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Mutations in the human *RAX* homeobox gene in a patient with anophthalmia and sclerocornea

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Anophthalmia and microphthalmia are among the most common ocular birth defects and a significant cause of congenital blindness. The etiology of anophthalmia and microphthalmia is diverse, with multiple genetic mutations associated with each of these conditions, along with potential environmental causes. Based on findings that mutations in the *Rx/Rax* homeobox genes in mice and fish lead to defects in retinal development and result in animal models of anophthalmia, we screened 75 individuals with anophthalmia and/or microphthalmia for mutations in the human *RAX* gene. We identified a single proband from this population who is a compound heterozygote for mutations in the *RAX* gene. This individual carries a truncated allele (Q147X) and a missense mutation (R192Q), both within the DNA-binding homeodomain of the RAX protein, and we have characterized the biochemical properties of these mutations *in vitro*. Parents and grandparents of the proband were found to be carriers without visible ocular defects, consistent with an autosomal recessive inheritance pattern. This is the first report of genetic mutations in the human *RAX* gene.

INTRODUCTION

Microphthalmia, anophthalmia and coloboma (MAC) represent a spectrum of structural eye malformations that result from developmental defects during ocular organogenesis at a rate of 1.9–3.5/10 000 live births (1,2). The combined occurrence rate for anophthalmia and microphthalmia is 1/10 000 births (3). These conditions are genetically heterogeneous, with potentially overlapping phenotypes resulting from mutations in any of a number of genes and varying phenotypes resulting from different mutations in a single gene. Mutations in several genes have been isolated from patients with both syndromic and non-syndromic anophthalmia. Mutations in at least two genes, *SOX2* and *PAX6*, cause anophthalmia in humans (4–6), although in the case of *PAX6*, heterozygous mutations can cause aniridia, Peter's anomaly, cataracts, corneal opacification or glaucoma (reviewed in 7) and mutations in both PAX6 alleles are necessary for anophthalmia (5,6). In addition, deletion of 14q22.3–23, which includes the *SIX6* gene, may lead to anophthalmia through haploinsufficiency of *SIX6* (8), although a *Six6* targeted mutation in mouse gives only a microphthalmia phenotype when both copies are mutated (9). Mutations in the *CHX10* (recessive) and *SHH* (dominant) genes correlate with cases of human microphthalmia (10,11). Another related defect is septo-optic dysplasia, where mutations in the *HESX1* gene have been identified (12,13). Finally, mutations in the *PAX2* and *SHH* genes have been found in patients with coloboma (11,14,15). In each syndrome, mutations in any one gene represent a small minority of the samples analyzed, suggesting that mutations in multiple genes are potentially

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responsible for the overlapping phenotypic effects seen in the MAC spectrum.

The *Rax* homeobox gene (also known as *Rx*, with the human gene designated *RAX* or *RX*) is expressed very early in retinal development and appears to direct the initial specification of retinal cell fate and the subsequent proliferation of retinal stem cells (16–24). We have previously demonstrated that the *Rax* homeobox gene is crucial for the proper formation of the optic vesicle during early mouse development and that deletion of *Rax* gene function in the mouse leads to non-viable anophthalmia (18,20). In addition, Rax gene family mutations have been identified in the zebrafish *Rx3* gene in the *chokh* mutant (23), in the medaka fish *Rx3* gene in the *eyeless* mutant (22) and in the mouse *Rax* gene in the *eyeless* mutation observed in the ZRDCT strain (21,25). The mouse mutant phenotypes present in the *Rax* knockout and *eyeless* mutant strains correlate well with defects seen in some patients with anophthalmia (26–28). To examine whether mutations in the human *RAX* gene are responsible for cases of anophthalmia and/or microphthalmia, we performed a molecular characterization of the human *RAX* locus and developed a screen for mutations in the RAX protein-coding region, which we employed to study an anophthalmia/microphthalmia patient population. Using this screen, we identified a bilaterally affected proband with right anophthalmia and left sclerocornea who carries two inherited abnormal alleles of the *RAX* gene. Mutations found in this proband affect DNA-binding affinity and nuclear localization. Together with the mutation data in animal models of anophthalmia, these findings demonstrate a crucial role for the *RAX* homeobox gene in human eye development.

