-4 fusion constructs (A2, A31, NK4HD1, NK4HDXNQ, and NK4HDSA; see below) or the GAL4-NK-1 fusion construct (GAL4-NK1HD).

Site-directed Mutagenesis—A PCR-based method was used to generate mutations within either the TN domain or the homeodomain. For the NK46Dtm construct that contains a mutation (Phe → Ala at aa 391 in the TN domain) within the DNA fragments (DDm, aa 1–43) were amplified by PCR with specific primers (primer NK4FA (5′-GTTCAAGATATCTGGAGAAGGGATGTCTGACCCGAGCCC-3′) and primer 9543), amplified DNA fragments were digested with EcoRI and XbaI. The corresponding region of the NKA6 construct was replaced by the DDm DNA fragments to generate the NK46DDm expression vector. For the A6-NK4HDXNQ construct that contains a mutation (Arg → Gln at aa 351, AAT → CAG) at the helix III region of the homeodomain, two separate DNA fragments (fragment A, aa 188–356; fragment B, aa 345–416) were amplified by PCR with specific primers (fragment A, primer 422 and primer 9542 (5′-GATGGAGGCTGCGAGTGAATCAGGCTCAGAC-3′); fragment B, primer 9541 (5′-CAAGTGAAGATTTGGTGCAGAGGAGCCGCTACATGCG-3′) and primer 9543) amplified DNA fragments were gel-eluted and subcloned into the EcoRI/XbaI sites of the pSG424 expression vector to generate the NK44HDXNQ plasmid. For the A6-NK4HDXNQ construct that contains a mutation (Ser → Ala at position 410, GTCGACGCGGCCGCTACATGCG-3′) and primer 9543)) and amplified DNA fragments were digested with EcoRI and XbaI, and then cloned into the EcoRI/XbaI sites of the pSG424 vector to generate the NK44HDXNQ plasmid. Mutations and open reading frames of the clones were confirmed by sequencing.

Expression Vectors, Cell Transfections, and CAT Assays—Cell growth, transfections into cells with indicated reporter and expression vectors using the calcium phosphate precipitation method, normalization of transfection efficiency with β-galactosidase activity, and CAT assays with a CAT enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) were performed as described (31). For the constructions of truncated NK-4 expression vectors (d8 and d6), DNA fragments (121 bp, from 5′-untranslated region to the first ATG codon) were amplified by PCR with specific primers (primers NK453 (5′-TAACTGCGAGCCCGCGCAGAGCCAGATTCCAAATTG-3′) and primer LE10 (5′-GATGGATTCACATCTGGCTGCAGAC-3′)) amplified DNA was digested with EcoRI and SaI, and then subcloned into the EcoRI/SalI sites of pBSVcat vector in order to generate the pKSI-NK4 plasmid. Subsequently, cDNA cloning of NK-4 from GAL4-NK-4 chimeras (A18 and A2 in Fig. 2A, d8 and d6 in Fig. 1, respectively), which were generated by digestion with EcoRI and SalI followed by ligation of EcoRI/XbaI sites of the pKSI-NK4 plasmid to generate the pKSI-NK4d8 and the pKSI-NK4d6 plasmids. These plasmids were digested with NotI, and DNA fragments were subcloned into NotI site of pRC-CMV expression vector (Invitrogen) to generate d8 or d6 expression vectors. Expression vector for full-length NK-4 (NK-4, CMV-NK-4) and reporter plasmids (P1, P1E2m, E2CAT, 1 The abbreviations used are: PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; GRO, Groucho; GST, glutathione S-transferase; bp, base pair; aa, amino acids; RD, repressor domain; HD, homeodomain; PAGE, polyacrylamide gel electrophoresis, TN, Timmam.
and E2mCAT) were described previously (31). The G5ExnXCAT reporter plasmid, which contains SV40 enhancer, was described previously (47). For the construction of the Groucho expression vector, PCR-amplified cDNAs were subcloned into the pKSII vector, and the resulting plasmid (pKS-GRO) was confirmed and used for the construction of the Groucho expression vector (pCI-CMV) by inserting cDNA into EcoRI/SalI sites of the pCI vector (Promega). For the construction of the Myc-tagged Groucho expression vector, the NcoI/XhoI DNA fragment from the pSPUTK-GRO was subcloned into the pcDNA3.1+ vector.

