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Cloning and chromosomal localization of the human *BARX2* homeobox protein gene

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Abstract

The human *BARX2* gene encodes a homeodomain-containing protein of 254 amino acids, which binds optimally to the DNA consensus sequence YYTAATGRTTTTY. *BARX2* is highly expressed in adult salivary gland and is expressed at lower levels in other tissues, including mammary gland, kidney, and placenta. The *BARX2* gene consists of four exons, and is located on human chromosome 11q25. This chromosomal location is within the minimal deletion region for Jacobsen syndrome, a syndrome including craniosynostosis and other developmental abnormalities. This chromosomal location, along with the reported expression of murine *barx2* in craniofacial development, suggests that *BARX2* may be causally involved in the craniofacial abnormalities in Jacobsen syndrome. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Chromosome 11; Jacobsen syndrome; Medullary thyroid carcinoma; Salivary gland

1. Introduction

Medullary thyroid carcinoma (MTC) is a tumor of the calcitonin-secreting thyroid parafollicular C-cells (reviewed by Ball, 1996). The differentiation status of MTC, as evidenced by its continued expression of the calcitonin (CT) gene, is associated with good prognosis. Thus, MTC patients whose tumors exhibit absent or patchy immunohistochemical staining for CT have significantly shorter survival than those patients whose tumors stain positively and homogeneously for CT. Using a cell culture model for human MTC, we have shown previously that these cells can be induced to differentiate by activation of the ras/raf signal transduction pathway (Nakagawa et al., 1987; Carson et al., 1995). This response includes morphological changes, cessation of cell growth, and increased transcription of the calcitonin gene.

The ras/raf pathway activates several protein kinase cascades, which can result in the subsequent activation of transcription factors; these, in turn, can alter gene expression and cell phenotype. To identify the transcription factors which mediate ras/raf dependent differenti-

Abbreviations: CT, calcitonin; IPTG, isopropyl-β-D-thiogalactopyranoside; MTC, medullary thyroid carcinoma; PAC, P1-derived artificial chromosome; RRE, ras/raf responsive element; YAC, yeast artificial chromosome.

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ation in MTC cells, we have examined the control of ras/raf mediated transcription of the calcitonin gene. Within the calcitonin gene promoter, we have identified a ras/raf responsive transcriptional element (RRE), between −206 and −132 base pairs upstream from the transcription startpoint (Thiagalingam et al., 1996). Within this element, two protein binding domains were identified; either of these domains is sufficient to confer responsiveness of a reporter gene to ras/raf activation. Using DNA affinity cloning, we have isolated genes for transcription factors binding to each of these domains. One of these proteins, RREB-1, is a zinc finger protein (Thiagalingam et al., 1996). Here, we report the cloning and chromosomal location of *BARX2*, the gene for a homeodomain protein which binds to this ras/raf element in the calcitonin gene promoter. The murine homolog, *barx2*, has been reported to be expressed in neural and craniofacial development (Jones et al., 1997). We find that the *BARX2* gene maps to human chromosome 11q25, a region of the genome consistently deleted in Jacobsen syndrome, which is characterized by craniofacial defects and other abnormalities. Thus, in addition to a possible role in differentiation of MTC, *BARX2* may be a candidate for involvement in Jacobsen syndrome.

2. Materials and methods

2.1. DNA affinity cloning

Construction of the TT cell λgt11 cDNA expression library has been described (Thiagalingam et al., 1996). Screening for CT gene binding proteins, by DNA affinity cloning methods (Vinson et al., 1988), was as described (Thiagalingam et al., 1996). In the present study, the probe was catenated, double-stranded oligonucleotide 7–8 (5′-ATCCATTTCCATCAATGACCTCAATGCAAATAC-3′), labeled by nick translation with α-^{32}P-dCTP. Screening of 10^6 plaques with oligonucleotide 7–8 yielded a positive clone. The cDNA insert of this clone was subcloned in pBluescript, and was used to probe a human placenta cDNA library in λgt10 to obtain the entire coding region. A 1.3 kb clone encoding full-length *BARX2* was obtained and subcloned into pBluescript. The GenBank accession number for the *BARX2* cDNA sequence is AF031924.

2.2. Northern and dot blot hybridization

Human salivary gland poly A+ mRNA (Clontech) and TT:ΔRaf-1:ER poly A+ mRNA (2 μg each) were electrophoresed, transferred to nylon membranes and hybridized with the 1.3 kb *BARX2* cDNA as described previously (Thiagalingam et al., 1996). A multiple tissue Northern blot and a human Master RNA dot blot were obtained from Clontech, and were hybridized with the *BARX2* cDNA.

2.3. DNA binding specificity

The DNA binding specificity of the λgt11 *BARX2* cDNA clone was initially examined by filter binding. The λgt11 *BARX2* clone was plated at a density of 400 plaques per 10 cm petri dish, using top agarose containing 10 mM IPTG to induce expression. Plaques were allowed to develop, and a nitrocellulose filter was placed on the agarose to adsorb the lacZ–BARX2 fusion proteins. The filter was then cut into three parts; each part was assayed for binding to a separate catenated double-stranded oligonucleotide probe, as described previously (Thiagalingam et al., 1996). The oligonucleotides used (described previously; Thiagalingam et al., 1996) were 1–2 (the upstream C-rich domain of the CT gene RRE), 7–8 (the CT gene RRE octamer domain probe used for screening for *BARX2*), and 5–6 (a random sequence oligonucleotide). The binding reaction and washing conditions were as described previously (Thiagalingam et al., 1996).

Determination of the BARX2 consensus DNA binding sequence by CASTing (Wright et al., 1991) was done as described previously (Thiagalingam et al., 1996). Briefly, DNA sequences were selected from a double-stranded degenerate oligonucleotide (5′-GAGATATTAGAATTCTACTC-N_{23}-GGTACATATACTCGAGT-3′) library, by binding to GST–BARX2, selection on glutathione–Sepharose, elution and PCR amplification. After five rounds of enrichment, DNA sequences which bound to GST–BARX2 were cloned into pBluescript and sequenced. DNA sequence alignments were done using the Clustal W program (Thompson et al., 1994). Conditions for gel mobility shift assays were as described previously (Thiagalingam et al., 1996). Here, 1 μg of thioredoxin–BARX2 was incubated with 1 ng of ^{32}P-end labeled oligonucleotide 7–8 (5′-ATCCATTTCCATCAATGACCTCAATGCAAATAC-3′) or oligonucleotide Barx2 cons (5′-GATCTTTCTTAATGGTTTTTCGA-3′).

