Transcriptional Antagonism between Hmx1 and Nkx2.5 for a Shared DNA-binding Site*

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The recently described Hmx family of homeodomain proteins is predominately expressed in discrete regions of developing sensory tissues. In this report, we have identified the preferred DNA-binding site of the murine Hmx3 homeodomain protein by the selection and amplification binding (SAAB) technique. The consensus Hmx-binding site contained the sequence 5'-CAAGTG-3', which differs from the 5'-TAAT-3' motif commonly associated with homeodomain proteins. Instead, the Hmx consensus is similar to the 5'-CAAGTG-3'-binding sites of Nkx2.1 and Nkx2.5 homeodomain proteins. Based on mutation studies, both the 5'-CAAG-3' core and the 3'-TG dinucleotide are required for high affinity binding by Hmx3 and the homologous Hmx1 protein. A critical determinant of this specificity is the glutamine at position 50 in the third helix of the Hmx homeodomain. Hmx1 binds to the 5'-CAAGTG-3' element with an apparent dissociation constant of 20 nM. Unexpectedly, the human Hmx1 protein specifically repressed transcription from a luciferase reporter gene containing 3 copies of the 5'-CAAGTG-3' sequence. In contrast, the Nkx2.5 protein transactivated this luciferase reporter. Interestingly, co-expression of Hmx1 and Nkx2.5 attenuated each other's activity, suggesting that genes containing the CAAGTG element can integrate signals from these proteins. Therefore, Hmx1 and Nkx2.5 proteins bind a unique DNA sequence and act as transcriptional antagonists.

Homeodomain genes are involved in a wide variety of developmental pathways (1–4). Mutation studies and expression patterns of several members of the homeodomain gene family have indicated a role in controlling specification of cranial structures, including development of neurons and sensory organs (2, 5–7). A novel homeodomain gene family, Hmx, was first identified by Stadler et al. (8) using low stringency screening of a human craniofacial cDNA library. Homologous Hmx genes have now been identified in a diverse number of species (9). There are three closely related members of the Hmx family in humans and mice, which are designated Hmx1, Hmx2, and Hmx3 (8, 9). The murine Hmx2 and Hmx3 genes were originally named Nkx5.2 and Nkx5.1, respectively, based on limited homology with the Nk homeodomain family of Drosophila (10, 11). However, the Hmx family is substantially different from the Nk family (9). Alignment of the Hmx3 homeodomain sequence to the four Drosophila Nk genes yielded 50–61% amino acid sequence identity, which is not substantially greater than seen when Hmx3 is compared with other homeodomains, such as Drosophila Antp (47%). Furthermore, a Drosophila Hmx homologue has been identified and found to have 94% amino acid identity with the murine Hmx3 homeodomain (9). Finally, there is little or no homology between the Nkx and Hmx proteins outside the homeodomains. Hence, we have used the Hmx nomenclature for these genes, as assigned by the human gene mapping nomenclature committee (9).

The Hmx genes are believed to be important regulators of development in sensory organs and neurons based on their expression patterns in the embryo (10, 12). The expression of murine Hmx3 is especially high in the otic vesicle, neuroectodermal cells of the central nervous system, neuronal derivatives of the neural crest, including the dorsal root ganglia and myenteric ganglia, and transiently in the second branchial arch. In particular, the predominant expression in the otic vesicle and postmitotic neurons has suggested an involvement in the development of the inner ear and specification of neuronal phenotype (13). The murine Hmx genes have very similar expression patterns with two chicken homologues, GH6 and SOHO-1 gene (14, 15), suggesting that the Hmx genes play an evolutionary conserved role during development. The chicken homologue of Hmx1 (GH6) has been reported to be expressed in the developing heart (14). Nkx2.5 is expressed early during heart morphogenesis and activates early cardiac gene expression (16–19). Hmx and Nkx genes are found in overlapping regions in vertebrate embryos (10, 12, 14). In particular, Nkx2.5 and chicken Hmx1 are both expressed in the developing heart myocardium (12, 14, 16). These results suggest that Hmx and Nkx2.5 proteins both regulate early transcription events in development.

