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DNA BINDING AND TRANSCRIPTIONAL PROPERTIES OF WILD-TYPE AND MUTANT FORMS OF THE HOMEODOMAIN PROTEIN MSX2

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SUMMARY: Msx2 is a mammalian homeodomain protein that is expressed during craniofacial development. A proline-to-histidine substitution at residue 148 of human Msx2 results in an autosomal dominant form of craniosynostosis. In this study, both wild-type and mutant Msx2 were shown to specifically bind to a DNA sequence previously identified as a high-affinity binding site for the related homeodomain protein Msx1. In co-transfection assays both wild-type and mutant Msx2 repressed reporter gene transcription in a dose-dependent but binding-site-independent manner. These results provide evidence that Msx2 is a transcriptional repressor and suggest that the mutant form of Msx2 may exert its pathophysiologic effects on craniofacial development by a gain-of-function mechanism.

The role of homeodomain proteins in the development of invertebrates (1) and vertebrates (2) is well established, as is their ability to bind to specific DNA sequences (3) and activate or repress gene transcription. In mammals, 38 different *HOX* genes encode proteins related to the *Drosophila* Antennapedia (Antp) protein and are involved in establishing the segmented body plan but are not involved in the development of cranial structures rostral to hindbrain rhombomere 3 (2). Two mammalian homeodomain proteins Msx1 and Msx2, which are related to the *Drosophila* muscle-segmentation homeobox (msh) protein, appear to play a role in craniofacial development based upon their expression patterns in mice (4-8). For both Msx1 and Msx2, the human and murine sequences are identical within the 60-amino-acid homeodomain and there are only 2 amino acid differences between the Msx1 and Msx2 homeodomains (9,10).

Mutations in genes encoding Msx1 and Msx2 have also demonstrated their role in craniofacial development. Mice homozygous for an inactivating mutation at the *Msx1* locus developed multiple craniofacial abnormalities including cleft palate (11). *Msx2* is expressed in the cranial sutures and a family has recently been described with an autosomal dominant form of craniosynostosis due to substitution of histidine for proline at residue 148 (P148H) of human Msx2 (10). The substitution is at position 7 of the homeodomain where proline is invariant in all

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known vertebrate and invertebrate Msx homologues (9,10), suggesting that this residue may play a key functional role. In this study, we have analyzed the DNA binding and transcriptional properties of wild-type and mutant (P148H) Msx2.

MATERIALS AND METHODS

Tissue Culture. Human 293 and Hep3B cells were cultured in Dulbecco's Modified Eagle Medium with high glucose and Minimum Essential Medium Eagle, respectively, supplemented with 10% fetal calf serum (BRL GIBCO). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Electrophoretic Mobility Shift Assay. 293 cells were plated at a density of 2 x 10^6 per 10-cm dish and 24 h later were transfected with 15 μg of expression plasmid (pCMV-Msx2.wt or pCMV-Msx2.mut) DNA by calcium phosphate co-precipitation. Cells were harvested 48 h after transfection and nuclear extracts were prepared as described (12). The double-stranded oligonucleotide containing the Msx binding site (MBS) consisted of the sequences 5'-GATCCACTAATTGGAGG-3' and 5'-GATCCCTCCAATTAGTG-3'. 5'-end-labelled MBS probe (10^4 cpm) was incubated with nuclear extract (5 μg) and analyzed as described (12). For competition experiments, excess unlabelled MBS or unrelated oligonucleotide was included in the binding reaction. The nucleotide sequences of the unrelated oligonucleotide were 5'-GATCGCCCTACGTGCTGTCTCA-3' and 5'-GATCTGAGACAGCACGTAGGGC-3'.

Co-transfection Assay. The reporter plasmids pMBS-PE-cat and pMBS-P-luc were constructed by ligating four copies of the double-stranded MBS oligonucleotide into the Bgl II site located 5' to the SV40 promoter sequences of pPE-CAT (pCAT-Control; Promega) and pP-luc (pGL2-Control; Promega), respectively. Hep3B cells were transfected by electroporation (12). In addition to reporter and expression plasmids, pSVβgal was co-transfected as a control for transfection efficiency and sonicated salmon sperm DNA was added to 350 μg of total DNA transfected per 4.5 x 10^6 cells. Cells were harvested 48 h after transfection and protein extracts were prepared by four rounds of freezing and thawing and assayed for luciferase, CAT, and β galactosidase activity (12).