2.4. Cell culture

The TT cell line of human MTC has been described (Nakagawa et al., 1987). TT:ΔRaf-1:ER is a subline of TT cells in which a fusion gene, containing the kinase domain of the *c-raf-1* gene and the hormone binding domain of the estrogen receptor, has been stably introduced by retroviral infection (Carson et al., 1995). In TT:ΔRaf-1:ER cells, activation of c-raf-1 by addition of 1 μM β-estradiol results in terminal differentiation of the cells, accompanied by cessation of growth and increased transcription of the CT gene (Carson et al., 1995).

2.5. Chromosomal localization

(i) *Somatic cell hybrids*. The NIGMS somatic cell hybrid mapping panel 2 was screened by PCR, using as primers 5′-GCGGCATATCGCTCCTCTCGGT-3′ and 5′-GCTCGGTGAAGATGGTGCGACT-3′, producing a 226 bp product from exon 2 (bases 296–521 in Fig. 2A). These primers were also used to obtain a PAC clone of *BARX2* by PCR screening of the RPCI-1 library (Genome Systems). A subclone of the PAC clone was partially sequenced to confirm that it contained *BARX2*.

(ii) *FISH*. DNA of the *BARX2* PAC clone was labeled with biotin-16-dUTP by nick translation. Human prometaphase spreads were fixed on slides and pretreated with RNase and pepsin as described (Lengauer et al., 1994). Biotinylated probe sequences were detected with avidin–DCS–fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA). Chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI). The resulting G-banding pattern and the signals were evaluated by standard epifluorescence microscopy (Nikon Eclipse 800; Lengauer et al., 1994). Photographs were taken using a cooled CCD camera (Photometrics, Tucson, AZ). The sequentially recorded gray-scale images were pseudocolored and merged, as described previously (Lengauer et al., 1994).

(iii) *YAC mapping*. YAC clones from a contig of distal 11q (Tunnacliffe et al., 1999) were tested as described previously (Penny et al., 1995) for the presence of *BARX2* by (a) PCR using primers specific for both the 5′ end and the 3′ end of the gene, and (b) direct hybridization using the full-length cDNA insert.

2.6. Genomic sequencing

A *BARX2* PAC clone, containing exons 1–4 of the *BARX2* gene, was partially sequenced, using exon-specific primers. Primers for intronic sequences were designed to amplify each exon from 100 ng of human genomic DNA (isolated from peripheral blood, using a Qiagen Blood and Cell Culture DNA kit) from normal subjects and trigonocephalic patients without 11q deletion. (For exon 1 and exon 4, one primer was based on untranslated cDNA sequences.) PCR amplifications (35 cycles of 95°C for 30 min, 57 or 60°C for 30 min, and 72°C for 30 min) were done in a 50 μl volume of 50 mM KCl, 20 mM trisCl, 2 mM $MgCl_2$, pH 8.4 at 25°C, containing 200 ng of each primer, and 1 unit of Taq DNA polymerase. The primer pairs used were: exon 1, 5′-CTCACCATGCATGCCACGCCG-3′ and 5′-GATCGCAAAGCACAGGCCACCTACACG-3′ (267 bp product); exon 2, 5′-CTGGCCTGCTTCCCCACACCGTTC-3′ and 5′-TGAGCCAAGGAGTGGACTCCGCCATAG-3′ (493 bp product); exon 3, 5′-AAGANAAGCGGTTTGGGGAAGACCTCGT-3′ and 5′-ATCCAACAGCTTCCCGCAAGCC-3′ (378 bp product); exon 4, 5′-CTGGAAGGTTTTCTCTCCCTACTCTCCC-3′ and 5′-CACTGCTGGAACAGATGGTTTAGATGCAACG-3′ (459 bp product). Annealing temperatures were 60°C for exons 1 and 2, and 57°C for exons 3 and 4. PCR products were gel purified and sequenced directly (JHMI DNA Analysis Facility), using one of the amplification primers as a sequencing primer.

3. Results

3.1. DNA affinity cloning of BARX2

We have been interested in obtaining the transcription factor(s) which interact with the ras/raf responsive element (RRE) of the CT gene. As described previously (Thiagalingam et al., 1996), this RRE is bipartite, containing a C-rich domain and a homeobox octamer containing domain. Previously, we had identified RREB-1, a zinc finger protein which binds to the C-rich region of the CT gene RRE (Thiagalingam et al., 1996). In order to identify the protein(s) which bind to the octamer containing domain, we screened a human MTC cell cDNA λgt11 expression library, by DNA affinity cloning, using oligonucleotide 7–8 (Thiagalingam et al., 1996), which contains the octamer binding domain of the CT gene RRE. Screening of 10^6 plaques yielded a

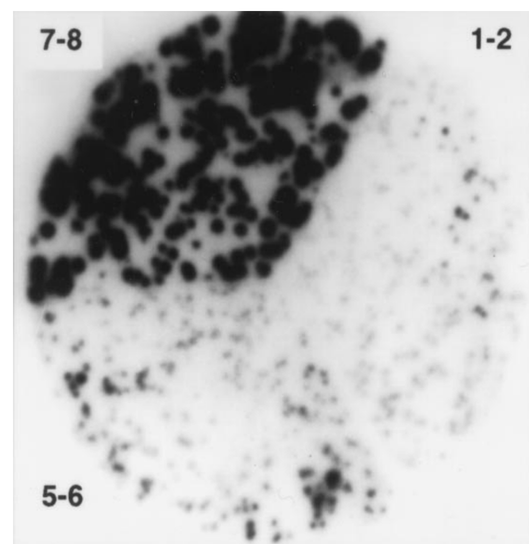

Fig. 1. Binding of lacZ–BARX2 to sequences from the human calcitonin gene ras/raf responsive element. The λgt11 phage containing a partial *BARX2* cDNA was plated, expressed as a lacZ fusion protein, and the fusion protein was transferred to a nitrocellulose filter. The filter was cut into three parts, which were separately incubated with double-stranded oligonucleotides 1–2, 5–6, and 7–8, as described in Materials and methods. Only oligonucleotide 7–8, the octamer containing domain of the CT gene RRE, was specifically bound by the lacZ–BARX2 fusion protein.