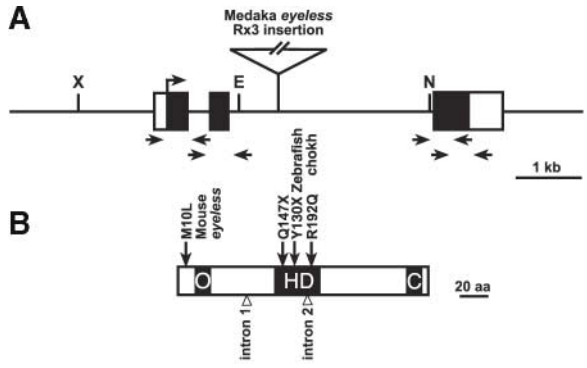

Figure 1. Position of *RAX* locus mutations. (**A**) Schematic of the human *RAX* locus containing three exons (boxes) that code for the RAX protein (black boxes, coding region, white boxes, untranslated region). The position of a >13 kb insertion in the *Rx3* gene that is responsible for the medaka *eyeless* mutant (22) is indicated in the homologous position of human *RAX* intron 2. Arrows below the line represent the position of primers used to amplify genomic DNA. E = *Eco*RI, N = *Not*I and X = *Xho*I restriction endonucleases. (**B**) Schematic of the RAX protein with the position of the Q147X and R192Q mutations indicated, along with that of the M10L mutation that has been correlated with the mouse *eyeless* mutation in the ZRDCT strain (21) and the zebrafish *chokh* mutation in the *Rx3* gene (23). Like other Rx/RAX proteins, the human RAX protein contains three highly conserved domains that are common in *paired*-like homeodomain proteins—the octapeptide (O), the homeodomain (HD), and the C-terminal domain (C; also known as the OAR or *paired*-tail domain). The positions of intron–exon splice junctions for the mature RNA are indicated.

RESULTS

To test whether the *RAX* gene has an important role in human ocular development and the conditions of anophthalmia and/or microphthalmia, we first isolated human genomic sequences encoding the *RAX* gene. Three overlapping genomic clones were obtained, representing approximately 22 kb of the *RAX* locus. Three regions within the human *RAX* locus cross-hybridized with mouse *Rax* cDNA clones. The genomic structure of the human *RAX* locus (Fig. 1A) is nearly identical to that found in mouse, including three coding exons and the position and relative size of the two introns. The sequence of these homologous regions revealed a protein-coding domain that is 86% identical to the predicted mouse Rax protein. The putative functional domains, such as the octapeptide, homeodomain, nuclear localization signal and the C-terminal domain (also called the OAR or *paired*-tail domain) are 100% identical to the mouse protein sequence (Fig. 1B; GenBank accession number NM 013833). The predicted protein sequence from the genomic clones is >99% identical to the previously characterized human *RAX* cDNA sequence (GenBank accession number AF 115392) (29), with a difference at amino acid 107 where glycine is found instead of tryptophan.

To determine whether mutations in the human *RAX* gene are correlated with defects found within the MAC spectrum of disease, we screened 75 patients who are either unilaterally or bilaterally affected with anophthalmia and/or microphthalmia using PCR-based genomic sequencing of the three *RAX* gene exons, along with the intron–exon borders. Two independent mutations were identified in a single proband within this patient population. The mutations were not observed in the remaining 74 patients or in our control population of 55 individuals with normal eye development, whereas two prevalent polymorphisms were detected in both patients and controls. The first of these polymorphisms occurs in exon 1 at amino acid 44, where both A and C are found in the third position of the GA codon, encoding either glutamic acid or aspartic acid (E44/D44), respectively. Glutamic acid is also present at this position in the mouse Rx protein, giving further support to the neutral effect of this polymorphism. The second polymorphism is found in exon 3 at amino acid 294 with both A and G occurring in the third codon position, and causes no change in the glutamine (Q294Q).