RESULTS

Analysis of DNA Binding Activity. The Msx2 homeodomain protein is a putative transcriptional regulatory factor but neither target genes for regulation by Msx2 nor DNA sequences that bind Msx2 have been identified. An *in vitro* binding site-selection assay has identified specific DNA sequences recognized by Msx1 (13). Because Msx1 and Msx2 differ at only 2 of 60 amino-acid residues in the DNA-binding homeodomain, it was possible that Msx2 would also recognize the Msx1 binding-site sequence. To test this hypothesis, a double-stranded Msx binding-site (MBS) oligonucleotide was synthesized for use as a probe in electrophoretic mobility-shift assays. Nuclear extracts were prepared from control 293 human embryonic kidney cells and 293 cells transfected with plasmids expressing, from a cytomegalovirus (CMV) promoter, wild-type or P148H mutant forms of Msx2. As shown in Fig. 1, the MBS probe detected minimal DNA-binding activity in control extracts (C), whereas nuclear extracts from cells expressing wild-type (W) or mutant (M) Msx2 protein demonstrated high levels of DNA binding activity which was sequence-specific, as it could be competed by 50- or 500-fold molar excess of unlabelled MBS oligonucleotide (m) but not by equivalent amounts of excess unlabelled oligonucleotide of unrelated sequence (u). These results indicate that Msx2 can bind to the same DNA sequence as Msx1 and that the P148H mutation has no demonstrable effect on the binding of Msx2 to the MBS probe.

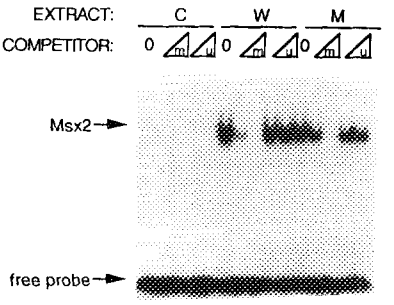

Fig. 1. Sequence-specific DNA-binding activity of wild-type and mutant Msx2. Nuclear extracts (5-μg aliquots) from 293 cells that were non-transfected controls (C) or transfected with plasmids expressing wild-type (W) or mutant (M) Msx2 were incubated with MBS probe DNA in the absence (0) or presence of 50- or 500-fold excess of unlabelled MBS (m) or unrelated (u) oligonucleotide and DNA binding activity was determined by electrophoretic mobility-shift assay.

Analysis of Transcriptional Properties. The properties of wild-type and mutant Msx2 were also characterized in co-transfection assays. We utilized a reporter plasmid, pPE-cat, in which *E. coli* chloramphenicol acetyltransferase (CAT) coding sequences were driven by the SV40 promoter (P) and enhancer (E). Four copies of the MBS oligonucleotide were inserted 5' to the promoter to generate pMBS-PE-CAT. Hep3B human hepatoblastoma cells were transfected with 20 μg of pMBS-PE-cat and 0 to 20 μg of the expression vector pCMV-Msx2.wt. Cell extracts were prepared 48 h after transfection and CAT protein was quantitated. Reporter gene transcription was repressed in a dose-dependent manner up to 15-fold in the presence of wild-type Msx2 (Fig. 2A). In these experiments, a second reporter gene, pSVßgal, consisting of *E. coli* ß-galactosidase coding sequences driven by the SV40 promoter-enhancer was also co-transfected as a control for transfection efficiency. Quantitation of of ß-galactosidase activity in cell extracts indicated a dose-dependent repression of pSVßgal by Msx2 (data not shown).

To determine whether repression of reporter gene transcription by Msx2 was dependent upon the presence of the Msx binding sites that had been cloned 5' to the promoter, Hep3B cells were co-transfected with pCMV-Msx2.wt and the parental reporter plasmid pPE-CAT. Despite the absence of the MBS oligonucleotide, CAT expression from pPE-CAT was also repressed by Msx2 in a dose-dependent manner (Fig. 2B). These results are consistent with the Msx2-mediated repression of pSVßgal which also did not contain any known Msx2 binding sites.

To determine the transcriptional properties of the P148H mutant form of Msx2, co-transfection assays were performed with pCMV-Msx2.mut and pMBS-PE-CAT. Mutant Msx2 protein also repressed CAT expression in a dose-dependent manner that was similar to the effect of the wild-type Msx2 protein (Fig. 2C). Thus, wild-type and mutant Msx2 proteins demonstrated similar properties in both DNA binding and transcriptional assays.

Transcriptional repression in co-transfection assays can occur due to squelching (14) in which over-expression of a transcription factor results in saturation binding of a limiting component of the transcription initiation complex. To determine whether this type of experimental artifact was responsible for the observed results, a reporter plasmid, pP-LUC, in which expression