A

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   1 AGGGGCCCAGGCCCCGCCGTCGCGCCAGCCCCGCGGCCCCAGCGGGCCGGGCACTCGCAACCGCGCT
  68 CGGGCGGCGGACGCTCGCGCCGGCTCACCATGCACTGCCACGCCGAGCTGAGGCTGAGCTCGCGGCC

                                        M   I   D   E   I   L   S   K     8
 135 AGCTCAAAGCAGCCAGGCGGCGCTACAAGACTTTC ATG ATC GAC GAG ATC CTC TCC AAG

      E   T   C   D   Y   F   E   K   L   S   L   Y   S   V   C   P   S    25
 194 GAG ACC TGC GAT TAC TTT GAG AAA CTT TCC CTC TAC TCC GTG TGC CCG TCG

      L   V   V   R   P   K   P   L   H   S   C   T   G   S   P   S   L    42
 245 CTG GTC GTG CGA CCC AAG CCC CTG CAT TCC TGT ACG GGC TCC CCT TCC CTG

      R   A   Y   P   L   L   S   V   I   T   R   Q   P   T   V   I   S    59
 296 CGG GCA TAT CCG CTC CTC TCG GTG ATC ACC CGC CAG CCC ACT GTC ATC TCC

      H   L   V   P   A   T   P   G   I   A   Q   A   L   S   C   H   Q    76
 347 CAC CTG GTC CCT GCC ACC CCG GGA ATC GCC CAG GCA CTG TCC TGC CAC CAG

      V   T   E   A   V   S   A   E   A   P   G   G   E   A   L   A   S    93
 398 GTC ACC GAG GCG GTC TCT GCT GAG GCC CCA GGG GGC GAG GCC CTA GCC AGC

      S   E   S   E   T   E   Q   P   T   P   R   Q   K   K   P   R   R   110
 449 AGC GAG TCA GAG ACG GAA CAG CCC ACG CCC CGA CAG AAG AAG CCC CGC CGG

      S   R   T   I   F   T   E   L   Q   L   M   G   L   E   K   K   F   127
 500 AGT CGC ACC ATC TTC ACC GAG CTG CAG CTC ATG GGC CTG GAG AAG AAA TTC

      Q   K   Q   K   Y   L   S   T   P   D   R   L   D   L   A   Q   S   144
 551 CAG AAG CAG AAG TAT TTG TCA ACC CCA GAC AGG TTG GAC TTG GCT CAG TCT

      L   G   L   T   Q   L   Q   V   K   T   W   Y   Q   N   R   R   M   161
 602 CTG GGA CTC ACT CAG CTG CAG GTG AAG ACC TGG TAT CAG AAT CGC AGG ATG

      K   W   K   K   M   V   L   K   G   G   Q   E   A   P   T   K   P   178
 653 AAA TGG AAG AAA ATG GTT CTT AAA GGT GGA CAG GAA GCA CCC ACA AAA CCC

      K   G   R   P   K   K   N   S   I   P   T   S   E   E   I   E   A   195
 704 AAA GGT CGC CCC AAG AAG AAC TCC ATC CCC ACA TCA GAA GAG ATT GAA GCT

      E   E   K   M   N   S   Q   A   Q   G   Q   E   Q   L   E   P   S   212
 755 GAA GAG AAG ATG AAC AGC CAG GCC CAG GGT CAG GAG CAG CTG GAG CCC TCT

      Q   G   Q   E   E   L   C   E   A   Q   E   P   K   A   R   D   V   229
 806 CAG GGG CAG GAG GAG CTC TGT GAA GCA CAG GAA CCG AAA GCA CGT GAT GTC

      P   L   E   M   A   E   P   P   D   P   P   Q   E   L   P   I   P   246
 857 CCC TTA GAG ATG GCA GAG CCA CCA GAC CCG CCC CAG GAG TTG CCA ATA CCC

      S   S   E   P   P   P   L   S   *                                   254
 908 TCT TCG GAA CCC CCA CCA TTA AGC TAA AGTAAAACCCTTTTGAGGGAAGAGGGAGACT
 966 GGGGAGAAGGGAAAAGAGAGAAGGCAGGGAGAGTAGGGAGAGAAAACCTTCCAGCAGCCCAGTAAAC
1033 TGCGGGCGAAGAGATCTACCCGTCTCCCTCCCTCCCACAGTTACCATTGAGCCTGTCATCGCAAGCA
1100 TTTGACAAAGACTTGCTTGTCTTGGGCCTGTCACCTCCTGAAAGGCTGCTTTAGCTGTGGATGCCCT
1167 TGATTAAGGGAGAGAGCGCCTAGGAGCTGCCTGCCCCAGCTGGGGTGACGGCTGTAGGGCTGGGTCT
1234 ATGTTGCAAGCCCTATATCCTAGCATGCAGTGGAAAGTGCTT
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Fig. 2. (**A**) Sequence of *BARX2* DNA. The nucleotide sequence of *BARX2* exhibits 86% homology with murine *barx2*. (**B**) The predicted protein sequence of *BARX2* also exhibits 86% identity with murine *barx2*. In the N-terminal portion, BARX2 lacks four amino acids encoded by the murine *barx2* gene. Asterisks indicate identical amino acids, while ':' and '.' indicate highly and moderately conserved amino acids, respectively. The homeodomain in human BARX2 encompasses amino acids 108–167.

cDNA whose product bound oligonucleotide 7–8, but not unrelated oligonucleotides (Fig. 1). This cDNA product was used to screen a human placenta cDNA library, yielding a 1.3 kb cDNA containing the complete coding sequence. The nucleotide sequence (Fig. 2A) predicts a protein of 254 amino acids (Fig. 2B), with a homeodomain closely related to the *bar* class of genes in Drosophila. Within the coding sequence, the cDNA has 86% homology, at both the nucleic acid and amino acid levels, with the murine *barx2* gene, a *bar* class