For the construction of the GST-NK-4 fusion expression vectors, DNA fragments of the corresponding regions of NK-4 were excised from GAL4-NK-4 fusion constructs (Fig. 2A) and subcloned into either the EcoRI site of the pGEX5X-1 plasmid or into the EcoRI/XbaI sites of the pGEX5X-I-xb plasmid containing an extra XbaI site. For the constructions of GST-TD and GST-TDm expression vectors, corresponding regions were amplified (primer 20 and primer 500) from NK4A6 and NK4A6TDm, respectively, and subcloned into pGEX5X-1. Expression and purification of the fusion proteins were performed as described previously (31, p300N(1–670), p300M(671–1184), and p300C(1135–2414) plasmids were described previously (48). For the constructions of the truncated p300 expression plasmids, the NK-4, and the Groucho expression plasmids used for in vitro translation (Figs. 4 and 5), DNA fragments of the corresponding regions of p300 and Groucho were amplified by PCR with specific primers (in the case of construction of the 2414-aa expression vector, the corresponding regions of the NK-4 were obtained from GAL4-NK-4 constructs) and subcloned into the EcoRI/SalI sites of the pSPUTK vector. Primer sets used for amplifications are as follows: p300(300–450), primer p300N1 (5′-GATGATATCCCAA-GATGGGTCAACAGCCA-3′) and primer p300C1 (5′-GATGATTCCAGCG-CAGACTGTGTTGACCCACC3′; p300; 450–4670), primer p300N2 (5′-GATGATTCCACCACCTAAGCTGTTAGTATG-3′), and primer p300C2 (5′-GATGATTCCAGCTGTTGACCGACCATCATG-3′; p300; 4670–1891), primer p300N3 (5′-GATGATATCCACGTTGCTGATGATGATGCTG-3′) and primer p300C3 (5′-GATGATTCCAGCTGTTGACCGACCATCATG-3′). GRO(1–719) expression vector (pCI-GRO) by inserting cDNA into the EcoRI/I sites of the pSPUTK vector. Primer sets used for amplifications are as follows: p300(300–450), primer p300N1 (5′-GATGATATCCCAA-GATGGGTCAACAGCCA-3′) and primer p300C1 (5′-GATGATTCCAGCG-CAGACTGTGTTGACCCACC3′; p300; 450–4670), primer p300N2 (5′-GATGATTCCACCACCTAAGCTGTTAGTATG-3′), and primer p300C2 (5′-GATGATTCCAGCTGTTGACCGACCATCATG-3′; p300; 4670–1891), primer p300N3 (5′-GATGATATCCACGTTGCTGATGATGATGCTG-3′) and primer p300C3 (5′-GATGATTCCAGCTGTTGACCGACCATCATG-3′).

