Characterization of Nkx3.2 DNA Binding Specificity and Its Requirement for Somitic Chondrogenesis*

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We have previously shown that Nkx3.2, a member of the NK class of homeoproteins, functions as a transcriptional repressor to promote somitic chondrogenesis. However, it has not been addressed whether Nkx3.2 can bind to DNA in a sequence-specific manner and whether DNA binding by Nkx3.2 is required for its biological activity. In this work, we employed a DNA binding site selection assay, which identified TAAGTG as a high affinity Nkx3.2 binding sequence. Sequence-specific binding of Nkx3.2 to the TAAGTG motif in vitro was confirmed by electrophoretic mobility shift assays, and mutagenesis of this sequence revealed that HRAGTG (where H represents A, C, or T, and R represents A or G) comprises the consensus DNA binding site for Nkx3.2. Consistent with these findings, the expression of a report gene containing reiterated Nkx3.2 binding sites was repressed in vivo by Nkx3.2 co-expression. In addition, we have generated a DNA nonbinding point mutant of Nkx3.2 (Nkx3.2-N200Q), which contains an asparagine to glutamine missense mutation in the homeodomain. Interestingly, despite being defective in DNA binding, Nkx3.2-N200Q still retains its intrinsic transcriptional repressor function. Finally, we demonstrate that unlike wild-type Nkx3.2, Nkx3.2-N200Q is unable to activate the chondrocyte differentiation program in somitic mesoderm, indicating that DNA binding by Nkx3.2 is critical for this factor to induce somitic chondrogenesis.

Endochondral ossification, which accounts for the majority of vertebrate skeletal formation, begins with chondrogenesis, in which a cartilaginous template is established prior to replacement by mature bone tissue (1). The cartilage of the axial skeleton originates from somites, paired blocks of mesodermal tissue symmetrically flanking the central vertebrate axis (1). Whereas the dorsal domain of each somite, the dermomyotome, is a progenitor tissue for skeletal muscle and dermis, the ventral domain of the somite, the sclerotome, differentiates into the cartilage template of the vertebrae and ribs (2, 3). Signals from the notochord are crucial for the induction of the sclerotome and subsequent formation of axial cartilage (4). Sonic Hedgehog (Shh), a secreted molecule expressed in both the notochord and floor plate of the neural tube, has been shown to be required for somitic chondrogenesis (5–7). In addition to Shh, bone morphogenetic proteins (BMPs) also play an important role in axial cartilage differentiation (8–11). However, it is currently unclear how BMP signal transduction pathways act to promote somitic chondrogenesis.

Nkx3.2, the vertebrate homologue of Drosophila bagpipe (NK3), is a member of the NK family of homeoproteins, which have been shown to be involved in cell fate specification and the differentiation of various organs (12, 13). Nkx3.2 is initially expressed in the sclerotomal portion of the somites, and its expression is maintained in developing cartilage (14). Nkx3.2-deficient mice lack the ventral medial region of their vertebrae (15–17), indicating a crucial role for this transcription factor to promote somitic chondrogenesis. We have previously shown that Shh signals can induce expression of Nkx3.2 in paraxial mesoderm and that forced expression of Nkx3.2 can activate somitic chondrogenesis in the absence of Shh signals (8, 10). Our prior findings also indicate that induction of chondrogenesis by Nkx3.2 requires both its transcriptional repressor activity and the presence of BMP signals (10). However, it has not been examined whether Nkx3.2 can bind to a specific DNA sequence(s) and, more importantly, whether the DNA binding activity of Nkx3.2 is required for its biological function. In this work, using a binding site selection assay, we both identify a high affinity DNA binding sequence for Nkx3.2 and determine the consensus DNA binding site for this transcription factor. Furthermore, we demonstrate that Nkx3.2 can bind to DNA in a sequence-specific manner both in vitro and in vivo and that the DNA binding activity of Nkx3.2 is essential for this factor to promote somitic chondrogenesis.