To begin to address the functional role of Hmx homeodomain proteins, we have identified the DNA-binding site of the Hmx1 and -3 proteins. We show that these Hmx proteins prefer the core binding sequence 5'-CAAG-3' and not the typical 5'-TAAT-3' core found among most homeodomain proteins (1–4). Instead, the Hmx-binding site resembles the consensus site found for Nkx2.5 protein (also called TINMAN) by Chen and Schwartz (20) and Nkx2.1 protein (also called thyroid transcription factor-1) by Damante et al. (21). We demonstrate that Hmx1 represses transcription of a luciferase reporter gene containing the Hmx preferred DNA-binding site. Since Nkx2.5 transactivates this reporter and the Nkx2.5 and Hmx proteins

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1 J. Murray, personal communication.
Hmx Homeodomain Activities

are co-expressed in some tissues, we propose that Hmx1 and Nkx2.5 may act as transcriptional antagonists.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Hmx1 and -3 Proteins—A fragment of the murine Hmx3 gene was PCR amplified from a genomic clone provided by Dr. Tom Lufkin (Mt. Sinai Medical Center). The primers were 5'-AACGAGCATTTCTTCCTGACATGG-3' and 5'-CCAGAGGACCGAATTCAGG-3' and nucleotides 1675 to 1695 (5'-gaattccCGGTCTCTCTGATG-3') with BamHI and EcoRI sites (lowercase) to facilitate subcloning into pGEX4T-3 glutathione S-transferase (GST) vector (Pharmacia) and was confirmed by DNA sequencing. The resulting plasmid, pGST-Hmx3, encodes amino acids 309-458, which contains the homeodomain 20 amino-terminal flanking residues, and the entire COOH-terminal region. To make plasmid pGST-Hmx1, pGST-Hmx3 Gln → Asn and antisense primer containing the point mutation (underlined), nucleotides 1423-1448, (5'-GGGATTTGGAACGACATCTGAC-3') was used with the previous 5' primer (nucleotides 1322-1325) to make a PCR megapimer. In the second PCR step the megamer was used with the previous 3' primer (nucleotides 1675-1695). The PCR profile was 94 °C, 2 min; 70 °C, 2 min, 72 °C, 3.5 min for 30 cycles using Pfu DNA polymerase (Stratagene). Hmx3 Gln → Asn DNA was cloned into pGEX4T-3 and confirmed by sequencing. Plasmid pGST-Hmx1 was made by a series of sequential subcloning of the Hmx1 coding region from pBSK II Hmx1 cDNA plasmid (9) into pGEX 6P-1 (Pharmacia). lon cleavage with 30 units of PreScission protease (Pharmacia) for 1 h at 25 °C in 10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 μg of poly(dI-dC), 120 ng of Hmx3 (thrombin-cleaved from GST or glutathione-eluted), or 300 ng of Hmx1 Protein was released by cleavage with 10 units of thrombin (same oligonucleotide as used in the EMSAs (5'-gatccAAAGCCTATACGATACG-3') ligated into the BamHI site upstream of the CMV promoter (TK) promoter in the TK-luciferase reporter construct (25). Hmx-TK-luc contains three inserts, 2 in the sense and 1 in the antisense orientation. A CMV β-galactosidase reporter plasmid (CLONTECH) was co-transfected in all experiments as a control for transfection efficiency.

Cell Culture, Transient Transfections, Luciferase, and β-Galactosidase Assays—COS-7 and HeLa cells were cultured and transfected as described (26) by the calcium phosphate method or electroporation. For calcium phosphate transfection, 5–10 μg of plasmid DNA was used. For electroporation, HeLa cells were mixed with 2.5 μg (or as indicated) of expression plasmids, 2.5 μg of reporter plasmid, and 0.5 μg of CMV β-galactosidase plasmid. HeLa cells were electroporated at 220 μF and 960 microfarads (Bio-Rad) placed in 60-mm culture dishes and fed with 5% fetal calf serum and Dulbecco’s modified Eagle’s medium. Cells were then lysed and assayed for reporter activities and luciferase activity by Bradford assay (Bio-Rad). Luciferase was measured using reagents from Promega. β-Galactosidase was measured using the Galacto-Light Plus reagents (Tropix Inc.).