RESULTS

NK-4 Can Act as either a Transcriptional Activator or Repressor and Contains Multiple Functional Domains—Previously, we showed that NK-4 is regulated by Twist and autoregulates its own promoter (31). We further characterized the properties of NK-4 as a transcription factor using the same reporters (Fig. 1, P1 and P1E2m) and truncated forms of the NK-4 expression vectors (Fig. 1, d8 and d6). As described previously (31), the P1 reporter contains NK-4's responsive elements (the E2 cluster) and is activated by NK-4. The P1E2m reporter contains mutated NK-4 binding sites but otherwise is exactly the same as the wild-type P1 reporter, which also contains several weak NK-4 binding sites. As shown in Fig. 1, we found that NK-4 can activate the P1 reporter (6-fold activation, lane 2). In contrast, NK-4 down-regulates the P1E2m reporter gene (3-fold repression, lane 6). In this case, NK-4 binds to weak binding sites and suppresses the P1E2m reporter gene. These results indicate that NK-4 can act either as a transcriptional activator or repressor, depending on the context of the reporters (P1 or P1E2m). Additionally, we demonstrate that this phenomenon is dependent on the functional domains of NK-4 (Fig. 1). For example, deletion of the amino-terminal region of NK-4 (Fig. 1, d8 construct) abrogates activation of the P1 reporter (Fig. 1, lane 3), indicating that the amino terminus (aa 1–110) of NK-4 is required for transcriptional activation. Indeed, this NK-4 mutant (d8) represses gene expression of both the P1 and the P1E2m reporter (Fig. 1, lanes 3 and 7). Further deletion of NK-4 (Fig. 1, construct d6) relieved this repression, irrespective of the reporter gene used (Fig. 1, lanes 4 and 8). These results suggest that the region following the amino terminus of NK-4 (aa 111–188) is required for the repressor activity of NK-4. Taken together, these results indicate that, depending on the context of the target genes (for example P1 or P1E2m; see lanes 2 and 6), NK-4 can act as either a transcriptional activator or repressor and that these different transcriptional activities are dependent on functional domains of NK-4.

In order to define the domains responsible for transcriptional activation and repression in great detail, various GAL4-NK-4 chimeras were generated by inserting DNAs coding for different regions of NK-4 into the pSG424 expression vector that contains a heterologous GAL4 DNA binding domain (Fig. 2A).
CAT activities from cells cotransfected with the indicated plasmids and a G5BCAT reporter plasmid were measured and compared with activities from cells cotransfected with pSG424 as a control (Fig. 2B). From this analysis, we found that the amino-terminal region of the NK-4 protein (construct A6, aa 1–110) contains a transcriptional activation domain, which is consistent with results described above (Fig. 1). Thus, this activation domain functions both in intact NK-4 and in a fusion form when tethered to the heterologous GAL4 DNA binding domain. Within this activation domain, the Gln, Asp, and Glu residues are enriched (24%). Of note is the presence of the TN domain (aa 39–47) that is well conserved in the NK-2 class homeoproteins (19) and the YYD tripeptide (aa 14–16) that is similar to the FYD motif that was found in the activation domains of MyoD, myogenin, and MRF (49). In fact, deletion of the first 13 amino acid residues within the activation domain (Fig. 2B, construct A10) did not affect transcriptional activation. However, further deletion of both the YYD motif and the TN domain (Fig. 2B, construct A14) markedly reduced transcriptional activation.

To map the repressor domain (RD) of NK-4, we constructed the G5EnXCAT reporter plasmid by inserting the SV40 enhancer into the G5BCAT reporter (Fig. 2A). The G5EnXCAT reporter alone showed very high CAT activity so that we could measure the repression activity by NK-4 more easily. The A1 construct showed strong repressor activity when cells were cotransfected with the G5EnXCAT reporter (Fig. 2C). Constructs A12 and A18, which contain a deletion of the activation domain, showed comparable repressor activities (Fig. 2C, lanes A12 and A18, respectively). Further deletion of NK-4 domains (constructs A19, A2, A23, and A24) revealed that both the region following the activation domain (RD, aa 110–188) and the homeodomain (HD) are important for repressor activity. Interestingly, either domain alone (constructs A31–A34) showed weak repressor activity. Strong repressor activity, however, was restored when both domains were combined (constructs A35 and A18). Thus, when fused to the heterologous GAL4 DNA binding domain, these domains (RD and HD) can act as a transcriptional repressor domain. These results are consistent with our observation that deletion of the RD (Fig. 1,
construct d6) caused a loss of repressor activity of NK-4 and support the idea that the RD is necessary for the repressor activity of intact NK-4. Within this region (aa 111–188), strong homology (69% identity) between Drosophila virilis NK-4 (DvNK-4) and NK-4 was observed. Taken together, these results indicate that NK-4 has both transcriptional activation and repressor domains.