EXPERIMENTAL PROCEDURES

Biological Materials and Antibodies—Fertilized chicken eggs were obtained from SPAFAS. Recombinant human BMP4 protein was a generous gift from Genetics Institute. Anti-GST monoclonal antibody was purchased from Sigma.

Oligonucleotides—Oligonucleotides for cyclic amplification of sequence of target (CAST) were as follows: N15-random oligonucleotide, GAGTCGACGAACTTACTGCTGNNN_GAGTCCCTCGAGAAGTGTCAAC; CAST top strand, GAGTCGACGAACTTACTGCTGNNN_GAGTCCCTCGAGAAGTGTCAAC; CAST bottom strand, GAGTCGACGAACTTACTGCTGNNN_GAGTCCCTCGAGAAGTGTCAAC; CAST bottom strand, GAGTCGACGAACTTACTGCTGNNN_GAGTCCCTCGAGAAGTGTCAAC.

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Characterization of Nkx3.2 DNA Binding

Recombinant GST-Nkx3.2-2HA protein was bacterially expressed and affinity-purified using glutathione-agarose beads following the manufacturer’s instructions provided by Amersham Biosciences. The affinity-purified GST-Nkx3.2-2HA protein was then immunopurified by anti-HA immunoprecipitation in immunoprecipitation buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM dithiothreitol, and 0.5% Nonidet P-40). This double-purified GST-Nkx3.2-2HA protein, immobilized on protein G-agarose beads, was used for the binding site selection assay.

The CAST reaction prior to electrophoresis. As expected, the addition of a 5'-end labeled probe containing the Nkx3.2 DNA-binding site resulted in a significant DNA-protein complex (Fig. 1), which we refer to as the Nkx3.2 consensus DNA-binding sequence.

Analysis of EMSA—Binding reactions for EMSA were done in 10 mM Tris (pH 7.5), 50 mM NaCl, 1.5 mM MgCl2, 2.5 mM dithiothreitol, 5% glycerol, 5 µg/ml poly(dI-dC)-poly(dI-dC), and 250 µg/ml bovine serum albumin. Reaction mixtures were incubated with various 32P-labeled probes (250,000 cpm/reaction). All of the EMSA probes were labeled by PCR using [α-32P]dCTP after annealing indicated primers. Each strand oligonucleotides described above.

To define a consensus sequence for Nkx3.2 DNA binding, the CAST (also known as selected and amplified binding sequence, or SAAB) assay was employed as previously described (22, 23, 25, 26). Recombinant GST-Nkx3.2-2HA protein was bacterially expressed and double-purified by GST purification and subsequent anti-HA antibody immunoprecipitation as described under “Experimental Procedures.” SDS-PAGE and Coomassie Brilliant Blue staining verified purification of GST-Nkx3.2-2HA to homogeneity (data not shown). This double-purified GST-Nkx3.2-2HA protein, immobilized on protein G-agarose beads, was used to select high affinity binding sequences from a pool of oligonucleotides containing 15 random base pairs flanked by 20 base pairs of invariant sequence. Five cycles of CAST were performed, and the isolated high affinity binding sequences were cloned and sequenced. Negative control experiments using GST protein were carried out in parallel and did not result in any significant sequence selection.

Alignment of 24 selected sequences obtained from the Nkx3.2 CAST, along with the percentage occurrence of base at each position, is shown in Fig. 1. Analysis revealed that the most frequently observed hexanucleotide motif (Fig. 1, open boxed) and the consensus sequence for Nkx3.2 DNA binding. A significant preference for G nucleotides (50%) was also observed at the position immediately 3' to this TAAGTG sequence (Fig. 1), which we refer to as the Nkx3.2 binding element (NBE).