**RESULTS**

Identification of the Hmx3 DNA-binding Site—To determine the DNA-binding site of Hmx3, the SAAB technique was used with bacterial expressed GST-Hmx3 protein containing the homeodomain and COOH-terminal region. Previous studies with other homeodomain proteins have shown that the information necessary for DNA binding is contained within the homeodomain region (3, 4). For example, the truncated homeodomain-containing Nkx2.1 protein binds with the same apparent Kd as the full-length protein (21). The sequences of oligonucleotides selected after 2, 3, and 5 sequential SAAB rounds are shown in Fig. 1A. As shown in Fig. 1B, a consensus containing 5′-CAA(T/G)-3′ was observed. Following the fifth round, there was a selection for oligonucleotides containing the consensus sequence 5′-CAAGTGCGTG-3′. In many cases, multiple copies of this sequence were found in the oligonucleotides. Each of the nucleotides within the consensus was highly conserved, with frequencies ranging from 75 to 100% occurrence (Fig. 1B). In particular, the 5′-CAAG-3′ core was very predominant, being present in all of the selected oligonucleotides.

2 The abbreviations used are: PCR, polymerase chain reaction; SAAB, selection and amplification binding; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; CMV, cytomegalovirus; luc, luciferase; HNF, hepatocyte nuclear factor.
**FIG. 1. Identification of Hmx3 binding sites.** A, alignment of the PCR product sequences after 2, 3, and 5 successive SAAB rounds (number of rounds is denoted by the prefix, e.g. 2-1 is from round 2). The best match and other conserved regions are indicated in **bold**. The common sequences contributed from the PCR primers are indicated by a and b. B, sequence of the consensus Hmx3 DNA-binding site determined from the PCR products after 5 SAAB rounds. The frequencies of each consensus nucleotide within the best match of each oligonucleotide, and from all the sites indicated in **bold** are shown. C, Hmx3 protein was incubated with the Nkx2.1 consensus sequence as the radioactive probe in the absence or presence of unlabeled oligonucleotides as competitor DNAs. Competitor oligonucleotides were used at 10-, 25-, and 50-fold molar excess concentrations. The free probe and bound complex are indicated. The sequences of the DNAs are shown at the bottom of the figure with the terminal partial BamHI sites in **lowercase**, and the CAAGTG motif separated by **vertical lines**.
These results indicate a strong preference for this consensus sequence. **Comparison of Hmx3 Binding to Nkx and Hmx Consensus Sites**—Because the Hmx and Nkx2.1 and 2.5 consensus elements both contain a 5'-CAAGT-3' motif, but the flanking sequences differed, we tested whether Hmx3 could also bind the Nkx2.1 element. Using a competitive EMSA DNA binding assay, we showed that the Hmx3 protein bound to DNA containing the Nkx2.1/2.5 consensus site and was efficiently competed by either the Nkx2.1/2.5 or the Hmx consensus motifs (Fig. 1C). Thus, Hmx3 binds both Nkx2.1/2.5 and Hmx consensus binding sites without preference for flanking sequences. As a control for binding specificity, Hmx3 binding to the 5'-CAAGTG-3' consensus sequence was not competed by an oligonucleotide containing an octamer consensus site recognized by the Oct-1 POU homeodomain. It should be noted that the 5'-CAAGTG-3' consensus site for Hmx and Nkx proteins also contains a consensus helix-loop-helix protein-binding site (5'-CANNTG-3') (27). While it was very unlikely that Hmx3 DNA binding activity was similar to helix-loop-helix proteins, we ruled out this formal possibility by demonstrating that Hmx3 did not bind to oligonucleotides containing two other 5'-CANNTG-3' motifs. Both the 5'-CAGCTG-3' and 5'-CAGCTG-3' elements recognized by the AP4 and muscle creatine kinase helix-loop-helix proteins, respectively, did not bind Hmx3 (data not shown). Thus, Hmx3 specifically binds the Hmx and Nkx2.1/2.5 motifs with comparable affinities.