The Homeodomain Is an Inhibitory Domain—As described above, although construct A1 contains a strong activation domain (aa 1–110) (Fig. 2B, lane A6), it still could not activate the G5BCAT reporter gene (Fig. 2B, lane A1). These results suggested that NK-4 contains inhibitory domains that suppress the transactivation function of NK-4. Since the homeodomain (in conjunction with the RD) showed repression of reporter gene expression, we suspected that the homeodomain might provide such an inhibitory function. In order to explore this possibility, various constructs were generated by the in-frame fusion of different domains of NK-4 to construct NK4A6 that contains the activation domain of NK-4 (aa 1–110) (Fig. 3A). As shown in Fig. 3, the carboxyl-terminal half of NK-4 including the homeodomain inhibited transactivation of the NK-4 activation domain, which is independent of DNA binding of the homeodomain (lane 7). C, mutation in the TN domain does not affect the repressor activity of the homeodomain (lanes 4–6). D, mutation in the helix 1 region of the homeodomain partially relieves the repressor activity of the homeodomain (lane 4). Results (lanes 6–8) also show that fusion of the homeodomain to the VP16 activation domain also inhibited the transactivation function of VP16. Effector plasmids used for cotransfection are indicated below each lane. E, GST pull-down assays showing that the TN domain interacts with the homeodomain. In vitro 35S-labeled NK-4 homeodomain (NK4HD; aa 297–364) was subjected to GST pull-down assays (upper panel) with glutathione-Sepharose-bound GST-TD (lane 3) or GST-TDm (lane 4). GST fusion proteins that were used for the GST pull-down assays are shown in the lower panel. Input samples (lane 1) contained 5% of the amount used for binding. GST, negative control with glutathione-Sepharose-bound glutathione S-transferase protein (lane 2).

2 C. Y. Choi, Y. H. Kim, and Y. Kim, unpublished results.
Functional Interaction of NK-4 with p300 and Groucho

p300 Augments NK-4-mediated Transactivation and Physically Interacts with NK-4—It was recently shown that the p300/CAF1 coactivator enhances transcription mediated by various transcription factors including CREB, AP-1, MyoD, and nuclear hormone receptors (58). Hence, we reasoned that p300 may be required for the transactivation of homeoproteins as well. In order to explore this possibility, we tested whether p300 can enhance NK-4-dependent transactivation using transient expression assays. For testing NK-4-dependent reporter gene activation, two different reporters containing either the wild-type NK-4 binding sites (E2 elements, E2CAT reporter) that were previously shown to be involved in autoregulation of NK-4 (31) or mutated NK-4 binding sites (E2m elements, E2mCAT reporter) were used for transfection. Cells were co-transfected with NK-4 in the presence (Fig. 4A, lanes 4 and 8) or absence (Fig. 4A, lanes 2 and 6) of the p300 expression vector, and CAT activities were measured. As shown in Fig. 4A, co-expression of p300 considerably increased CAT activity (lane 4) compared with expression of NK-4 alone (lane 2). However, this coactivation by p300 was not observed with the E2mCAT reporter that lacks NK-4-responsive elements (Fig. 4A, lane 8), suggesting that p300 enhanced transcriptional activation mediated by NK-4.

Next, we tested whether NK-4 can physically interact with p300. To this end, HeLa nuclear extracts were mixed with various GST-NK-4 proteins bound to glutathione-Sepharose beads, and any co-precipitated p300 was separated on SDS-PAGE and detected by Western blot analysis with an anti-p300 antibody. As shown in Fig. 4B, the p300 protein can bind to the full-length NK-4 protein (lane 3) and binds more strongly to the amino-terminal half of NK-4 (lane 4), which includes an activation domain. p300 also interacted weakly with the carboxy-terminal half of NK-4 that contains the homeodomain (Fig. 4B, lane 5).