To confirm that Nkx3.2 can specifically bind to the NBE sequence in vitro, either purified GST or GST-Nkx3.2-2HA recombinant proteins were incubated with 32P-labeled NBE probe (containing three reiterated of the sequence TAAGTG; 3X-TAAGTG) and analyzed by EMSA. Whereas GST failed to form a complex with the NBE probe, incubation of this probe with GST-Nkx3.2-2HA resulted in a significant DNA-protein complex (Fig. 2A, compare lanes 2 and 3). Since complexes of three distinct electrophoretic mobilities were repeatedly observed following incubation of GST-Nkx3.2-2HA with the NBE probe, which contained three reiterated of the TAAGTG sequence, we surmise that these distinct complexes may represent DNA-protein complexes containing different numbers of Nkx3.2 molecules bound to the DNA probe. To further confirm the identity of these shifted bands, anti-GST antibody was added to the binding reaction prior to electrophoresis. As expected, the addition of the anti-GST antibody decreased the mobility of the GST-
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Nkx3.2-NBE complex and diminished the total amount of this DNA-protein complex (Fig. 2A, lane 5).

To determine whether Nkx3.2 binds to the NBE (TAAGTG) in a sequence-specific manner, the binding affinity of Nkx3.2 to either the “wild-type” NBE probe or two different mutant NBE probes, NBE-m1 (containing three reiterations of the sequence TAGATG) and NBE-m2 (containing three reiterations of the sequence TAGGCG), was analyzed. Purified recombinant GST-Nkx3.2-HA protein was incubated with the ATP-labeled NBE probe either in the absence or the presence of excess cold competitors, and the DNA/protein interaction was analyzed by EMSA. Nkx3.2 binding to the hot NBE probe (Fig. 3B, lane 2) was nearly completely extinguished in the presence of a 25-fold excess of cold NBE competitor (TAAGTG) (Fig. 3B, lane 3). In contrast, a 25-fold excess of cold competitors containing either CAAGTG, TAATGG, or TAAATG sequences failed to compete for Nkx3.2 binding to the hot NBE probe as effectively as did the cold NBE probe (Fig. 3B, compare lanes 3, 5, 7, and 9). A 100-fold excess of all of the tested cold competitors successfully abolished Nkx3.2 DNA binding to the hot NBE probe (Fig. 3B, lanes 4, 6, and 8). As a negative control, a competition EMSA using the NBE-m2 sequence as a cold competitor was carried out. Unlike the NBE or NBE-related sequences, neither a 25-fold nor a 100-fold excess of the cold NBE-m2 competitor significantly diminished Nkx3.2 binding to the hot NBE probe (Fig. 3B, compare lanes 2, 3, and 4). Together, these findings indicate that Nkx3.2 can also bind to various NBE-related sequences but with relatively lower affinities compared with the NBE sequence.

Systematic Characterization of Nkx3.2 Interaction with Sequences Deviating from the NBE—Because all Nkx3.2 DNA binding sites identified by the CAST technique contained A, G, and T at positions 3, 4, and 5, respectively (Fig. 1), it seems likely that these invariant residues are critical for interaction of Nkx3.2 with DNA. However, to systematically examine whether other nucleotides are necessary to support high affinity interaction of Nkx3.2 with the NBE sequence, we incubated Nkx3.2 with a hot NBE probe (TAAGTG) and added a 100-fold molar excess of oligonucleotides differing from the NBE sequence by a single base pair substitution in either the first, second, third, or sixth positions. We found that NBE-related oligonucleotides containing T in the first position competed slightly better for Nkx3.2 interaction than those containing either A or C in this position (Fig. 3D, lanes 3, 4, and 6), whereas oligonucleotides containing a G in this position failed to significantly compete for Nkx3.2 binding (Fig. 3D, lane 5). NBE-related oligonucleotides containing A in the second position competed slightly better for Nkx3.2 interaction than those containing a G in this position (Fig. 3D, lanes 7 and 9), whereas oligonucleotides containing either a C or T in this position failed to significantly compete for Nkx3.2 binding (Fig. 3D, lanes 8 and 10). Thus, Nkx3.2 can interact with sequences displaying some nucleotide variation at positions 1 and 2 of the NBE. In contrast, the binding site specificity for positions 3 and 6 is apparently invariant, since Nkx3.2 only bound to sequences containing A and G at these respective positions in the NBE (Fig. 3D, lanes 11–18). Consistent with our statistical analyses of the CAST results, as shown in Fig. 1, TAAGTG was clearly the strongest binding sequence for Nkx3.2. Further-

Fig. 1. Nkx3.2 consensus DNA binding sequence. Selected and amplified Nkx3.2 binding sequences are aligned, and the most frequently observed TAAGTG sequence is shown in an open box. The percentage occurrence of each base at each position is also calculated and displayed.
more, these findings allow us to more broadly define the Nkx3.2 DNA binding consensus as HRAGTG (where H represents A, C, or T, and R represents A or G; see Fig. 3E).