**Mutations in the CAAG Core and Flanking TG Dinucleotide Reduce Hmx3 Binding**—The binding of Hmx3 to the CAAG core was compared with the canonical TAAT sequence found in the Antennapedia class of homeodomains (28). Hmx3 binding to the competitor was greatly reduced when the 5'-CAAG-3' sequence was mutated to a typical homeodomain-binding site (Fig. 2A). An even greater effect was seen when the competitor binding site was mutated at both the core and flanking dinucleotide 5'-TAATCA-3' (Mu10) (Fig. 2A). Similarly, mutation to 5'-TAATTC-3' (Hmx Mu11), which matches a consensus Idx-binding site (29), greatly reduced Hmx3 binding to the competitor (data not shown). Finally, complete removal of the CAAG core (Hmx Mu3) completely eliminated binding to the competitor (Fig. 2B). These results demonstrated that the 5'-CAAG-3' core is critical for Hmx3 binding activity.

The relative difference between Mxs (TAATTG) and Hmx Mu10 (TAATCA) competitions indicated that the 3'-TG dinucleotide contributes to Hmx3 binding. This contribution was confirmed by the reduced Hmx3 binding to the competitor seen upon mutation of the TG in the context of the 5'-CAAG-3' core sequence (Hmx Mu2, CAAGGT) (Fig. 2A). To further test the importance of the TG dinucleotide in the absence of the 5'-CAAG-3' core, the TG was mutated to a GG (Fiz) or AT (Hmx Mu9) following a 5'-TAAT-3' core. Hmx3 did not bind either of these DNAs in competition assays (Fig. 2A). Quantitation of the effect of each competitor on Hmx3 binding is shown in Fig. 2B.

We also confirmed that sequences outside the 5'-CAAG-3' and flanking TG dinucleotide motif are not required for Hmx3 binding. This was important to test since the SAAB consensus protein had contained an additional four nucleotides (CGTG) downstream of the CAAGTG motif. Changes in these nucleotides (Hmx Mu4) did not noticeably affect Hmx3 binding activity within the parameters of the competition assay (10–50-fold molar excess) (Fig. 2A). This is consistent with Hmx3 binding to the Nkx2.1/2.5 consensus sequence, which also lacks the 3'-CGTG (Fig. 1C). Taken together these results show that

**Fig. 2. Both the 5'-CAAG-3' core and 3'-TG dinucleotide are required for Hmx3 binding.** A, Hmx3 DNA binding activity was measured by EMSA with the Nkx2.1 probe in the presence or absence of competitor DNAs at 10-, 25-, and 50-fold molar excess. The free probe and bound complexes are indicated. The oligonucleotide sequences are shown below the autoradiogram, with differences from the Hmx3 oligonucleotide underlined. The terminal partial BamHI sites are in lowercase. B, quantitation of the binding efficiency of Hmx3 from the EMSA experiments. Dried gels were quantitated using the InstantImager (Packard). The bound DNA radioactivity was measured and the inhibition of bound complex from 50-fold excess of each competitor DNA was determined. The values are normalized to 100% binding without competitor DNA, with the means and standard deviations from two to six independent shown.

Both the 5'-CAAG-3' and the 3'-TG dinucleotide are the key components of the DNA-binding site.

**Mutation in the Recognition Helix of Hmx3 Reduces DNA Binding Activity**—It has been shown that the 3'-dinucleotide of the DNA-binding site can confer binding specificity that is determined by the amino acid at position 50 of the classical TAAT binding homeodomain proteins (4, 30). In addition, Da mante et al. (21) demonstrated that the glutamine at position 50 is important for recognition of the TG dinucleotide in the Nkx2.1 consensus DNA-binding site, 5'-CAAGTG-3'. Hmx3 also contains this glutamine in the highly conserved third helix, suggesting that this residue may play a similar role in Hmx3. We made the corresponding mutation in Hmx3 changing the glutamine (Q) to a lysine (K). The glutamine to lysine mutation decreased binding to undetectable levels (Fig. 3A). As a control, the mutant protein could bind to a bicoid element (5'-TAATCC-3') (Fig. 3B). The lysine at position 50 is important for recognition of the CC dinucleotide in the bicoid element (4, 31). GST-Hmx3 Gln → Lys mutant protein binding to the
The glutamine at position 50 in the homeodomain is required for Hmx3 binding to the 5'-CAAGTG-3' sequence. A, GST-Hmx2 and GST-Hmx3 Gln → Lys DNA binding activity was measured by EMSA with the Nxx2.1 probe. Several concentrations of each protein were used for binding: 0.1, 0.5, 1 μg of GST-Hmx3, and 1, 5, 10 μg of GST-Hmx3 Gln → Lys. The free probe and bound complexes are indicated. B, GST-Hmx3 Gln → Lys DNA binding activity was measured by EMSA with the bicoid probe (5'-TAATCC-3'). Approximately 120 ng (0.1 μg) of GST-Hmx3 and GST-Hmx3 Gln → Lys (1.0 μg) proteins were used to determine binding activity. Binding activity was measured in the presence and absence of competitor DNA at 50-fold molar excess (Bic, bicoid competitor). C, Western blot of bacterial expression GST-Hmx3 and GST-Hmx3 Gln → Lys proteins. Equal volumes of GST affinity purified fusion proteins (4 μg) were resolved on a 12.5% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride filter, and immuno-blotted using an antibody against a COOH-terminal peptide of Hmx3.