The single proband with *RAX* gene mutations has clinical anophthalmia in the right orbit, with an ocular remnant observed at birth. The left eye has sclerocornea with persistent fetal vasculature and retinal detachment (Fig. 2A). A CT scan shows the extent of orbital defects (Fig. 2B). Sequence analysis from the DNA of the proband revealed a premature termination codon within exon 2 in one allele of the *RAX* gene (Fig. 3D). At this location, the patient carries a C>T nonsense mutation, changing a conserved glutamine at position 147 to a stop codon (Q147X) and truncating the protein within helix 1 of the homeodomain (Fig. 1B). The Q147X mutation eliminates helices 2 and 3 of the homeodomain, the nuclear localization signal, the Rx-domain and the C-terminal domain from the protein, and therefore would be expected to eliminate RAX function completely (see below). This nonsense mutation also destroys a *Pvu*II restriction endonuclease site, making this

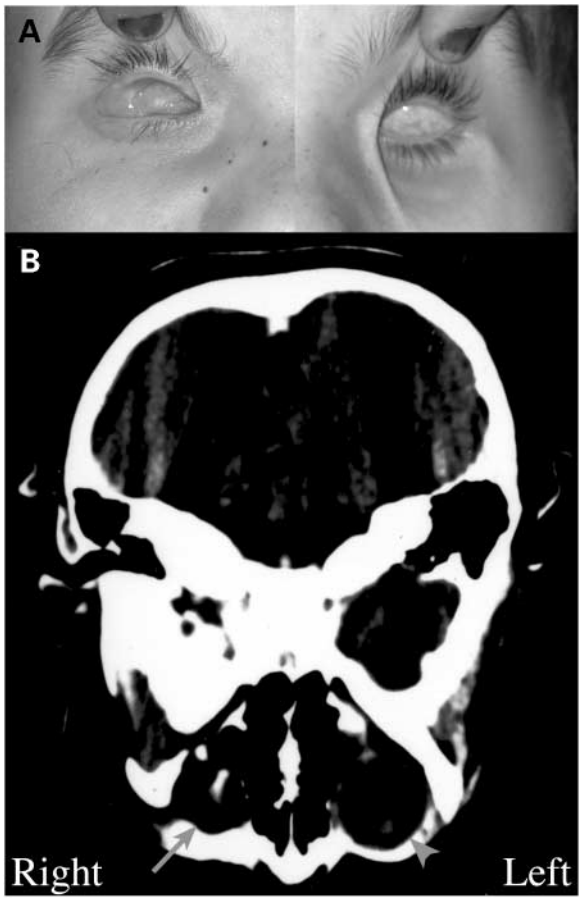

Figure 2. Ocular phenotype of proband. (**A**) Orbits of proband, showing absence of ocular tissue OD (his right) and sclerocornea OS (his left). Note that the tissue seen in the right orbit is a dermal fat graft placed at 2 years of age. (**B**) CT scan of proband showing anophthalmic orbit (gray arrow) and other orbit (gray arrowhead). Note that the plane of the scan suggests bony defects that are not present in the patient.

enzyme digestion a diagnostic screen for the mutation (Fig. 3B). Sequence analysis and *Pvu*II digestion of DNA samples from family members identified the father and paternal grandmother as heterozygous carriers of this allele (Fig. 3A and B). They both report normal ocular development and vision, suggesting that a single functional copy of the *RAX* gene is sufficient to direct normal eye development. This finding is consistent with our mouse *Rax*-mutation model (18).

The proband was found to carry a second mutation within exon 3 on the other allele of the *RAX* gene (Fig. 3C and E). The G>A missense mutation changes a highly conserved arginine at position 192 to glutamine (R192Q). Genetic analysis revealed that the mother and maternal grandfather are heterozygous carriers of this allele (Fig. 3A and C). Both possess normal vision and ocular development, also suggesting that a single copy of the R192Q mutation in the presence of a normal *RAX* allele is not sufficient to cause abnormal ocular development.

