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Screening Downstream Genes of a Homeobox Gene by Differential Display Using a Knockout Mouse

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Summary. **The products of homeobox genes are DNA-binding transcription factors. However, little is known about downstream genes whose activities are regulated directly or indirectly by the homeobox genes. In the current study, we tested a differential display (DD) method using the tissue of a knockout mouse in order to identify the downstream genes of a homeobox gene *Msx1* systematically. Our previous *in situ* hybridization analysis of a *Msx1* deficient mouse showed that *Msx1* induced by epithelial bone morphogenetic protein 4 (BMP4) and fibroblast growth factors (FGFs) induces *Bmp4*, the HMG box gene *Lef1*, and the heparan sulfate proteoglycan syndecan-1 in the tooth mesenchyme. Although it is a very powerful approach for identifying downstream genes of a homeobox gene to test whether the candidate gene's expression is affected in the knockout mouse, this approach is not directly applicable to the identification of unknown genes downstream of *Msx1*. In the current study, we performed DD using total RNA from E14.5 *Msx1* mutant mandibles and were able to obtain four novel downstream genes of *Msx1* from 20 cDNA clones verified by Northern blot hybridization and semiquantitative RT-PCR. Despite several problems inherent to this method, we concluded that DD analysis using the tissue of a knockout mouse is a useful systematic approach for the identification of downstream genes of a homeobox gene.**

Key words—homeobox gene, downstream genes, differential display, *Msx1*, knockout mice.

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INTRODUCTION

The epithelial-mesenchymal interaction plays a key role in vertebrate organogenesis. The *Msx* family of vertebrate homeobox genes was originally isolated[1,2] by homology to the *Drosophila msh (muscle segment homeobox)* gene[3]. So far three *Msx* class genes *Msx1*[1,2], *Msx2*[4,5], and *Msx3*[6,7] have been identified in vertebrate. *Msx1* and *Msx2* are generally expressed in overlapping or related patterns in many tissues which employ epithelial-mesenchymal interactions during organogenesis, including the brain, limb, heart, and the developing mandible and tooth[1,2,4,5,7-13]. The patterns of expression of *Msx1* and *Msx2*, and their correlation with inductive epithelial-mesenchymal interactions, suggest that they could function as primary mediators of inductive signals transmitted between epithelial and mesenchymal layers in various tissues[14-16]. To test the hypothesis that *Msx1* is directly involved in mediating inductive signals, we generated *Msx1* deficient mice by gene targeting and examined the phenotypic consequences of its deficiency during mouse development. *Msx1* deficient mice exhibit marked abnormalities in craniofacial development including a complete cleft of the secondary palate, an arrest in tooth development at the E13.5 bud stage, and a deficiency of alveolar mandible and maxilla[17]. To understand the function of *Msx* genes during organogenesis, it is very important to determine the molecular signaling pathways in which the *Msx* genes function. Recently a part of signaling pathways upstream of *Msx1* and *Msx2* was elucidated. *Msx1* and *Msx2* expression can be induced in the dental mesenchyme by bone morphogenetic protein 4(BMP4)[18]

and in the limb bud mesenchyme by BMPs and fibroblast growth factors (FGFs)[19,20] which are produced in each epithelium. However, there has been little progress in characterizing the molecular processes downstream of homeobox genes including *Msx1* and *Msx2*. In a previous study, we examined the expression of several potential *Msx1* downstream genes in *Msx1* mutant tooth germs by *in situ* hybridization and showed that *Msx1* induced by epithelial BMP4 and FGFs induces *Bmp4*, the HMG box gene *Lef1*, and the heparan sulfate proteoglycan syndecan-1 in the tooth mesenchyme[21]. Although it is a very powerful approach for identifying downstream genes of *Msx1* to test whether the candidate gene's expression is affected in *Msx1* deficient mice, this approach is not directly applicable to the identification of unknown genes downstream of *Msx1*. Differential display (DD)[22-24] is currently the method of choice among many investigators for identifying differentially expressed mRNAs because it identifies mRNA independent of prevalence, requires small amounts of RNA, identifies both increases and decreases in mRNA levels and has rapid output[25]. To systematically identify both known and unknown genes downstream of a homeobox gene, we tested a DD method using the tissue of *Msx1* knockout mouse. We used the E14.5 *Msx1* mutant mandible for the analysis because the mandible—including tooth germs—manifests obvious developing abnormalities at the E14.5 stage in *Msx1* mutants and allowed us to obtain four novel genes downstream of *Msx1*. Although there were several problems with this method, we concluded that DD analysis using the tissue of a knockout mouse is a useful systematic approach for the identification of downstream genes of a homeobox gene.