Hmx1 Binds the 5'-CAAGTG-3' Element with a Similar Specificity as Hmx3—To determine if the binding characteristics of the homologous human Hmx1 protein were similar to Hmx3 we used a competitive EMSA DNA binding assay as described above. Hmx1 was chosen so as to extend our findings to another member of the Hmx family. Human Hmx1 has 92% amino acid identity to murine Hmx3 in the homeodomain. We demonstrate that the Hmx1 protein bound to DNA containing the Nkx2.1/2.5 site and was efficiently competed by the Nkx and Hmx consensus elements, but not other motifs (Fig. 4A). Hmx1 had the same DNA binding specificity as shown for the truncated Hmx3 protein (Fig. 4B).

The binding affinity of Hmx1 to the 5'-CAAGTG-3' sequence was measured by EMSA (Fig. 4C). The apparent dissociation constant (K_D) was calculated using different protein and probe concentrations by Scatchard analysis as 20 nM (Fig. 4C). This K_D is higher than those reported for Nkx2.1 (3 nM), Antennapedia (1.2 nM), and Engrailed (1–2 nM) (4, 20, 21). The K_D using the GST-Hmx3 fusion protein was 1.4 nM, which is very similar to the reported K_D values of other homeodomain proteins (Fig. 4D). We have seen that the GST moiety can affect binding of the Pitx2 homeodomain protein. GST-Pitx2 has an apparent K_D of ~0.5 nM while the non-fusion purified Pitx2 protein demonstrated a K_D of 50 nM (26).

Hmx1 Specifically Represses Transcription from a Promoter Containing the 5'-CAAGTG-3' sequence—To determine if Hmx1 could regulate a reporter gene containing the 5'-CAAGTG-3' sequence we co-transfected an expression vector encoding human Hmx1 (CMV-Hmx1) with a luciferase reporter containing three Hmx-binding sites (Hmx-TK-luc) into HeLa cells. As a control for transfection efficiency, a CMV containing three Hmx-binding sites (Hmx-TK-luc) was co-transfected with the CMV vector containing Hmx1 (Fig. 5A). In the absence of the Hmx sites, there was only marginal repression of the reporter by Hmx1 (Fig. 5A). Thus, Hmx1 specifically represses the promoter activity of a reporter containing the 5'-CAAGTG-3' sequence.

For comparison, we asked if Nkx2.5 could transactivate the 5'-CAAGTG-3' reporter under our conditions. We found that Nkx2.5 caused a 3-fold stimulation of this reporter and had no effect on the reporter without the 5'-CAAGTG-3' elements (Fig. 5B). This is consistent with published reports that Nkx2.1 and Nkx2.5 are transcriptional activators (20, 21). Thus, Hmx1 represses, while Nkx2.5 activates, transcription via the 5'-CAAGTG-3' element.