Nkx3.2 Can Regulate the Expression of an NBE-driven Reporter Gene in Vivo—Since we have shown that Nkx3.2 can bind to the NBE in vitro, we next examined whether Nkx3.2 can regulate the expression of an NBE-containing reporter gene in vivo. Either an empty vector (pCS2) or an expression vehicle encoding Nkx3.2 (pCS2-Nkx3.2) was co-transfected into NIH-3T3 murine fibroblasts with either the pGL3P luciferase control reporter (Promega) or the NBE-pGL3P luciferase reporter, which contains three NBE sites upstream of the SV40.
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basal promoter and luciferase reporter gene. Whereas Nkx3.2 only modestly repressed the expression of a co-transfected pGL3P luciferase reporter construct (~1.5-fold on average) (Fig. 4A, compare lanes 1 and 2), Nkx3.2 significantly repressed the expression of the NBE-pGL3P luciferase reporter by ~10-fold (Fig. 4A, compare lanes 3 and 4). These results suggest that Nkx3.2 can repress the expression of a reporter gene in vivo in an NBE-dependent manner. We surmise that the repression of the parental pGL3P reporter by Nkx3.2 may reflect low affinity binding sites for Nkx3.2 that are fortuitously present in this construct.

To investigate whether the repression of the NBE-pGL3P luciferase reporter by Nkx3.2 is indeed mediated by direct binding of Nkx3.2 to the NBE sites, the NBE-m1-pGL3P and NBE-m2-pGL3P luciferase reporters, which contain mutant NBE sequences that do not bind to Nkx3.2 (Figs. 2 and 3C), were analyzed in similar reporter assays. Unlike the NBE-pGL3P luciferase reporter, neither the NBE-m1-pGL3P nor the NBE-m2-pGL3P luciferase reporter exhibited any significant repression beyond the intrinsic response of the parental pGL3P luciferase reporter to co-transfected Nkx3.2 (Fig. 4A, compare lanes 2, 6, and 8). These results indicate that Nkx3.2-mediated repression of the NBE-pGL3P luciferase reporter is dependent on Nkx3.2 binding to the NBE sequences.

To further confirm the in vivo interaction between Nkx3.2 and the NBE sequence, we employed Nkx3.2-VP16, in which the strong transcriptional activation domain of HSV-VP16 (30) is fused to the carboxyl terminus of Nkx3.2. Either the pCS2 expression vehicle or pCS2 encoding either Nkx3.2 or Nkx3.2-VP16 was co-transfected into NIH-3T3 cells along with the NBE-pGL3P luciferase reporter. Whereas wild-type Nkx3.2 repressed the expression of the NBE-pGL3P luciferase reporter as expected (Fig. 4B, compare lanes 1 and 2), Nkx3.2-VP16 strongly activated the NBE-pGL3P luciferase reporter (Fig. 4B, compare lanes 1 and 3). These results verify that Nkx3.2 directly binds to NBE sites and regulates transcription in vivo.

Nkx3.2-N200Q Is a DNA Nonbinding Mutant—A large number of homeoproteins have been shown to bind to DNA via their homeodomains and regulate target gene transcription (31, 32). Among the highly conserved amino acids in the homeodomain, a specific asparagine residue (position 51 of the homeodomain), which is part of the recognition helix and makes crucial hydrogen bonds with DNA base pairs during homeodomain/DNA interactions, has been suggested to be essential for the DNA binding activity of various homeoproteins (33, 34). Since a missense mutation of this asparagine to glutamine has been shown to eliminate DNA binding activity in other homeoproteins (35, 36), including those of NK family proteins (37, 38), we attempted to generate a DNA nonbinding mutant of Nkx3.2 (termed Nkx3.2-N200Q) by introducing the same point mutation at the corresponding position (residue 200) of Nkx3.2.