The R192Q mutation occurs in a region of the RAX protein that acts as both a nuclear localization signal and as a DNA-binding domain; a second nuclear localization signal exists in the amino-terminal portion of the homeodomain (30,31). In order to determine the mechanism by which this allele caused a RAX-mediated defect in ocular development, we compared each of the mutant proteins to the wild-type RAX protein for their ability to localize to the nucleus and to bind DNA *in vitro.* For testing the subcellular localization of the mutant proteins, the R192Q and Q147X mutations were generated in the context of the wild-type *RAX* cDNA clone, and mutant and wild-type clones were fused with an amino-terminal FLAG-epitope tag to allow protein detection. Each plasmid was transiently transfected into Cos-7 cells and the location of the recombinant RAX protein was examined by immunofluorescence using an anti-FLAG antibody and propidium iodide as a nuclear stain. The R192Q mutant protein behaves indistinguishably from that of wild-type RAX protein, with both proteins restricted to the nucleus (Fig. 4A and B). Therefore, it appears that the R192Q mutation does not affect the ability of the mutant RAX protein to enter the nucleus. As expected from its truncation upstream of the second nuclear localization domain, the Q147X mutant protein is excluded from the nucleus in a majority of the transfected cells (Fig. 4C).

Because arginine 192 corresponds to amino acid 57 of the homeodomain, falling within DNA-recognition helix 3, we sought to determine whether the R192Q mutation influenced DNA binding. A positively charged amino acid at this position is extremely well conserved, with 98% of homeodomain proteins carrying either arginine (R) or lysine (K) at this position. The crystal structure of the *Paired* homeodomain protein reveals that R57 makes electrostatic contacts with the phosphate backbone of the DNA recognition sequence for homeodomain binding (32), as does the analogous K57 of the *engrailed* homeodomain (30). Based on these studies, a change in the charge of R192 of the RAX protein is predicted to reduce its DNA-binding affinity. Similar electrostatic changes at arginine 53 of the homeodomain in the *CHX10* and *HESX1* genes disrupt the DNA-binding activity of these proteins in microphthalmia and septo-optic dysplasia patients, respectively (10,13), and in a cataract-causing mutation of the bHLH protein, L-Maf (33).

To test the DNA-binding ability of the R192Q and Q147X mutations, wild-type and mutant FLAG-RAX proteins were synthesized *in vitro* and used in electrophoretic mobility shift assays (Fig. 5). To assay RAX binding, we used radioactive oligonucleotides containing the photoreceptor-conserved element as probe (PCE I or Ret1) (34), which acts as a RAX binding target (29). Unlabeled oligonucleotides (PCE I and the unrelated COUP-TF binding site) were used to test the specificity of RAX binding. We found that unlabeled COUP-TF oligonucleotides failed to compete with RAX protein for the PCE I probe, while unlabeled PCE I oligonucleotides do compete with probe, showing that binding activity in RAX-containing lysates is specific for the PCE I binding site. When R192Q RAX protein lysates are used in the binding assay, a 10-fold reduction in the amount of retarded probe is observed when compared to wild-type RAX protein, suggesting that the R192Q mutation contributes to the phenotype of the proband by reducing the occupancy of RAX binding sites (Fig. 5). As expected from the position of the truncation, Q147X RAX protein fails to show DNA-binding activity.