B

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hbarx2     1 MIDEILSKETCDYFEKLSLYSVCPSLVVRPKPLHSCTGSPSLRAYPLLSVITRQPTVISH
mbarx2     1 MIDEILSKETCDYFEKLSLYSVCPSLVVRPKPLHSCTGSPSLRAYPLPSVITRQPTVISH
             *********************************************** ************

hbarx2    61 LVPATPGIAQALSCHQVT----EAVSAEAPGGEALASSESETEQPTPRQKKPRRSRTIFT
mbarx2    61 LVPTGSGLTPVLTRHPVAAAEAAAAAAETPGGEALASSESETEQPTPRQKKPRRSRTIFT
             ***: .*:: .*: * *:     *.:**:*******************************

hbarx2   117 ELQLMGLEKKFQKQKYLSTPDRLDLAQSLGLTQLQVKTWYQNRRMKWKKMVLKGGQEAPT
mbarx2   121 ELQLMGLEKKFQKQKYLSTPDRLDLAQSLGLTQLQVKTWYQNRRMKWKKMVLKGGQEAPT
             ************************************************************

hbarx2   177 KPKGRPKKNSIPTSEEIEAEEKMNSQAQGQEQLEPSQGQEELCEAQEPKARDVPLEMAEP
mbarx2   181 KPKGRPKKNSIPTSEEIEAEEKMNSQAQSQELLESSERQEEPCDTQEPKACLVPLEVAEP
             ****************************.** **.*: *** *::*****  ****:***

hbarx2   237 PDPPQELPIPSSEPPPLS
mbarx2   241 IHQPQELSEASSEPPPLS
              . ****. .********
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Fig. 2. (*continued*)

homeodomain protein expressed in murine craniofacial and neural development (Jones et al., 1997). There is complete amino acid identity within the homeodomain with murine *barx2*. We therefore propose to call this human gene *BARX2*.

3.2. DNA binding specificity of BARX2

The DNA sequence binding specificity of *BARX2* was determined by CASTing (Wright et al., 1991). GST–BARX2 fusion protein was bound to a pool of random double-stranded oligonucleotides, and the bound oligonucleotides were eluted and amplified by PCR. After five rounds of enrichment for oligonucleotides which can bind GST–BARX2, the selected DNA sequences were cloned in pBluescript and sequenced. The consensus BARX2 binding sequence appears to be YYTAATGRTTTTY (Fig. 3A), which is closely related to the BARX2 binding sequences in the *NCAM* and *NCAM–LI* genes (Jones et al., 1997), but somewhat more divergent from the sequence in the human calcitonin gene promoter (oligonucleotide 7–8; see Materials and methods), which was used as an affinity probe to isolate *BARX2*. The BARX2 consensus binding sequence contains an octamer sequence, TAATGRTT, typical of homeobox protein binding sites, and this octamer is flanked by pyrimidine-rich bases. We designed an oligonucleotide, Barx2 cons (GATCTTTCTTAATGGTTTTCGA) containing the CASTing-derived BARX2 consensus DNA binding site, for use in a gel mobility shift assay. Fig. 3B shows that, in the conditions of this gel mobility shift assay, oligonucleotide Barx2 cons, based on the BARX2 consensus binding sequence, was bound much more efficiently by BARX2 than was oligonucleotide 7–8, from the human calcitonin gene promoter. Similarly, in competition experiments, Barx2 cons binding to BARX2 was competed by excess cold Barx2 cons, but not by oligonucleotide 7–8 (Fig. 3C).

3.3. Expression pattern of BARX2

The expression pattern of *BARX2* was examined. Using commercial multiple tissue dot blots and Northerns of human poly A+ RNAs, we found that expression of *BARX2* was tissue specific; the highest expression was seen in adult salivary gland mRNA (Fig. 4), with expression also detectable in placenta, pancreas, mammary gland, kidney and trachea (data not shown).

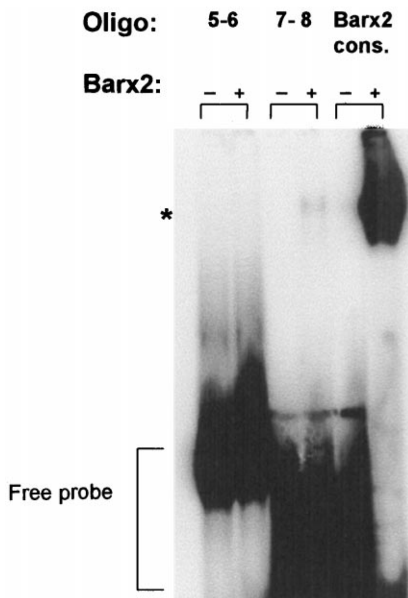
BARX2 appears as a 2.2 kb mRNA species on Northern blots, somewhat larger than the 1.7 kb transcript reported for murine *barx2* (Jones et al., 1997). In MTC cells, the level of *BARX2* mRNA was not increased by activation of c-raf-1 (Fig. 4), which induces terminal differentiation of MTC cells (Carson et al., 1995). Since portions of the salivary gland and craniofacial structures are derived from the same region of the neural crest (Nakamura, 1982), this adult salivary gland expression may be consistent with the report of murine *barx2* expression in fetal craniofacial structures (Jones et al., 1997).

3.4. The BARX2 gene is localized to chromosome 11q25, within the minimal deletion domain for Jacobsen syndrome

Using PCR screening of a somatic cell hybrid panel (NIGMS Panel 2, Coriell Cell Repository, Camden, NJ), we found that *BARX2* was on human chromosome 11 (data not shown). Further examination of a cell

A

A	7	6	0	95	95	0	0	26	5	0	5	6	6
C	36	19	0	0	0	0	0	0	0	5	0	0	35
G	14	12	0	5	5	0	84	69	5	5	21	22	6
T	43	63	100	0	0	100	16	5	90	90	74	72	53
cons.	Y	Y	T	A	A	T	G	R	T	T	T	T	Y