MATERIALS AND METHODS

RNA preparation and genotyping

E14.5 embryos were collected from matings of *Msx1* (+/−)×*Msx1* (+/−) mice, taking the day of vaginal plug discovery as day 0.5. The mandibles of E14.5 embryos were dissected, immediately freezed on dry ice, and stored at -80℃. Total RNAs of the mandibles were isolated from pooled 6-10 mandibles of either wild-type or *Msx1* mutant embryos by a modified acid-guanidine thiocyanate-phenol-chloroform method using TRIzol (GIBCO-BRL) reagents, according to the manufacturer's instructions. Genomic DNAs were isolated from tails of E14.5 embryos. For *Msx1* genotyping, 250-500 ng of genomic DNA was analyzed by PCR using (in a single reaction) the two forward primers 5'- CCAGCATGCACCTACGCAA-3' (wild-type *Msx1* sequence) and 5'- TCTGGACGAAGAGCATCAGG-3' (*neo* sequence in the mutant), and the reverse primer 5'-AGCAGGCGGCAACATGGGTT-3' (wild-type *Msx1* sequence). The primers amplify a 270 bp fragment from the wild-type allele and a 490 bp fragment from the mutant allele. Following an initial denaturation step, samples were subjected to PCR using 45 cycles of 94℃ for 1 min, 58℃ for 2 min, and 72℃ for 2 min, followed by analysis on a 1.4% agarose gel.

Differential display

Total RNAs were digested with RNase-free DNase I(Boehringer) according to the manufacturer's protocol. DD was carried out using the Differential Display Kit (Display Systems Biotech). DNase I-treated total RNA (300 ng) was used for the reverse transcription reaction (final volume, 30μl) with 20μM dNTPs, 0.4 units/μl RNase inhibitor (TOYOBO), 2.5μM downstream primer, 27 units of Superscript II reverse transcriptase (GIBCO-BRL) at 42℃ for 60 min according to the manufacturer's specifications. PCR was performed (final volume, 20μl) with 1 μl reverse transcription, 10mM Tris/HCl (pH8.3), 50 mM KCl, 3 mM $MgCl_2$, 2μM dNTPs, 0.5μM upstream primer, 2.5μM corresponding downstream primer, 0.1μl [α^{-35}S] dATP (1000Ci/mmol) (Amersham), and 1 unit Taq DNA polymerase (TAKARA) using the 40 cycles of 94℃ for 30 sec, 40℃ for 1 min, 72℃ for 1 min followed by an extension step of 72℃ for 5 min in a PTC100 thermocycler (MJ Research). In the current study, we utilized nine downstream primers No. 1 (5'-T11AA-3'), No. 2 (5'-T11AC-3'), No. 3 (5'-T11AG-3'), No. 4 (5'-T11CA-3'), No. 5 (5'-T11CC-3'), No. 6 (5'-T11CG-3'), No. 7 (5'-T11GA-3'), No. 8 (5'-T11GC-3'), and No. 9 (5'-T11GG-3') and 16 upstream primers No. 1 (5'-GATCATAGCC3'), No. 2 (5'-CTGCTTGATG-3'), No. 3 (5'-GATCCAGTAC-3'), No. 4 (5'-GATCGCATTG-3'), No. 5 (5'-AAACTCCGTC-3'), No. 6 (5'-TGGTAAAGGG-3'), No. 7 (5'-GATCATGGTC-3'), No. 8 (5'-TTTTGGCTCC-3'), No. 9 (5'-GTTTTCGCAG-3'), No. 10 (5'-TACCTAAGCG-3'), No. 11 (5'-GATCTGACAC-3'), No. 12 (5'-GATCTAACCG-3'), No. 13 (5'-TGGATTGGTC-3'), No. 14 (5'-GGAACCAATC-3'), No. 15 (5'-GATCAATCGC-3'), and No. 16 (5'-TCGGTCATAG-3'). Samples (4μl) were run on 6% polyacrylamide /8M urea sequencing gel. The gel was dried on Whatman paper without fixing and subjected to autoradiography for 2 to 3 days. Putative differentially expressed bands were excised, resuspended in 1 ml TE for 15 min at room temperature, and boiled for 10