Hmx1 and Nkx2.5 Act as Transcriptional Antagonists—Since Nkx2.5 (Timman) and Hmx1 are expressed in the developing heart and both bind the same core DNA element (5'-CAAGTG-3') we then asked if these factors had an antagonistic effect on transcription. The Nkx2.5 and Hmx1 expression vectors were co-transfected along with the 5'-CAAGTG-3' TK-luc reporter plasmid. Co-transfection of equal amounts of each vector resulted in an overall 2-fold repression of transcription (Fig. 6). This is an intermediate value between the 3-fold activation by Nkx2.5 alone and the 4-fold repression by Hmx1 alone. These results indicate that Hmx1 can antagonize Nkx2.5 activation of the reporter plasmid, and conversely Nkx2.5 can attenuate Hmx1 repression. To vary the relative amounts of Hmx1 protein compared with Nkx2.5 in the transient transfection assay, we varied the amount of expression vector DNA. Antagonism was observed even with lower levels of Hmx1 expression vector DNA (Fig. 6). These results suggest that the relative levels of Nkx2.5 and Hmx1 proteins may regulate the activity of genes containing the CAAGTG element.

DISCUSSION

This study represents the first molecular/biochemical characterization of members of the Hmx homeodomain family. Hmx 1 and Hmx 3 strongly prefer the 5'-CAAGTG-3' DNA-binding site in contrast to the 5'-TAAT-3' motif preferred by nearly all other metazoan homeodomain proteins. The usual bias among homeodomain proteins for a 5'-TAAT-3' core was recently confirmed by Wilson et al. (30) using the SAAB selection strategy similar to the one used in this study. For Hmx proteins, both the 5'-CAAGTG-3' core and the 3'-flanking TG dinucleotide contribute to binding specificity. This binding site has a striking similarity to the consensus sites identified for the Nkx2.5 and Nkx2.1 proteins. Chen and Schwartz (20) used a similar SAAB methodology with Nkx2.5 to identify a high affinity 5'-TNAGTG-3' motif and lower affinity 5'-TAAT-3' containing motifs. Similarly, Damante et al. (21) have shown that Nkx2.1 bound the core consensus sequence 5'-CAAGTG-3', which is also recognized by the Nkx2.5 protein (20). Thus, Hmx binds to
the same 5'-CAAGTG-3' sequence as Nkx2.1 and 2.5 proteins. DNA binding specificity of homeodomains is dictated mostly by residues in the recognition helix and the NH2-terminal arm (21, 30, 32–34). We have shown that the glutamine at position 50 of the Hmx recognition helix is essential for binding. The position 50 residue has been shown to be critical for recognizing the 3'-dinucleotide of the DNA binding sequence of both TAAT-binding and CAAG-binding proteins (4, 21, 30). For example, conversion of a glutamine to lysine at position 50 in the Ftz homeodomain changed the recognition sequence from 5'-TAATGG-3' to 5'-TAATCC-3' (32–34). The latter sequence is bound by the Bicoid protein, which contains a lysine at position 50. Consequently, Hmx requires the same residue that has also been identified as a critical determinant in other groups of homeodomain proteins. Recently, a detailed set of experiments was performed to determine the amino acids required for binding to the 5'-CAAGTG-3' sequence by Nkx2.1 (35). This study demonstrated that the amino acid in position 54 of the homeodomain is involved in the recognition of the guanosine at the 3' end of the core sequence 5'-CAAG-3'. The authors further demonstrated that the 5' cytosine is recognized by the amino acids located in positions 6, 7, and 8 of the NH2-terminal arm. Comparison of the Nkx and Hmx sequences supports and extends the conclusions that these residues contribute to binding to the 5'-CAAGTG-3' sequence. Specifically, Nkx2.1 has a tyrosine at position 54 that is conserved among Nkx2 family members. In contrast, all the Hmx family members contain an asparagine at this position. These residues are similar in that both have bulky polar side chains. The Nkx2.1 residues at positions 6, 7, and 8 are valine, leucine, phenylalanine, while Hmx proteins contain threonine, valine (isoleucine in one case), phenylalanine at these positions. Between the two families, positions 7 and 8 are identical or homologous, while the residues at position 6 are structurally quite different. Hence the difference in position 6, together with the different, albeit similar, residues at position 54, suggests that there is some flexibility in the binding determinants of the CAAG-binding group of homeodomain proteins.