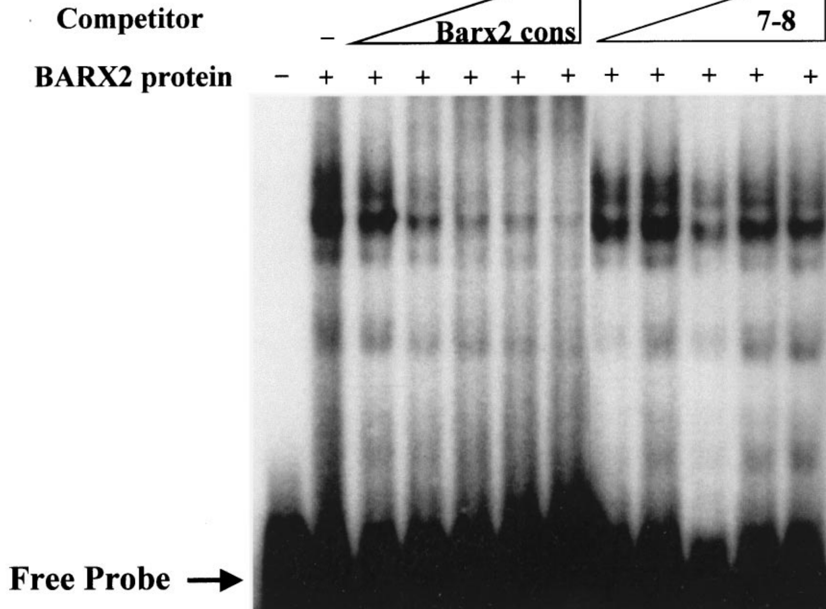


Fig. 3. Specificity of binding of BARX2. (**A**) Selection of high affinity binding sites by CASTing. GST–BARX2 was incubated with a double-stranded degenerate oligonucleotide, and bound oligonucleotides were recovered and amplified after glutathione–Sepharose purification (Thiagalingam et al., 1996). After five rounds of enrichment, the selected oligonucleotides were cloned into pBluescript and 19 clones were sequenced. Sequences were aligned by Clustal W. (**B**) Gel mobility shift assay. 1 μg of thioredoxin–BARX2 was incubated with 1 ng of oligonucleotide 7–8 (from the human calcitonin gene RRE) or oligonucleotide Barx2 cons (based on the BARX2 consensus binding sequence). As a negative control, BARX2 was incubated with unrelated oligonucleotide 5–6. Specific BARX2–DNA complexes are indicated by an asterisk. BARX2 binds well to its consensus sequence, and also binds to the calcitonin gene RRE sequence, but not to the unrelated oligonucleotide. (**C**) Competition of binding to oligonucleotide Barx2 cons. Binding of thioredoxin–BARX2 with oligonucleotide Barx2 cons was done as in (**B**), except that the thioredoxin–BARX2 was pre-incubated for 15 s with 10–800 ng cold competitor oligonucleotide Barx2 cons or oligonucleotide 7–8. The strong binding of oligonucleotide Barx2 cons can only be competed by excess cold Barx2 cons.

hybrid containing all of human chromosome 11, except for 11q23-ter, showed that the *BARX2* gene was absent, suggesting that *BARX2* may be within the interval from 11q23-qter (data not shown). This localization was confirmed by FISH, which showed that *BARX2* is at 11q25 (Fig. 5A). We have further localized *BARX2* to a YAC contig previously shown to map within this region (Fig. 5B).

Using cDNA-based primers to sequence a PAC template, we determined the positions of the introns of *BARX2* (Fig. 6). The cDNA sequences are contained within 4 exons. Intron 1 is located after base 281, intron 2 after base 582, and intron 3 after base 667. The splice donor and acceptor sequences are good matches with described canonical consensus splice sequences (Shapiro and Senapathy, 1987). Our sequences for *BARX2* coding sequences and flanking intronic sequences match those recently published by Hjalt and Murray (1999).

Homeobox containing genes have been shown to be involved in normal vertebrate development, and mutation or deletion of specific homeobox containing genes has been shown to be responsible for over a dozen human developmental syndromes (reviewed in Mark et al., 1997). Therefore, we considered whether the expression pattern and chromosomal location of *BARX2* might suggest its involvement in a known syndrome of abnormal development. Murine *barx2* has been reported to be expressed in craniofacial and neural development (Jones et al., 1997). This expression pattern, along with our data localizing *BARX2* to human chromosome 11q25, suggested that *BARX2* might be involved in Jacobsen syndrome, a rare congenital disorder which

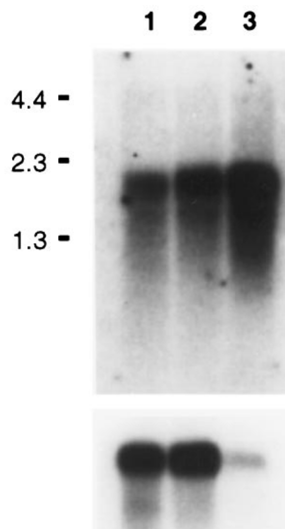


Fig. 4. Expression of *BARX2*. Northern blot showing expression in poly A+ RNA from TT:raf-1:ER cells without β-estradiol (lane 1), TT:Δraf-1:ER cells exposed to 1 μM β-estradiol for 48 h to activate Δc-raf-1 and induce cell differentiation (lane 2) and human salivary gland (lane 3). The filter was probed with the 1.3 kb h*BARX2* cDNA. No change in *BARX2* expression is seen upon exposure of TT:Δraf-1:ER cells to estradiol. RNA markers (in kb) are indicated. The lower panel shows hybridization to GAPDH as a measure of poly A+ RNA loading.

includes abnormalities in craniofacial development. Jacobsen syndrome is typically characterized by a number of craniofacial abnormalities (craniosynostosis with trigonocephaly, upslanting palpebral fissure slant, short nose and long philtrum, retrognathia, and lowset or malformed ears), as well as growth and psychomotor retardation, and, less commonly, cardiac defects and thrombocytopenia (Jacobsen et al., 1973; Jones et al., 1995; Penny et al., 1995). Jacobsen syndrome is consistently associated with loss of one copy of the end of chromosome 11q; the breakpoint is often near fragile site FRA11b, resulting in deletion from 11q23–11qter (Lewanda et al., 1995). Smaller deletions of 11q have also been seen in Jacobsen syndrome; the minimal deletion still associated with the typical features of the syndrome encompasses 18cM (sex average; Dib et al., 1996) from marker D11S1351 to the telomere (Lengauer et al., 1994). While the large deletions in Jacobsen syndrome suggest that the overall phenotype results from haploinsufficiency of several genes in this region, the expression pattern of murine *BARX2* (Jones et al., 1997) suggests that *BARX2* may be a candidate for involvement in the craniofacial abnormalities in this syndrome.