min in 1 ml 100 mM NaCl/TE followed by incubation at room temperature overnight. The eluted cDNA fragment (5μl) was re-amplified (final volume, 50μl) with the same primer set (0.2μM each), 10mM Tris/HCl (pH8.3), 50mM KCl, 3mM $MgCl_2$, 100μM dNTPs, 2.5 units Taq DNA polymerase using the same PCR condition. The re-amplified cDNA fragments were subcloned into pGEM-T vector (Promega).

Northern blot analysis

Total RNA (10μg) was denatured in formamide and formaldehyde at 55℃ and separated in formaldehyde-containing gel as described[26]. RNA was blotted onto a Hybond N nylon membrane (Amersham), and the membrane was baked, hybridized, and washed as directed by the manufacturer. The subcloned cDNA fragment was labeled using a Multiprime DNA Labeling System (Amersham) and [α-^{32}P] dCTP (3000Ci/mmol) (NEN) and used as a probe.

DNA sequence analysis

DNA sequencing was performed by the dideoxy nucleotide sequencing method. Sequence searches of GenBank were carried out using the BLAST program.

RT-PCR analysis

DNase I-treated total RNA (1μg) was reverse transcribed using 200 units of Superscript II reverse transcriptase and 250 ng of random hexamer primers (TAKARA) in a 20μl reaction at 42℃ for 60 min according to the manufacturer's instructions. cDNA (4μl) was subjected to semiquantitative PCR in a 25μl reaction containing 10 mM Tris/HCl (pH8.3), 50mM KCl, 1.5mM $MgCl_2$, 200μM dNTPs, 1μl [α-^{32}P] dCTP (3000Ci/mmol), 20 pM of the corresponding primer set, and 1 unit Taq DNA polymerase. After an initial denaturation step of 94℃ for 4 min, the reaction was cycled between 94℃ for 1 min, 60℃ for 2 min, and 72℃ for 2 min. We usually used 24 cycles of amplification for samples and 20 cycles for the amplification of murine β-actin gene as a standard. The PCR primers for β-actin gene were as follows: 5'-CCTAAGGCCAACCGTGAAAAGATG-3' (forward) and 5'-ATGGATGCCACAGGATTCCATACC-3' (reverse). The primers amplify a 470 bp fragment. The PCR product (5μl) was electrophoresed using a 6% polyacrylamide gel, dried, and subjected to autoradiography.

RESULTS

Differential display

We performed 144 sets of DD reactions using 16 upstream primers in combination with nine downstream primers. Eighty-two differentially amplified PCR fragments were identified in the first round DD reactions. To verify if these 82 cDNA fragments really had been differentially expressed, we carried out the second round DD analysis with interesting primer combinations and newly prepared reverse transcriptions. As a result, 27(33%) of 82 cDNA fragments obtained in the first round analysis were found to be false positive. Of 55 candidate fragments, 27 fragments were decreased and 28 were increased in density in the *Msx1* mutant. The patterns of amplified cDNA fragments are indicated in Fig. 1A and 2A. Twenty cDNA fragments which were randomly selected from the 55 candidate clones were re-amplified and subcloned into the pGEM-T vector.

Northern blot analysis

The 20 cloned cDNA fragments were used as the probes for Northern blot analysis to confirm their differential expression. Two clones were found to be differentially expressed at significant levels (Fig. 1B). Five clones showed unaltered expressions, and the remaining 13 clones (65%) did not produce any detecable signals in either the wild type or *Msx1* mutant.