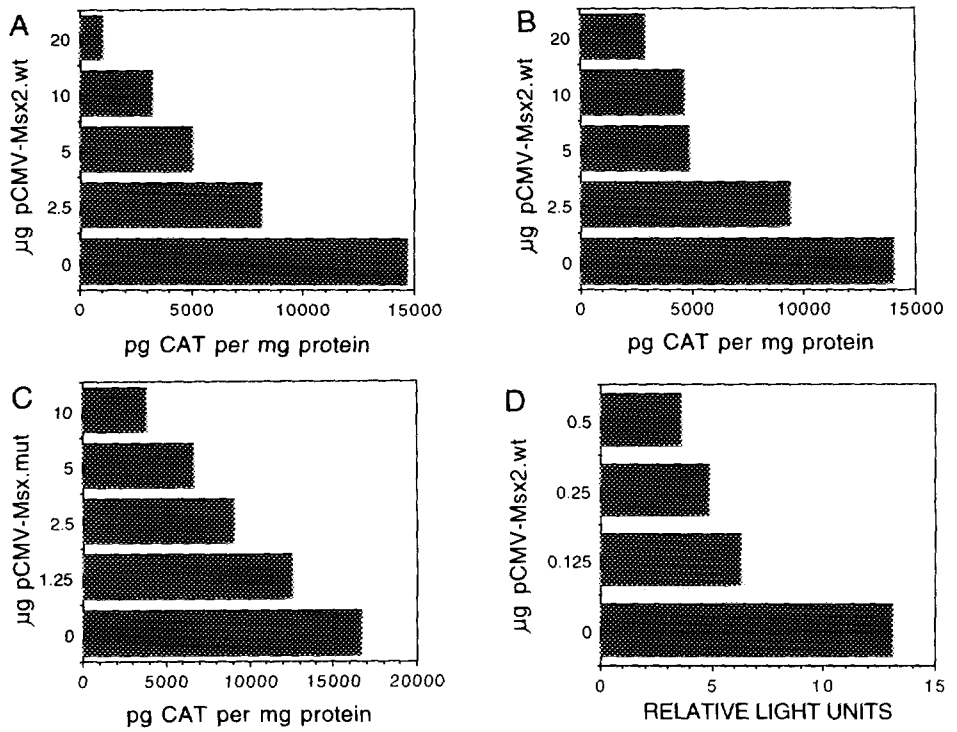

Fig. 2. Effect of wild-type and mutant Msx2 on reporter gene expression. Hep3B cells were co-transfected with the indicated amount of expression plasmid pCMV-Msx2.wt (A, B, D) or pCMV-Msx2.mut (C) and 20 μg of reporter plasmid pMBS-PE-CAT (A, C) or pPE-CAT (B) or 5 μg of pMBS-P-LUC (D). CAT protein (A-C) or luciferase activity in relative light units (D) was measured in cellular extracts. Mean results from two independent experiments are shown.

of firefly luciferase (LUC) was driven by the SV40 promoter, was utilized. Four copies of the MBS oligonucleotide were inserted 5' to the promoter to generate pMBS-P-LUC. Because of the sensitivity of the luciferase assay, a detectable signal could be obtained from cell extracts transfected with only 2 μg of pMBS-P-LUC and 0 to 0.5 μg of pCMV-Msx2.wt. Transfection of only 0.125 μg of the Msx2 expression plasmid resulted in a 50% decrease in luciferase activity (Fig. 2D). These results provide evidence that squelching is not a cause of the observed repression. Furthermore, repression occurred in the absence of the SV40 enhancer, suggesting that Msx2 may interfere with the function of one or more factors that bind (directly or indirectly) to the SV40 basal promoter.

DISCUSSION

In this study several properties of wild-type and mutant Msx2 were determined. First, Msx2 can bind to the same DNA sequence as Msx1. Second, Msx2 can repress transcription of CAT or LUC reporter genes driven by SV40 basal promoter sequences in the presence or absence of the SV40 enhancer. This repression is dose-dependent and occurs with low levels of transfected Msx2 expression plasmid. Third, repression of reporter gene transcription does not

require the presence of Msx binding sites in *cis*. While this manuscript was in preparation, Msx1 was also shown, in the absence of cognate binding sites, to repress transcription *in vivo* and *in vitro* from promoters that either contained or lacked a canonical TATA box (15). Multiple regions of Msx1, both within and outside the homeodomain, contributed to the repression and Msx1 interacted *in vitro* with complexes containing the TATA binding protein and basal transcription factor IIA (15). It appears possible, therefore, that both Msx1 and Msx2 may contribute to craniofacial developmental processes by functioning as transcriptional repressors.

In the case of Msx2, a naturally-occurring mutation (P148H) is responsible for a human malformation syndrome, the most prominent aspect of which is the presence of craniosynostosis (7). The molecular pathophysiology of this condition remains undetermined. The P148H missense mutation could affect protein stability or the binding of Msx2 to DNA or to other proteins. DNA binding assays presented above (Fig. 1) indicate that the mutation does not affect binding of Msx2 to the MBS oligonucleotide and is also unlikely, therefore, to have significant effect on protein stability. Co-transfection experiments (Fig. 2), which indirectly assay protein-protein interactions that play a crucial role in transcriptional activation and repression (14), demonstrated that wild-type and mutant forms of Msx2 both functioned as repressors of transcription. These results suggest that the P148H mutation is not a classic loss-of-function allele but may instead represent a gain-of-function mutation such that, compared to wild-type Msx2, the ability of the mutant Msx2 protein to interact with target DNA sequences or other transcriptional regulatory proteins may be altered quantitatively or qualitatively. A gain-of-function mechanism is consistent with the autosomal dominant inheritance of the craniosynostosis phenotype. One means of testing this gain-of-function hypothesis will be to determine the phenotypes of transgenic mice that express wild-type and mutant forms of Msx2.

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