Since Jacobsen syndrome is invariably accompanied by a large 11q deletion, it would be unlikely to find a mutation in *BARX2* in Jacobsen syndrome. Instead, we have begun to examine the *BARX2* gene in patients with some of the features of Jacobsen syndrome, but lacking a cytogenetically visible deletion of chromosome 11q. Thus, we have sequenced the *BARX2* exon sequences amplified from nine patients with trigonocephaly, but without other features of Jacobsen syndrome. No mutations of *BARX2* were seen in these patients (data not shown).

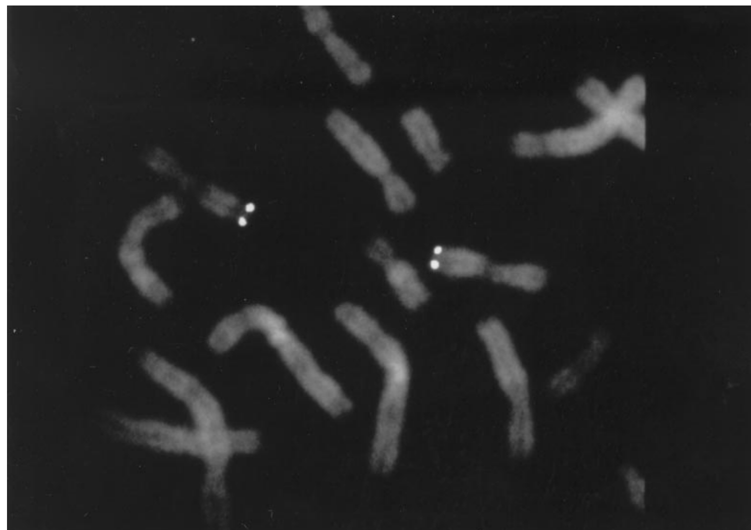
4. Discussion

The BARX2 protein may participate in ras/raf signaling, since it binds to the octamer containing domain of the bipartite CT gene RRE, which augments the ras/raf response of the CT gene promoter in human medullary thyroid carcinoma cells (Thiagalingam et al., 1996). Other homeobox proteins have been reported to mediate or modify the effects of ras in other systems. In *Caenorhabditis elegans*, ras-dependent induction of vulval differentiation appears to be mediated by the homeobox protein lin-39, a sex combs reduced homolog, and antagonized by the homeobox protein mab-5, an antennapedia homolog (Clandinin et al., 1997). In ras-transformed 3T3 fibroblasts, interference with expression of the homeobox protein MSX-2 expression results in reversion of the transformed phenotype; this suggests that MSX-2 may function downstream of ras in cell transformation (Takahashi et al., 1996). Finally, and similar to our findings, Gutierrez-Hartmann and colleagues have characterized a ras-responsive element in the prolactin gene promoter (Bradford et al., 1995). Like the CT gene RRE, the prolactin gene RRE is bipartite, with an ets1 site juxtaposed to a GHF-1/Pit-1 POU homeobox protein site.

Our data (see Figs. 1 and 3) indicate that BARX2 can specifically bind different DNA sequences, with differing efficiency. Such differences might reflect different stringency of control of BARX2 target genes in vivo. However, BARX2 binding may be modified in vivo by combinatorial interactions between BARX2 and other proteins; such interactions have been described for several homeobox proteins, and these interactions can modify the DNA sequence specificity, the binding affinity, or the transcription activating ability of the homeobox proteins (Takahashi et al., 1996; Knoepfler et al., 1997; Wu et al., 1997).

As mentioned above, genetic lesions in homeobox genes underlie many human developmental syndromes (reviewed in Mark et al., 1997). These include Waardenburg syndrome (commonly associated with facial and pigmentary abnormalities and cochlear deafness), Boston-type craniosynostosis, and Rieger syndrome (associated with eye and tooth abnormalities); these syndromes are due to loss of one functional copy of the homeobox genes *PAX3*, *MSX2*, and *PITX2*, respectively (Baldwin et al., 1992; Jabs et al., 1993; Semina et al., 1996). Since complete deletion of one copy of the gene can result in the clinical syndrome, it

A



B

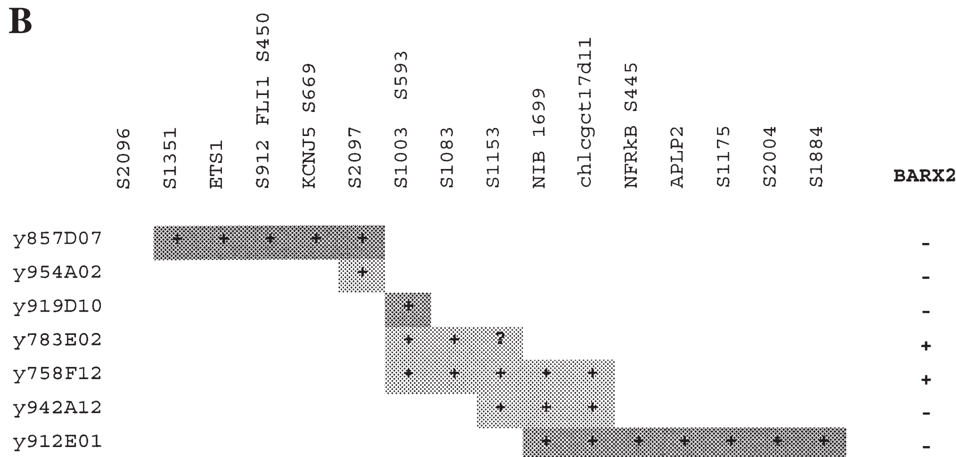


Fig. 5. Chromosomal location of *BARX2*. (**A**) Chromosomal FISH. The hbarx2 PAC clone, labeled with biotin-16-dUTP, was hybridized with normal human prometaphase spreads, and detected with avidin–DCS–fluorescein isothiocyanate, as described in Materials and methods. The only detectable signal is at 11p25. (**B**) YAC contig mapping. A YAC contig for distal chromosome 11q, assembled by STS content mapping (Tunnacliffe et al., 1999), was screened for *BARX2* by PCR and cDNA hybridization. Positive and negative results are indicated, showing the location of the *BARX2* gene close to marker D11S1083. Marker prefixes 'D11S' are abbreviated 'S'.