DNA sequence analysis

All cDNA clones except five clones with unaltered expressions were sequenced. The size of the cDNA clones ranged from 300 bp to 500 bp. Sequence searches of GenBank revealed that neither of these 15 cDNAs had any significant homology with known genes.

RT-PCR analysis

To select differentially expressed genes from the 13 cDNA clones which gave no signal on Northern blots, we performed semiquantitative RT-PCR analysis. PCR primers (20 mer each) were synthesized according to the sequence data of the clones. Two clones were found to be differentially expressed at significant levels (Fig. 2B). The other clones did not reveal differential expressions.

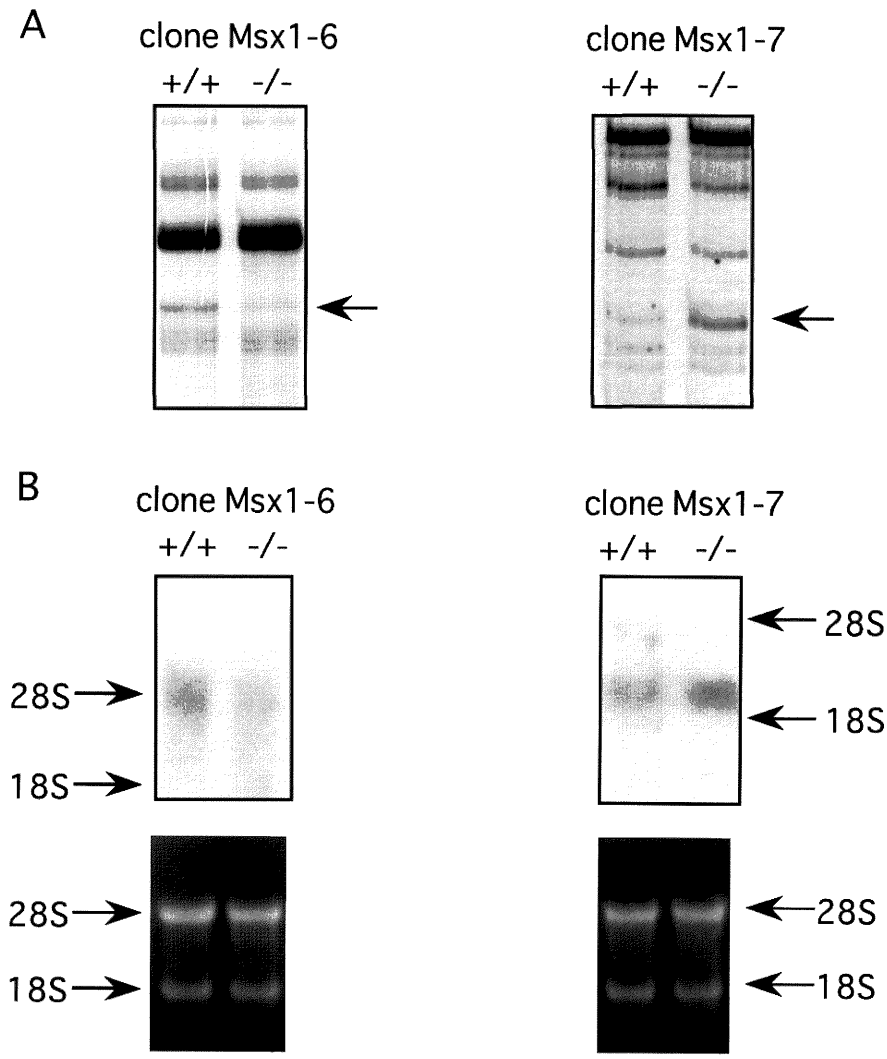

Fig. 1. Identification of novel downstream genes of *Msx1* by differential display and Northern blot analysis. **A.** Autoradiograph of radiolabeled differential display products using total RNA from E14.5 wild-type (*left lanes*) and *Msx1* mutant (*right lanes*) mandibles. cDNA fragments of interest are indicated by arrows. **B.** Northern blot analysis of clones *Msx1-6* and *Msx1-7*. Ten microgram of total RNA from E14.5 wild-type (*left lanes*) and *Msx1* mutant (*right lanes*) mandibles was analyzed. Ethidium bromide staining of 28S and 18S rRNAs was used to normalize RNA loading.