We have demonstrated that Hmx1 can specifically repress transcription of a reporter gene containing 3 copies of the 5'-CAAGTG-3' sequence. Furthermore, we have shown that Hmx1 and Nkx2.5 act as transcriptional antagonists. The degree of Nkx2.5 transactivation that we observed was similar to previous results with the Nkx2.5 protein on multimers of its binding site (20). Since both Nkx2.5 and chicken Hmx1 (GH6) have overlapping expression patterns during heart development (12, 14), these proteins may also differentially regulate genes containing the 5'-CAAGTG-3' element. There is precedence for the regulation of transcription during differentiation by transcriptional antagonists. A well studied example is the transcriptional antagonism between the homeodomain proteins Ftz and Engrailed, where Engrailed represses or
Fig. 5. Transcriptional repression of a 5′-CAAGTG-3′-containing luciferase reporter by Hmx1. A. HeLa cells were co-transfected with either Hmx-TK-luciferase reporter gene containing three copies of the Hmx binding sequence (striped boxes) or the parental TK-luciferase reporter without the Hmx sites. The cells were co-transfected with either the CMV-Hmx1 expression plasmid (+) or the CMV plasmid without Hmx1 (−). To control for transfection efficiency, all transfections included the CMV β-galactosidase reporter. Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown relative to the TK-luc without Hmx1 control (mean ± S.E. (n = 8) from four independent experiments). All luciferase activities were normalized to β-galactosidase activity. The mean TK-luciferase activity without Hmx1 expression was about 1600 light units per 20 μg of protein, and the β-galactosidase activity was about 15,000 light units per 20 μg of protein. B. HeLa cells were co-transfected with either Hmx-TK-luciferase reporter gene containing three copies of the Hmx binding sequence (striped boxes) or the parental TK-luciferase reporter without the Hmx sites. The cells were co-transfected with either the Nkx2.5 expression plasmid (+) or a CMV plasmid without Nkx2.5 (−). Activities were normalized as described in panel A, from three independent experiments (n = 6).

Fig. 6. Transcriptional antagonism of the 5′-CAAGTG-3′-containing luciferase reporter by Nkx2.5 and Hmx1. HeLa cells were co-transfected with the Hmx-TK-luciferase reporter gene and either the Nkx2.5 expression plasmid (7.5 μg), the CMV-Hmx1 expression plasmid (0–7.5 μg). The total amount of DNA was held constant by addition of the empty CMV vector (−). To control for transfection efficiency, all transfections were normalized to β-galactosidase from a co-transfected CMV β-galactosidase reporter. Cells were incubated for 24 h, then assayed for luciferase and β-galactosidase activities. The activities are shown relative to Hmx-TK-luc without Hmx1 or Nkx2.5 expression (mean ± S.E. (n = 6) from three independent experiments).

activity by competing for HNF-3β binding to a shared DNA element.

In addition to our experimental system, there are now at least three reports of 5′-CAAGTG-3′ type elements controlling gene transcription in response to Nkx2.1 and Nkx2.5. A 5′-CAAGTG-3′ response element for Nkx2.1 has been identified in the rat thyroglobulin promoter near the TATA box (21). Recently, targets for Nkx2.5 have been identified in the atrial natriuretic factor promoter (39) and the cardiac α-actin gene (40). The observation that natural Nkx-target genes can be regulated by 5′-CAAGTG-3′ elements strengthens the likelihood that Hmx proteins will also regulate genes containing this element. Furthermore, the shared DNA binding specificity and overlapping expression patterns suggest that the Hmx and Nkx gene families may recognize and coordinately regulate overlapping sets of target genes during specification of cardiac and neuronal phenotypes.