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                   281                                    282
exon1-CATTCCTGTACGGgtaagaac...cctccctgcttgttttccagGCTCGCCCT-exon2

                   582                                    583
exon2-CAACCCCAGACAGgtgaggac...ttgtatcttgtgccttctagGTTGGACTT-exon3

                   667                                    668
exon3-ATGGAAGAAAATGgtaagaaa...aacaatttttctcacgtagGTTCTTAAA-exon4
```

Fig. 6. Exon–intron boundaries of the human *BARX2* gene. Exon sequences are shown in upper case, and introns in lower case. The base numbering for the exons is according to the cDNA sequence in Fig. 2.

appears that many of these syndromes result from haploinsufficiency, rather than a dominant negative effect. However, the gene targets for these homeobox proteins have not yet been well characterized.

The possible involvement of *BARX2* in Jacobsen syndrome, as suggested by the expression pattern of murine *BARX2* and by the location of the *BARX2* gene in the minimal deletion region for Jacobsen syndrome, suggests a possible interaction between ras/raf signaling and homeobox proteins in craniofacial development. Previous studies of craniofacial abnormalities, especially craniosynostosis, provide ample, albeit circumstantial,

precedent for this possibility. Crouzon craniofacial dysostosis (OMIM 123500) has been shown to result from activating mutations in fibroblast growth factor receptor-2 (Jabs et al., 1994); this activation of fibroblast growth factor receptor has been shown to induce the ras/raf signal transduction pathway (Neilson and Friesel, 1995). Boston-type craniosynostosis (OMIM 123101) has been shown to be due to mutations in the MSX-2 homeobox gene (Jabs et al., 1993). As mentioned above, there is evidence that MSX-2 may interact with the ras/raf signal transduction pathway as a downstream effector. Transgenic animals, with either overexpression or impaired expression of MSX-2, developed craniofacial abnormalities (Liu et al., 1995; Foerst-Potts and Sadler, 1997; Winograd et al., 1997). Speculatively, *BARX2* may also function as a necessary downstream effector to mediate ras/raf signals in normal craniofacial development.

Abnormalities in expression or structure of homeobox-containing genes have been reported in several types of cancer. These abnormalities, including activating chromosomal translocations within homeobox-containing genes, have been extensively documented in hematopoietic malignancies (reviewed in Look, 1997). In solid tumors, evidence for the involvement of homeobox-containing genes is also extensive; for example, *cdx2* expression is decreased in colon carcinoma relative to normal colon (Mallo et al., 1997), and heterozygous deletion of *cdx2* results in adenomatous polyposis in mice (Chawengsaksophak et al., 1997). In alveolar rhabdomyosarcoma, activation of *PAX3* or *PAX7*, by fusion with the transactivation domain of *FKHR*, is a common event in tumorigenesis (Barr, 1999). In this context, it is tempting to speculate what possible role *BARX2* might play in MTC. As mentioned earlier, heterogeneous or absent production of calcitonin is associated with poor prognosis in MTC. *BARX2* may participate in maintenance of differentiation of thyroid C-cells in both the normal and neoplastic state, and that one marker of this differentiation is expression of the calcitonin gene. Loss of *BARX2*-mediated differentiation may result in loss of expression of the calcitonin marker, as well as other *BARX2* target genes; such changes may result in MTC tumor progression.

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References

Baldwin, C.T., Hoth, C.F., Amos, J.A., da Silva, E.O., Milunsky, A., 1992. An exonic in the HuP2 paired domain gene causes Waardenburg's syndrome. Nature 355, 637–638.

Ball, D.W., 1996. Clinical manifestations of multiple endocrine neoplasia type 2. In: Nelkin, B.B. (Ed.), Genetic Mechanisms in Multiple Endocrine Neoplasia Type 2. R.G. Landes, San Antonio, TX, pp. 1–20.

Barr, F.G., 1999. The role of chimeric paired box transcription factors in the pathogenesis of pediatric rhabdomysarcoma. Cancer Res. 59, 1711s–1715s.

Bradford, A.P., Conrad, K.E., Wasylyk, C., Wasylyk, B., Gutierrez-Hartmann, A., 1995. Functional interaction of c-Ets-1 and GHF-1/Pit-1 mediates Ras activation of pituitary-specific gene expression: mapping of the essential c-Ets-1 domain. Mol. Cell. Biol. 15, 2849–2857.

Carson, E.B., McMahon, M., Baylin, S.B., Nelkin, B.D., 1995. Ret gene silencing is associated with Raf-1-induced medullary thyroid carcinoma cell differentiation. Cancer Res. 55, 2048–2052.

Chawengsaksophak, K., James, R., Hammond, V.E., Kontgen, F., Beck, F., 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature 386, 84–87.

Clandinin, T.R., Katz, W.S., Sternberg, P.W., 1997. *Caenorhabditis elegans* HOM-C genes regulate the response of vulval precursor cells to inductive signal. Dev. Biol. 182, 150–161.

Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J., Weissenbach, J.A., 1996. A comprehensive genetic map of the human genome based on 5264 microsatellites. Nature 380, 152–154.

Foerst-Potts, L., Sadler, T.W., 1997. Disruption of Msx-1 and Msx-2 reveals roles for these genes in craniofacial, eye, and axial development. Dev. Dynam. 209, 70–84.

Hjalt, T.A., Murray, J.C., 1999. The human *BARX2* gene: genomic structure, chromosomal localization, and single nucleotide polymorphisms. Genomics 62, 456–459.