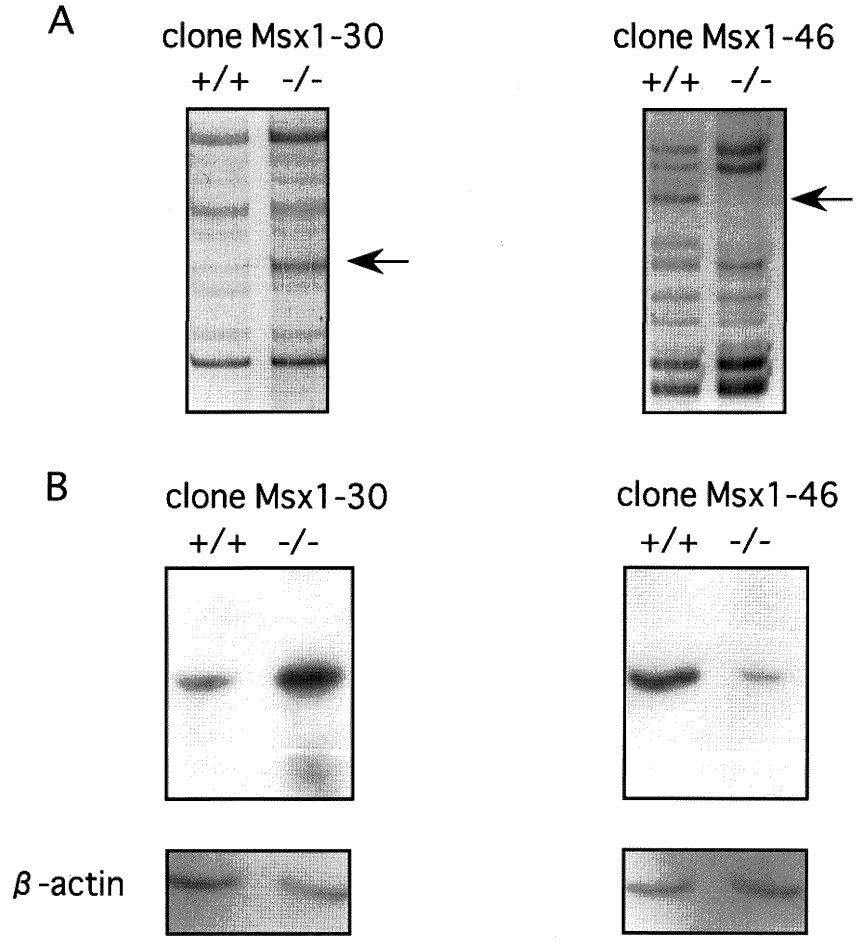

Fig. 2. Identification of novel downstream genes of *Msx1* by differential display and RT-PCR analysis. **A.** Autoradiograph of radiolabeled differential display products using total RNA from E14.5 wild-type (*left lanes*) and *Msx1* mutant (*right lanes*) mandibles. cDNA fragments of interest are indicated by arrows. **B.** RT-PCR analysis of clones *Msx1-30* and *Msx1-46*. We used 24 cycles of amplification for the samples and 20 cycles for the amplification of murine β-actin gene as a standard.