To investigate whether Nkx3.2-N200Q can bind to DNA, bacterially expressed and purified GST-Nkx3.2-N200Q recombinant protein was incubated with 32P-labeled NBE probe and analyzed by EMSA. Purified GST and GST-Nkx3.2 recombinant proteins were also included in the experiment as negative and positive controls, respectively. As expected, the NBE probe failed to interact with the parental GST protein but formed stable complexes with GST-Nkx3.2 (Fig. 5A, lanes 2 and 3). Consistent with previous observations with other homeoproteins (35–38), GST-Nkx3.2-N200Q was unable to bind to the NBE probe (Fig. 5A, lane 4). This result not only verifies that Nkx3.2-N200Q is a DNA nonbinding mutant but also suggests that Nkx3.2 binds to DNA in a manner that is structurally similar to that of other homeoproteins (33, 34).

Nkx3.2-N200Q Is Unable to Repress the NBE Reporter but Retains Intrinsic Transcriptional Repressor Activity—Since Nkx3.2 represses the NBE reporter by directly binding to the NBE (Fig. 4), we next examined whether the DNA nonbinding Nkx3.2-N200Q mutant can regulate the expression of the NBE-pGL3P luciferase reporter. Either the parental pCS2 expression vehicle or pCS2 encoding either wild-type Nkx3.2 or Nkx3.2-N200Q was co-transfected into NIH-3T3 cells along with the NBE-pGL3P luciferase reporter. In contrast to Nkx3.2 WT (Fig. 5B, lane 2), Nkx3.2-N200Q was unable to repress expression of the NBE-pGL3P luciferase reporter (Fig. 5B, lane 3). Thus, the Nkx3.2-N200Q mutant has lost its ability to repress the NBE reporter, most likely due to its inability to bind DNA.

To confirm that the inability of Nkx3.2-N200Q to repress the NBE reporter is due to a specific defect in DNA binding, we evaluated the transcriptional repressor activity of Nkx3.2-N200Q when fused to the DNA binding domain of GAL4 (GAL4-Nkx3.2-N200Q). A 5×-GAL4-pGL3E luciferase reporter, which contains five GAL4 binding sites and the E1b TATA box 5′ to the luciferase gene and the SV40 enhancer 3′ to the gene (20), was transfected into NIH-3T3 cells in the presence of an expression vector encoding either the GAL4 DNA binding domain, GAL4-Nkx3.2-WT, or GAL4-Nkx3.2-N200Q. Consistent with our prior findings (10), transfection of GAL4-
Nkx3.2-WT significantly repressed the expression of the 5'-GAL4-pGL3E luciferase reporter (Fig. 5C, lane 2). Interestingly, GAL4-Nkx3.2-N200Q similarly displayed efficient transcriptional repressor activity (Fig. 5C, lane 3), indicating that the N200Q missense mutation introduced into the homeodomain of Nkx3.2 altered only its DNA binding activity without compromising its intrinsic transcriptional repressor activity.