To delineate the NK-4 binding domains of p300, in vitro translated [35S-labeled] p300 was subjected to in vitro pull-down assays with the GST-NK-4 (aa 1-416) protein (Fig. 4C, lanes 1-9). Full-length NK-4 strongly binds to p300C (aa 1135-2414) that contains the carboxy-terminal half of p300 (Fig. 4C, lane 9). NK-4 also binds weakly to p300N (aa 1-670) (Fig. 4C, lane 3) but not to p300M (aa 671-1194) (Fig. 4C, lane 6). Indeed, further delineation of the NK-4 binding domains of p300 using the truncated GST-NK-4 (aa 1-264) (Fig. 4C, lanes 10-18) revealed that the C/H3 region (aa 1620-1891) of the p300 is important for strong interaction with NK-4 (Fig. 4C, lane 18). Both p300 (aa 300-450) and p300 (aa 451-670), which include the C/H1 and the KIX domain, respectively, also weakly interact with GST-NK-4 (aa 1-264) (Fig. 4C, lanes 12 and 15). Fig. 4D shows the NK-4-binding domains for p300 along with the binding domains of p300 for other transcription factors. Taken together, these results indicate that p300 interacts with NK-4 and augments NK-4-mediated transactivation.

Functional Interaction of NK-4 with the Groucho Corepressor—As described above, NK-4 can act as an active transcriptional repressor (Fig. 1) for which the homeodomain is required (Fig. 2C and Fig. 3). As is the case for other active transcriptional repressors, NK-4 may recruit corepressors for its repression activity. Groucho proteins are well-known corepressors that are required for transcriptional repression by several distinct types of active transcriptional repressors (59). Among homeodomain proteins that act as transcriptional repressors, Engrailed was recently shown to interact with Groucho (60, 61). Like NK-4 and NK-3, Engrailed also contains the eh-1 domain, which is similar to the TN domain. Hence, we tested whether Groucho can act as a corepressor for NK-4.

As shown in Fig. 5A, coexpression of Groucho greatly enhanced the repressor activity of GAL4-NK-4 (lanes 6-8), whereas the effect of Groucho on GAL4 alone was negligible (lanes 2-4), suggesting that Groucho may act as a transcriptional corepressor for NK-4. In order to test whether NK-4 can directly interact with Groucho, we transfected cells with the Myc-tagged Groucho expression vector. Nuclear extracts from transfected cells were mixed with a GST-NK-4 protein bound to glutathione-Sepharose beads, and any co-precipitated Groucho was separated on SDS-PAGE and detected by Western blot analysis with an anti-Myc antibody. As shown in Fig. 5B, Groucho protein can bind to the full-length NK-4 protein.

Using full-length NK-4 and various in vitro translated Groucho proteins, we mapped the NK-4-binding domain of Groucho (Fig. 5C). Indeed, NK-4 can bind efficiently to the full-length Groucho protein (Fig. 5C, lane 3). Also, we found that the amino-terminal region of the Groucho protein (residues 1-192)
is required for the interaction with NK-4 (Fig. 5C, lane 9). The WD repeat region does not show any interaction with NK-4 under high stringency washing conditions (Fig. 5C, lanes 13–15). Next, we further delineated the Groucho-binding domains of NK-4 using in vitro pull-down assays with a variety of in vitro translated NK-4 and GST-Groucho(1–719). As expected, Groucho can bind strongly to full-length NK-4 (Fig. 5D, lane 3). Deletion of either the RD (construct 188–416) or the homeodomain (construct 1–267) significantly weakened the binding of NK-4 to Groucho (Fig. 5D, lanes 9 and 12). Also, whereas the construct (110–364) containing both the RD and the homeodomain showed a strong interaction with Groucho (Fig. 5D, lane 27), constructs containing either the homeodomain or the RD showed weak interaction with Groucho (Fig. 5D, lanes 24 and 21) Thus, for a strong interaction of NK-4 with Groucho, both the RD and the homeodomain are required, which is consistent with the result that both the RD and the homeodomain are required for the strong repressor activity of NK-4 when fused to the heterologous GAL4 DNA binding domain (Fig. 2C). Interestingly, deletion of the TN domain (construct 46–416) does not affect NK-4 binding to Groucho (Fig. 5D, lane 6). Taken together, the data show that Groucho directly interacts with NK-4 and enhances NK-4-mediated repression, suggesting that Groucho can act as a corepressor for NK-4.