DISCUSSION

The products of the homeobox genes are DNA-binding transcription factors[27]. Homeodomain-DNA-affinity cleaving analysis using *Msx1* homeodomain[28] and oligonucleotide-binding analysis using the *Msx1* protein[29] have been identified as the consensus homeodomain-binding sequence (C/G)TAATG *in vitro*. This sequence has been found in the promoters of the gene encoding signaling molecule Wnt1[28] and the osteocalcin gene which is expressed specifically in mature osteoblasts during bone development[30]. In spite of the related expression patterns of these genes to *Msx1* expression, it is difficult to conclude that these genes are target genes of *Msx1* protein because there is no evidence that this simple consensus sequence actually accounts for the specificity of the DNA binding *in vivo*. This is one of the reasons why there has been little progress in identifying the downstream genes, including target genes, whose activities are regulated directly or indirectly by a homeobox gene. However, gene knockout has recently made it possible to test whether candidate genes function downstream of a homeobox gene *in vivo*. By testing candidate genes using *Msx1* deficient mice, we demonstrated that the three genes *Bmp4*, *Lef1*, and syndecan-1 are downstream genes of *Msx1* in developing tooth germs[21]. Although this approach provides a convincing answer, it can not be applied to unknown genes. To systematically identify downstream genes of a homeobox gene, we tested an approach of DD using the tissue of a knockout mouse. Subtractive hybridization (SH)[26,31] and DD are by far the most commonly used methods for identifying differentially expressed mRNAs. In comparing the two methods, we chose DD for the following reasons[25]: 1)SH requires 1 to 5 μg of poly (A) RNA whereas DD only needs $\leqq$1/50 of this amount to screen $>$10,000 cDNAs. 2)SH identifies abundant differentially expressed mRNAs whereas DD identifies mRNAs dependent not on a prevalence but a primer sequence. 3)SH is only a one-way comparison and identifies a mRNA with large changes ($\geqq$10 fold) whereas DD simultaneously identifies both increases and decreases ($\geqq$two fold) of a particular mRNA. 4)SH is technically difficult and time-consuming whereas DD is easy and has rapid output. However, DD can generate many false positives. According to the Differential Display Kit manual (Display Systems Biotech), 20-40% of the fragments in the first round DD drop out in the second round DD. In this study, 33% of differetially amplified fragments in the first round DD dropped out in the second round DD. Therefore the DD analysis should be repeated at least two times under the same conditions in order to reduce the number of false positive cDNA fragments. The major obstacle of DD is not the technique itself but rather the post-DD process of discriminating between false positives and the truly differentially expressed cDNAs[32]. The methods for verification such as Northern blot analysis, RNase protection assay, semiquantitative RT-PCR, and *in situ* hybridization are not optimal for large scale screening operations because they are arduous and/or require large amounts of RNA. First we tested two newly-reported methods which would allow one to screen putative positives from DD analysis using micrograms of total RNA because RNAs were limited in our study. These were differential screening methods with the use of cDNA probes generated from either total RNAs[33] or amplified RNAs[34]. However, we could not obtain any reliable or reproducible results by either of these methods (data not shown). Consequently, we chose Northern blot analysis and semiquantitative RT-PCR for verification. Although Northern blot analysis did require large amounts of RNAs, we could not verify 65% of candidate clones by this method because of the absence of signals. We think that it would be better to choose semiquantitative RT-PCR in the first place as a verification method if the source of the tissue is very limited Another problem in the current approach was that we could not detect any difference in the expression of either *Bmp4* and *Lef1* between the wild-type and *Msx1* mutant when we performed RT-PCR analysis using total RNA from a whole mandible (data not shown). *In situ* hybridization analysis revealed that the expression of either *Bmp4* or *Lef1* was not reduced at the *Msx1* mutant mandible except for the developing tooth mesenchyme (I Satokata et al, unpublished data). This result suggests that our current method may underestimate the differential expressions of some genes if we use the additional tissue of a knockout mouse for the analysis. However, we can also expect that the positive clones identified by this method may show more distinct differential expression in the limited area of the mutant tissue which we use for the analysis. Although there are several problems in this approach, we conclude that DD analysis using the tissue of a knockout mouse is a useful systematic approach for the identification of downstream genes of a homeobox gene because 20% of verified cDNA clones turned out to be truly differentially expressed cDNAs in this study. We are now verifying the remaining 35 cDNA clones, cloning full-length cDNAs of the positive clones, and examining their

expression patterns in the mandible and the other organs. Although there is no successful report so far, DD analysis using the tissue of a knockout mouse may become a popular method for the identification of downstream genes of a transcription factor and bring results in the study of signaling pathways in which transcription factors such as homeobox genes are involved.

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