The DNA Binding Activity of Nkx3.2 Is Required to Induce Somitic Chondrogenesis—Prior work has indicated that Nkx3.2 function as a transcriptional repressor to promote somitic chondrogenesis (8, 10). However, it has not been characterized whether this activity of Nkx3.2 is dependent on direct binding to DNA. Since Nkx3.2-N200Q is specifically defective in its DNA binding (Fig. 5, A and B) but not in its intrinsic transcriptional repressor activity (Fig. 5C), we sought to evaluate whether Nkx3.2-N200Q would be competent to induce somitic chondrogenesis. Explants of psm dissected from Hamburger and Hamilton stage 10 chick embryos were infected with nondefective avian retroviruses (RCAS) (21) encoding either GFP, Nkx3.2-WT, or Nkx3.2-N200Q and cultured in the presence of BMP signals. After 6 days in culture, the explants were harvested, and expression of chondrogenic differentiation markers such as aggrecan, epiphycan, collagen IX, and Sox9 (Fig. 6, B–E, respectively) was assayed by RT-PCR as previously described (8, 10). Glyceraldehyde-3-phosphate dehydrogenase expression was also analyzed in these various cultures as a control for explant viability (Fig. 6A). As expected, forced expression of Nkx3.2-WT induced robust expression of the chondrogenic marker genes in the psm explant cultures (Fig. 6, lane 2). Whereas Nkx3.2-WT and Nkx3.2-N200Q were expressed at equivalent levels (Fig. 6F), overexpression of Nkx3.2-N200Q, which is unable to bind to DNA (Fig. 5A) but retains its transcriptional repressor activity (Fig. 5C), failed to significantly activate the chondrocyte differentiation program (Fig. 6, lane 3). These results indicate that the DNA binding activity of Nkx3.2 is essential for this factor to promote the chondrocyte differentiation program in somitic tissue.

**Fig. 5.** Nkx3.2-N200Q is unable to bind to the NBE but retains intrinsic repressor activity. A, purified recombinant GST (lane 2), GST-Nkx3.2-WT (lane 3), or GST-Nkx3.2-N200Q (lane 4) proteins were incubated with 32P-labeled NBE probes and processed for EMSA. B, Nkx3.2-N200Q is unable to repress the NBE reporter. Either the pcS2 expression vector (lane 1), pcS2-Nkx3.2-WT (lane 2), or pcS2-Nkx3.2-N200Q (lane 3) was co-transfected into NIH-3T3 cells along with the NBE-pGL3P luciferase reporter gene. 40 h post-transfection, cell extracts were made and assayed for luciferase activity. C, GAL4-Nkx3.2-WT is able to repress the 5'-GAL4 reporter. NIH-3T3 cells were co-transfected with a pCS2 expression vector (lane 1), pCS2-Nkx3.2-WT (lane 2), or pCS2-Nkx3.2-N200Q (lane 3). 40 h post-transfection, cell extracts were made and assayed for luciferase activity.
Nkx2.1 (28), TAAGTG for Nkx3.1 (22), TNAAGTG for Nkx2.5 (23), and AAAGTG for ceh-22, the Caenorhabditis elegans
NK-2 homologue (39). Interestingly, we have found that Nkx3.2 can also bind to various NBE related sequences in vitro by
EMSA assays (Fig. 3). Furthermore, we have shown that expression of a reporter gene containing reiterated NBE sites can be
repressed by co-transfected Nkx3.2 in vivo (Fig. 4). Together, these results suggest that the NBE and/or NBE-related
sequences may serve as Nkx3.2 binding sites that mediate Nkx3.2-dependent transcriptional repression in vivo.

Structural Basis of Nkx3.2 DNA Binding Is Very Similar to That of Other Previously Characterized Homeoproteins—
A large number of homeoproteins including NK family members contain a highly conserved asparagine residue in the third
helix of the homeodomain (33, 34). Nkx3.2 also contains this conserved asparagine at residue 200. Similar to other previously
characterized homeoproteins (35–38), an asparagine to glutamine substitution at this position of Nkx3.2 eliminated its
ability to bind to the NBE sequence (Fig. 5, A and B). Furthermore, we have also found that an adenine base at the third
position of the NBE is essential for interaction with Nkx3.2 (Fig. 3D). This invariant adenine base at the third position of
homeodomain binding sites has been shown to make a crucial contact with the highly conserved asparagine residue in the
third helix of the homeodomain (33, 34). Together these findings strongly suggest that Nkx3.2 binds to DNA in a manner
that is structurally very similar to other previously characterized homeodomain-containing proteins.