Jabs, E.W., Muller, U., Li, X., Ma, L., Luo, W., Haworth, I.S., Klisak, I., Sparkes, R., Warman, M.L., Mulliken, J.B., Snead, M.L., Maxson, R., 1993. A mutation in the homeodomain of the human *MSX2* gene in a family affected with autosomal dominant craniosynostosis. Cell 75, 443–450.

Jabs, E.W., Li, X., Scott, A.F., Meyers, G., Chen, W., Eccles, M., Mao, J.I., Charnas, L.R., Jackson, C.E., Jaye, M., 1994. Jackson–Weiss and Crouzon syndromes are allelic with mutations in fibroblast growth factor receptor 2. Nat. Genet. 8, 275–279.

Jacobsen, P., Hauge, M., Henningsen, K., Hobolth, N., Mikkelsen, M., Philip, J., 1973. An (11;21) translocation in four generations with chromosome 11 abnormalities in the offspring. A clinical, cytogenetical, and gene marker study. Human Hered. 23, 568–585.

Jones, C., Penny, L., Mattina, T., Yu, S., Baker, E., Voullaire, L., Langdon, W.Y., Sutherland, G.R., Richards, R.I., Tunnacliffe, A., 1995. Association of a chromosome deletion syndrome with a fragile site within the proto-oncogene CBL2. Nature 376, 145–149.

Jones, F.S., Kioussi, C., Copertino, D.W., Kallunki, P., Holst, B.D., Edelman, G.M., 1997. *Barx2*, a new homeobox gene of the Bar class, is expressed in neural and craniofacial structures during development. Proc. Natl. Acad. Sci. USA 94, 2632–2637.

Knoepfler, P.S., Calvo, K.R., Chen, H., Antonarakis, S.E., Kamps, M.P., 1997. Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a–Pbx1. Proc. Natl. Acad. Sci. USA 94, 14 553–14 558.

Lengauer, C., Henn, T., Onyango, P., Francis, F., Lehrach, H., Weith, A., 1994. Large-scale isolation of human 1p36-specific P1 clones and their use for fluorescence in situ hybridization. Genet. Anal. Tech. Appl. 11, 140–147.

Lewanda, A.F., Morsey, S., Reid, C.S., Jabs, E.W., 1995. Two craniosynostotic patients with 11q deletions, and review of 48 cases. Am. J. Med. Genet. 59, 193–198.

Liu, Y.H., Kundu, R., Wu, L., Luo, W., Ignelzi, M.A., Snead, M.L., Maxson, R.E., 1995. Premature suture closure and ectopic cranial bone in mice expressing *Msx2* transgenes in the developing skull. Proc. Natl. Acad. Sci. USA 92, 6137–6141.

Look, A.T., 1997. Oncogenic transcription factors in the human acute leukemias. Science 278, 1059–1064.

Mallo, G.V., Rechreche, H., Frigerio, J.M., Rocha, D., Zweibaum, A., Lacasa, M., Jordan, B.R., Dusetti, N.J., Daghorn, J.C., Iovanna, J.L., 1997. Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Downregulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. Int. J. Cancer 74, 35–44.

Mark, M., Rijli, F.M., Chambon, P., 1997. Homeobox genes in embryogenesis and pathogenesis. Pediatr. Res. 42, 421–429.

Nakagawa, T., Mabry, M., de Bustros, A., Ihle, J.N., Nelkin, B.D., Baylin, S.B., 1987. Introduction of v-Ha-ras oncogene induces differentiation of cultured human medullary thyroid carcinoma cells. Proc. Natl. Acad. Sci. USA 84, 5923–5927.

Nakamura, H., 1982. Mesenchymal derivatives from the neural crest. Arch. Histol. Jpn. 45, 127–138.

Neilson, K.M., Friesel, R.E., 1995. Constitutive activation of fibroblast growth factor receptor-2 by a point mutation associated with Crouzon syndrome. J. Biol. Chem. 270, 26037–26040.

Penny, L.A., dell'Aquila, M., Jones, M.C., Bergoffen, J.A., Cunniff, C., Fryns, J.P., Grace, E., Graham, J.M., Kousseff, B., Mattina, T., Syme, J., Voullaire, L., Zelante, L., Zengerhain, J., Jones, O.W., Evans, G.A., 1995. Clinical and molecular characterization of patients with distal 11q deletions. Am. J. Human Genet. 56, 676–683.

Semina, E.V., Reiter, R., Leysens, N.J., Alward, W.L., Small, K.W., Datson, N.A., Siegel-Bartelt, J., Bierke-Nelson, D., Bitoun, P., Zabel, B.U., Carey, J.C., Murray, J.C., 1996. Clinical and molecular characterization of patients with distal 11q deletions. Am. J. Human Genet. 14, 392–399.

Shapiro, M.B., Senapathy, P., 1987. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 15, 7155–7174.

Takahashi, C., Akiyama, N., Matsuzaki, T., Takai, S., Kitayama, H., Noda, M., 1996. Characterization of a human MSX-2 cDNA and its fragment isolated as a transformation suppressor gene against v-Ki-ras oncogene. Oncogene 12, 2137–2146.

Thiagalingam, A., de Bustros, A., Borges, M., Jasti, R., Compton, D., Diamond, L., Mabry, M., Ball, D.W., Baylin, S.B., Nelkin, B.D., 1996. RREB-1, a novel zinc finger protein, is involved in the differentiation response to Ras in human medullary thyroid carcinomas. Mol. Cell. Biol. 16, 5335–5345.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tunnacliffe, A., Jones, C., Le Paslier, D., Todd, R., Cherif, D., Birdsall, M., Devenish, L., Yousry, C., Cotter, F.E., James, M.R., 1999. Localization of Jacobsen syndrome breakpoints on a 40 Mb physical map of distal chromosome 11q. Genome Res. 9, 44–52.

Vinson, C.R., LaMarco, K.L., Johnson, P.F., Landschulz, W.H., McKnight, S.L., 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. Genes Dev. 2, 801–806.

Winograd, J., Reilly, M.P., Roe, R., Lutz, J., Laughner, E., Xu, X., Hu, L., Asakura, T., van der Kolk, C., Strandberg, J.D., Semenza, G.L., 1997. Perinatal lethality and multiple craniofacial malformations in MSX2 transgenic mice. Human Mol. Genet. 6, 369–379.

Wright, W.E., Binder, M., Funk, W., 1991. Cyclic amplification and selection of targets (CASTing) for the myogenin consensus binding site. Mol. Cell. Biol. 11, 4104–4110.

Wu, L., Wu, H., Ma, L., Sangiorgi, F., Wu, N., Bell, J.R., Lyons, G.E., Maxson, R., 1997. Miz1, a novel zinc finger transcription factor that interacts with Msx2 and enhances its affinity for DNA. Mech. Dev. 65, 3–17.