Cloning and chromosomal localization of the human \textit{BARX2} homeobox protein gene

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Abstract

The human \textit{BARX2} gene encodes a homeodomain-containing protein of 254 amino acids, which binds optimally to the DNA consensus sequence YYTAATGRTTTTY. \textit{BARX2} is highly expressed in adult salivary gland and is expressed at lower levels in other tissues, including mammary gland, kidney, and placenta. The \textit{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 \textit{barx2} in craniofacial development, suggests that \textit{BARX2} may be causally involved in the craniofacial abnormalities in Jacobsen syndrome. © 2000 Elsevier Science B.V. All rights reserved.

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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. 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-
2.2. Northern and dot blot hybridization and TT:

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′-ATCCATTTCCATCAATGACCTCAATGCA-3′), labeled by nick translation with α-32P-dCTP. Screening of 106 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′-GA-GATATTAGAATTTCACTC-N23-GGTACATATACGAGT-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 32P-end labeled oligonucleotide 7−8 (5′-ATCCATTTCCATCAATGACCTCAATGCA-3′) or oligonucleotide Barx2 cons (5′-GATATTAGAATTTCACTC-N23-GGTACATATACGAGT-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′-GGCCGTATCGTCTCTCGG-3′ and 5′-GCTGCTGTAAGATGTTGCT-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 \( \text{BARX2} \) by PCR screening of the RPCI-1 library (Genome Systems). A subclone of the PAC clone was partially sequenced to confirm that it contained \( \text{BARX2} \).

(ii) FISH. DNA of the \( \text{BARX2} \) PAC clone was labeled with biotin-16-dUTP by nick translation.

3. Results

3.1. DNA affinity cloning of \( \text{BARX2} \)

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 \( \lambda gt11 \) 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 226 bp product from exon 2 (bases 296-521 in Fig. 2A). These primers were also used to obtain a PAC clone of \( \text{BARX2} \) by PCR screening of the RPCI-1 library (Genome Systems). A subclone of the PAC clone was partially sequenced to confirm that it contained \( \text{BARX2} \).

2.6. Genomic sequencing

A \( \text{BARX2} \) PAC clone, containing exons 1-4 of the \( \text{BARX2} \) 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°C for 30 min, and 72°C for 30 min) were done in a 50 \( \mu \)l 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′-CTCAGATGAGCCAGG-3′ and 5′-GATC- GCAGACACGCACCTACACG-3′ (267 bp product); exon 2, 5′-TCGCTGTTCTCCACACCG-3′ and 5′-GAGGTTGTTGACTCCGCC-3′ (493 bp product); exon 3, 5′-AAGAN-

AAAGGTCTTGAGGAGACCTCGT-3′ and 5′-ATC- CAAACAGCTTCCCGCAAGCC-3′ (378 bp product); and 5′-ATGCAACG-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.

![Fig. 1. Binding of lacZ-BARX2 to sequences from the human calcitoni- nin gene ras/raf responsive element. The \( \lambda gt11 \) phase containing a partial \( \text{BARX2} \) 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.](image-url)
Fig. 2. (A) Sequence of BARX2 DNA. The nucleotide sequence of BARX2 exhibits 86% homology with murine barx2. 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
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 contains an octamer sequence, 5'-TTAATGRTTT-3', typical of homeobox protein binding sites, and this octamer is flanked by pyrimidine-rich bases. We designed an oligonucleotide, Barx2 cons (GATCTTTCTT-3') 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 Northern blots of human poly A+ RNAs, we found that expression of BARX2 was tissue specific; the highest expression was seen in adult salivary gland mRNA. The consensus BARX2 binding sequence appears to be YYTAATGRTTTY (Fig. 3A), which is closely related to the BARX2 binding sequences in the NCAM and NCAM-L1 genes (Jones et al., 1997), but somewhat more divergent from the sequence in the human calcitonin gene (NCAM-L1) genes (Jones et al., 1997), but somewhat more divergent from the sequence in the human calcitonin gene promoter (sequence contains an octamer sequence, 5'-TTAATGRTTT-3', typical of homeobox protein binding sites, and this octamer is flanked by pyrimidine-rich bases. We designed an oligonucleotide, Barx2 cons (GATCTTTCTT-3') 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).

3.4. 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
Fig. 3. Specificity of binding of BARX2. (A) Selection of high a

sequence 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-pter (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
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 (Cladimir 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). 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; Di 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.
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 (Tunnicliffe 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’.

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 (Neelson and Friedel, 1995). Boston-type craniofocal dysostosis (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 craniofocal abnormalities (Liu et al., 1995; Foerst-Potts and Sadler, 1997; Winogrod et al., 1997). Speculatively, BARX2 may also function as a necessary downstream effector to mediate ras/raf signals in normal craniofocal 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, cd2 expression is decreased in colon carcinoma relative to normal colon (Mallo et al., 1997), and heterozygous deletion of cd2 results in adenosomatous polyposis in mice (Chawengsaksophak et al., 1997). In alveolar rhabdomyosarcoma, activation of PAX3 or PAX7, by fusion with the transcriptional factor FKHR, is a common event in tumorigenesis (Barr, 1999). In this context, it is tempting to speculate that 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 marker, as well as other BARX2 target genes; such changes may result in MTC tumor progression.

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