It is currently unclear whether Nkx3.2 binds to DNA as a monomer or dimer (or multimer). However, several lines of
evidence suggest that Nkx3.2 may bind to DNA as a monomer. First, none of the sequences selected by Nkx3.2 in the CAST
technique displayed any apparent palindromic or repetitive sequence pattern, which frequently is observed in the binding
sites for dimeric (or multimeric) proteins (26, 40, 41). Instead, Nkx3.2 binds to a nonpalindromic hexanucleotide sequence
motif. Second, we have found that the DNA binding characteristics of Nkx3.2 resemble those of other well characterized
homeodomain-containing proteins, which have been shown to make intimate contacts with the DNA via the helix-turn-helix
motif of the homeodomain as a monomer (33, 34). Therefore, it seems most likely that Nkx3.2 similarly binds to its target
sequences as a monomer. However, it is certainly possible that association of Nkx3.2 with other proteins in vivo may alter
the DNA binding specificity of Nkx3.2 and thereby target it to sites other than simple NBE sequences.

Nkx3.2 Promotes Somitic Chondrogenesis via Its Direct Bind-
ing to DNA—We demonstrated that a DNA nonbinding mutant form of Nkx3.2, Nkx3.2-N200Q, failed to activate the chondro-
cytes differentiation program in presomitic mesodermal explant cultures (Fig. 6), indicating that Nkx3.2 must directly bind to
DNA to promote somitic chondrogenesis. Since we have found that the intrinsic transcriptional repressor activity of Nkx3.2-
N200Q remains intact (Fig. 5C), it seems most likely that Nkx3.2-N200Q fails to induce somitic chondrogenesis due to its
specific inability to recognize its target genes. The dependence of Nkx3.2 prochondrogenic activity on DNA binding is of
importance, since it has been shown that a number of transcriptional regulators can execute their biological activity via pro-
tein-protein interactions with transcriptional partners in the absence of direct DNA binding (42–46).

Nkx3.2 Activates Chondrogenesis by Repressing Antichon-
drogenic Genes—It has been previously been shown that BMP signals can antagonize sclerotomal gene expression in paraxial
mesoderm and elicit the expression of lateral plate mesodermal

markers such as GATA-4, -5, and -6 (9, 47–49). However, after
prior exposure to Shh, paraxial mesodermal cells interpret
subsequent BMP signals as prochondrogenic cues (9). In this
case, BMP signals strongly promote somitic chondrogenesis,
and the induction of GATA family members by BMP signaling
is blocked (9). Thus, Shh signaling alters the cellular response of
paraxial mesodermal cells to subsequent BMP signals. Since
Nkx3.2 is a transitory repressor induced by Shh in somitic
mesoderm (8, 10), it may play an important role in preserving
a prochondrogenic cellular environment by repressing an anti-
chondrogenic gene(s) upon exposure to secondary BMP signal-
ing. We have previously shown that Shh can induce Nkx3.2 in
somitic mesoderm and that forced expression of Nkx3.2 in
somites induces the expression of Sox9, which can in turn
activates the chondrogenic differentiation program in somitic
mesodermal cells (8, 10). Therefore, our current hypothesis is
that Nkx3.2 induces somitic chondrogenesis by repressing the
expression of an antichondrogenic gene(s) that blocks the ex-
pression or function of various prochondrogenic genes such as
Sox family proteins (50–57).

The findings of the present study indicate that the prochon-
drogenic activity of Nkx3.2 requires direct DNA binding by this
transcription factor and suggest that antichondrogenic gene(s)
repressed by Nkx3.2 should contain NBE-like sequences regu-
larizing its expression. Interestingly, we have found that
forced expression of Nkx3.2 in somitic tissue will block the
induction of GATA-4, -5, and -6 by BMP signals and that
repression of GATA-6 expression by Nkx3.2 requires an NBE-
like sequence in the GATA-6 promoter. Thus, members of the
GATA gene family are potential direct targets of Nkx3.2 in vivo
and therefore could potentially play a role in modulating chon-
drogenesis. Present work in our laboratory is focused on deter-
mining whether GATA family members or other targets of
Nkx3.2 are negative regulators of chondrogenesis.

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