**DISCUSSION**

NK-4 plays a key role in cardiogenesis (19), yet the regulatory mechanisms of gene transcription by NK-4 are poorly understood. In the present study, we have characterized functional domains of NK-4 that are required for transcriptional activity of NK-4. Our results indicate that NK-4 can act either as a transcriptional activator or repressor that utilizes separate functional domains. Furthermore, we provide the first evidence of NK-4 interactions with the p300 coactivator and the Groucho corepressor.

Other classes of transcription factors recruit coactivators for their transcriptional activation (58), and coactivators have been shown to form a multimeric activation complex with
P/CAF and p300/CBP (62, 63). As a potential coactivator for the NK-4 homeoprotein, we have demonstrated that p300 augments NK-4-mediated transcriptional activation in cultured cells (Fig. 4A). Consistent with these data, expression of viral protein E1A alleviated this effect,2 and p300 physically interacted with NK-4 both in vivo and in vitro (Fig. 4, B and C). These results support the notion that p300 can act as a coactivator for homeoproteins. The interaction of p300 with NK-4 is mediated by common domains of p300, such as C/H3, which was shown to act as binding sites for many other transcription factors, including viral proteins (Fig. 4D) (58). Thus, there may be a competition between NK-4 and other factors for binding to p300. It has recently been shown that the Drosophila CBP can act as a coactivator of Ci, a transcription factor homologous to the Gli family of proteins (64), and that Drosophila CBP is necessary for Dorsal-mediated activation of the twi promoter (65). These results indicate a common role for CBP as a coactivator of different transcription factors and suggest that the Drosophila CBP/p300 may also act as a coactivator for homeoproteins during Drosophila embryogenesis. Nevertheless, it is still conceivable that homeoproteins may also recruit specific coactivators for their transactivation function, because CBP/p300 represents a common coactivator that is required in addition to distinct coactivators for the function of different classes of transcription factors in mammals (66). Thus, it will be interesting to see whether specific coactivators of homeodo-

FIG. 5. Interaction of NK-4 with the Groucho corepressor. A, Groucho enhances the repressor activity of GAL4-NK-4. Cells were cotransfected with the G5EnXCAT reporter and the indicated expression vector (0.5 μg each) in the presence of increasing amounts of Groucho expression vector (GRO; lanes 2 and 6, 0.5 μg/transfection; lanes 3 and 7, 1 μg/transfection; lanes 4 and 8, 2 μg/transfection), and CAT activities were measured. The normalized CAT activity obtained from transfection with G5EnXCAT and pSG424 (GAL4 alone) was divided by the corresponding value obtained with a test expression vector, and -fold repression of the GAL4 and GAL4-NK-4 by Groucho is shown (averages of three sets of independent experiments). B, physical interaction of NK-4 with the Groucho corepressor. Cells were transfected with a Myc-tagged Groucho expression vector. Nuclear extracts were prepared and mixed with GST-NK-4 fusion protein (lane 3) or GST alone as control (lane 2) bound to glutathione-Sepharose beads. The co-precipitated Myc-Groucho (GRO) was separated on SDS-PAGE and detected by Western blot analysis with anti-Myc antibody. Lane 1 (Input) contained 4% (20 μg) of the amounts used for binding. C, analysis of NK-4-interaction domain of Groucho. The GST-NK-4 (NK4, aa 1–416) was expressed, bound to glutathione-Sepharose beads, and subjected to pull-down assays with in vitro translated, 35S-labeled various Groucho protein. Numbers indicate amino acid positions of the coding region used for in vitro translational. Input samples contained 5% of the amount used for binding. A schematic diagram of Groucho indicating interaction domains with NK-4 is shown in the lower panel. Regions of Groucho used for pull-down assays with NK-4 are indicated below the diagram, and NK-4 binding (+, interaction; −, no interaction) is shown. Numbers indicate amino acid residues. Q, GP, and SP, glutamine-rich, glycine and proline-rich, and serine and proline-rich regions, respectively; WD, WD motif. D, mapping Groucho interaction domain of NK-4. In vitro pull-down assays were performed with different in vitro 35S-labeled NK-4 proteins and glutathione-Sepharose-bound GST-GRO (GRO, aa 1–719). Input samples contained 5% of the amount used for binding. The lower panel shows a schematic diagram of NK-4 indicating interaction domains with Groucho. TN, TN domain; +, weak interaction; ++, strong interaction.
main transcription factors exist.