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REFERENCES

1. Scott, M. P., Tamkun, J. W., and Hartzel, G. W. (1989) Biochim. Biophys. Acta 989, 25–48
2. McGinnis, W., and Krumlauf, R. (1992) Cell 68, 283–302
3. Kornberg, T. B. (1993) J. Biol. Chem. 268, 26813–26816
4. Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G., and Wuthrich, K. (1994) Cell 78, 211–223
5. Gruss, P., and Walther, C. (1992) Cell 69, 719–722
6. He, X., Treacy, M. N., Simmons, D. M., Ingraham, H. A., Swanson, L. W., and Rosenfeld, M. G. (1989) Nature 339, 551–554
7. Herr, W., and Cleary, M. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2320–2324
8. Stadler, H. S., Padanilam, B. J., Beutow, K., Murray, J. C., and Solursh, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 11579–11583
9. Stadler, H. S., Murray, J. C., Leyssens, N. J., Goodfellow, P. J., and Solursh, M. (1995) Mamm. Genome 6, 383–388
11642

Hmx Homeodomain Activities

10. Bober, E., Baum, C., Braun, T., and Arnold, H. (1994) *Dev. Biol.* 162, 288–303
11. Kim, Y., and Nirenberg, M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* 86, 7716–7720
12. Rinkwitz-Brandt, S. R., Justus, M., Oldenettel, I., Arnold, H. H., and Bober, E. (1995) *Mech. Dev.* 52, 371–381
13. Hadrys, T., Braun, T., Rinkwitz-Brandt, S., Arnold, H., and Bober, E. (1998) *Development* 125, 33–39
14. Stadler, H. S., and Sotursch, M. (1994) *Dev. Biol.* 161, 251–262
15. Deitche, D. L., Fekete, D. M., and Cepko, C. L. (1994) *J. Neurosci.* 14, 486–498
16. Lints, T. J., Parsons, L. M., Hartley, L., Lyons, I., and Harvey, R. P. (1993) *Development* 119, 419–431
17. Davis, R. L., Cheng P-F., Lassar, A. B., and Weintraub, H. (1990) *Cell* 60, 733–746
18. Hayashi, S., and Scott, M. P. (1990) *Cell* 63, 883–894
19. Miller, C. P., McGhee, R. E., and Habener, J. F. (1994) *EMBO J.* 13, 1145–1156
20. Wilson, D. S., Chen, G., Jun, S., and Desplan, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6886–6891
21. Hanes, S. D., and Brent, R. (1989) *Cell* 57, 1275–1283
22. Jaynes, J. B., and O’Farrell, P. H. (1988) *Nature* 336, 744–749
23. Han, K., Levine, M. S., and Manley, J. L. (1989) *Cell* 56, 573–583
24. Duncan, S. A., Navas, M. A., Dufort, D., Rossant, J., and Stoffel, M. (1998) *Science* 281, 692–695
25. Tverberg, L. A., and Russo, A. F. (1993) *J. Biol. Chem.* 268, 15965–15973
26. Amendt, B. A., Sutherland, L. B., Semina, E., and Russo, A. F. (1998) *J. Biol. Chem.* 273, 20066–20072
27. Davis, R. L., Cheng P-F., Lassar, A. B., and Weintraub, H. (1990) *Cell* 60, 733–746
28. Miller, C. P., McGhee, R. E., and Habener, J. F. (1994) *EMBO J.* 13, 1145–1156
29. Wilson, D. S., Chen, G., Jun, S., and Desplan, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6886–6891
30. Wilson, D. S., Chen, G., Jun, S., and Desplan, C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 6886–6891
31. Driever, W., and Nusslein-Volhard, C. (1988) *Nature* 337, 138–143
32. Percival-Smith, A., Muller, M., Affolter, M., and Gehring, W. J. (1990) *EMBO J.* 9, 1967–1974
33. Hanes, S. D., and Brent, R. (1989) *Cell* 57, 1275–1283
34. Treisman, J., Gonczy, P., Vashishtha, M., Harris, E., and Desplan, C. (1989) *Cell* 57, 553–562
35. Damante, G., Pellizzari, L., Esposito, G., Fogolari, F., Viglino, P., Fabbro, D., Tell, G., Formisano, S., and Di Lauro, R. (1994) *Nucleic Acids Res.* 22, 3075–3083
36. Duncan, S. A., Navas, M. A., Dufort, D., Rossant, J., and Stoffel, M. (1998) *Science* 281, 692–695
37. Durocher, D., Chen, C., Ardati, A., Schwartz, R. J., and Nemer, M. (1996) *Mol. Cell. Biol.* 16, 4648–4655
38. Chen, Y., Bei, M., Woo, I., Satekata, I., and Maas, R. (1996) *Dev.* 122, 3035–3044