Groucho proteins can act as corepressors for specific active repressors such as Hairy-related proteins and Runt domain proteins (59, 60). For the interactions between Groucho and these class repressors, the WD repeat region of Groucho and the four-amino acid WRWP (WRPY) motif of repressors are important (67). As shown in Fig. 5, we have demonstrated that Groucho enhances the repressor activity of NK-4 and directly interacts with NK-4. These results suggest that Groucho can act as corepressor for NK-4. Furthermore, we found that Groucho can act as a corepressor for NK-4.5 In both cases, the homeodomains are required for the strong interaction with Groucho (Fig. 5C). The WD repeat domain of Groucho appears not to be important for the interaction with NK-4 under our experimental conditions (Fig. 5D). Instead, the amino-terminal region of Groucho, which was assigned to a transcriptional repressor domain, is required for the interaction (Fig. 5D). The Engrailed homeodomain also interacts with Groucho (61) via the eh1 domain, which is related to the TN domain of the NK-2 class. However, the deletion of the TN domain from the NK-3 and NK-4 proteins does not affect interactions with Groucho. Thus, NK class homeodomain proteins have a unique feature in this sense, suggesting a different regulatory role for the TN domain in protein-protein interaction. We have recently shown that the NK class of homeoproteins can interact with homeodomain-interacting protein kinases (HIPKs) that can act as co-repressors of homeoproteins (47). Indeed, we found that homeodomain-interacting protein kinases (HIPKs) also interact with the Groucho corepressor.3

We have demonstrated that NK-4 can act either as a transcriptional activator or repressor by recruiting the p300 coactivator and the Groucho corepressor, respectively. It was recently shown that Dorsal, which plays an important role for the body axis formation of Drosophila, recruits Drosophila CBP and Groucho for its transcriptional activity (65, 68). Thus, our results raise one interesting question as to how NK-4 can be switched from a transcriptional activator to repressor. As shown in Fig. 6, the context of the target gene promoter could be critical for determining whether activation or repression occur. For example, Dorsal can interact with Twist for the activation of the target gene (69), whereas DSP1, an HMG-like protein, can switch a Dorsal activator to a transcriptional repressor (70). Likewise, NK-4 may interact with other cofactors, thereby acting as either an activator or repressor. As was seen in the D-mef-2 activation in cardiac cells, a GATA site binding factor collaborates with NK-4 (37, 42). Also, we found that NK-4 can interact with other transcription factors such as Twist and NK-3 in cultured cells.4 Thus, it is conceivable that, depending on gene promoters that contain different cis-acting regulatory elements, NK-4 can interact with other cofactors, thereby providing different protein-binding interfaces to recruit either coactivator or corepressor complexes. Since a corepressor complex often contains histone deacetylase activity (71), it will be of great interest to see whether the NK-4 homeodomain transcription factor, which interacts with the Groucho corepressor, recruits histone deacetylases.

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