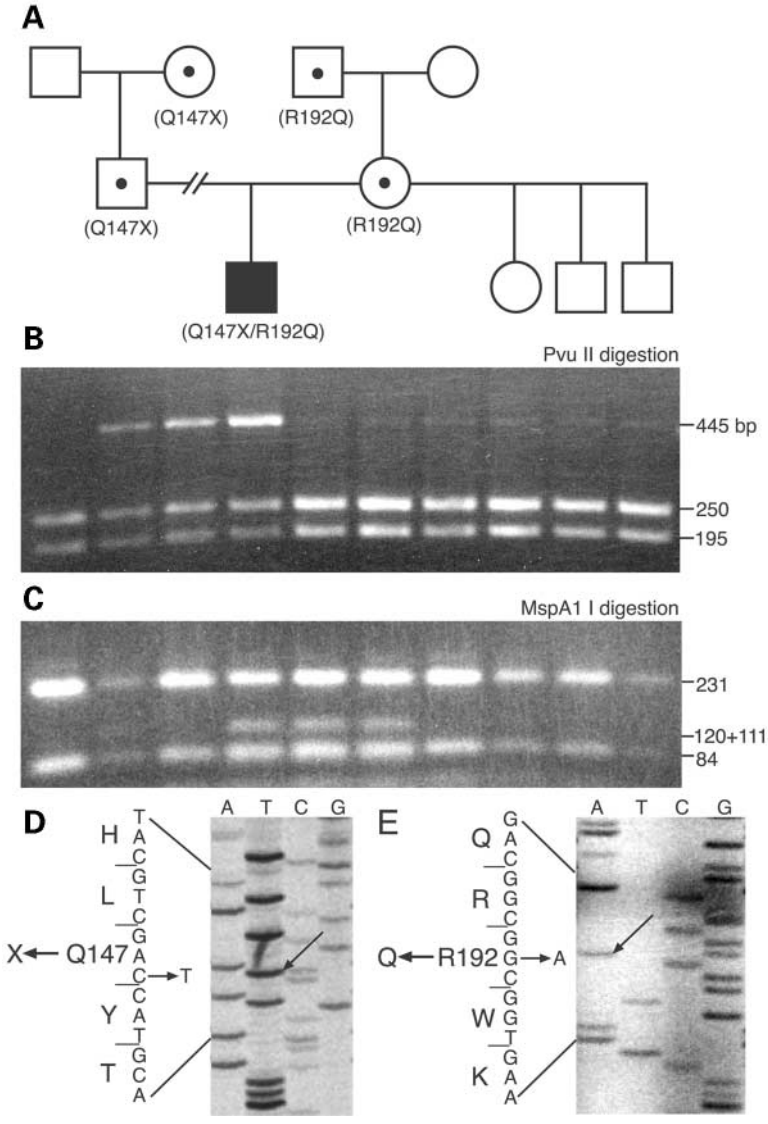

Figure 3. Pedigree and mutation detection in proband's family. (**A**) Pedigree showing recessive inheritance pattern of Q147X (paternal lineage) and R192Q (maternal lineage) mutations. The proband is a compound heterozygote for the two mutations. (**B**) *Pvu*II-digestion of PCR-amplified exon 2 in the pedigree. The Q147X mutation causes the loss of the *Pvu*II site, (C>T)AGCTG. The uncut band is 445 bp, while *Pvu*II digestion cuts this band into 250 and 195 bp bands. Note that each digestion sample is positioned under the appropriate individual in the pedigree shown in (A). (**C**) *MspA1*I-digestion of PCR-amplified exon 3 in the pedigree. The R192Q mutation introduces an *MspA1*I site, C(C>A)GCGG. The normal pattern of *MspA1*I digestion gives bands of 231 and 84 bp. The R192Q mutation causes the 231 bp band to be cleaved into 111 and 120 bp bands. (**D**) Sequencing gel of DNA sample from proband, showing heterozygosity at amino acid 147. The DNA sequence and accompanying translation are shown to the left. Arrow denotes the aberrant band. (**E**) Sequencing gel of DNA sample from proband, showing heterozygosity at amino acid 192. Arrow again shows the aberrant band.

DISCUSSION

Through our mutational screen of patients with anophthalmia and microphthalmia, we have identified *RAX* gene mutations in a proband that were not found in the other 74 patients analyzed or in the 55 control samples. The proband is a compound heterozygote, with mutations in both alleles of the *RAX* gene. This finding is consistent with the autosomal recessive inheritance seen in some familial cases of anophthalmia and microphthalmia (35–37). Given the similarity to the mouse *Rax* mutant phenotypes (18,21), we conclude that the net effect of these two mutations was sufficient to cause the anophthalmia/sclerocornea phenotype observed in the proband.

The Q147X mutation causes premature truncation of the RAX protein and removes the ability of the mutant protein to interact with DNA and to localize properly to the nucleus. Despite the premature termination codon position matching the criteria for nonsense-mediated decay (38), we observe significant protein accumulation in Cos-7 cells following transfection with the Q147X FLAG-*RAX* plasmid (Fig. 4C). This finding suggests either that the *RAX* gene is not subject to nonsense-mediated decay, at least in this cellular context, or the quantity of *RAX* RNA produced by the CMV early

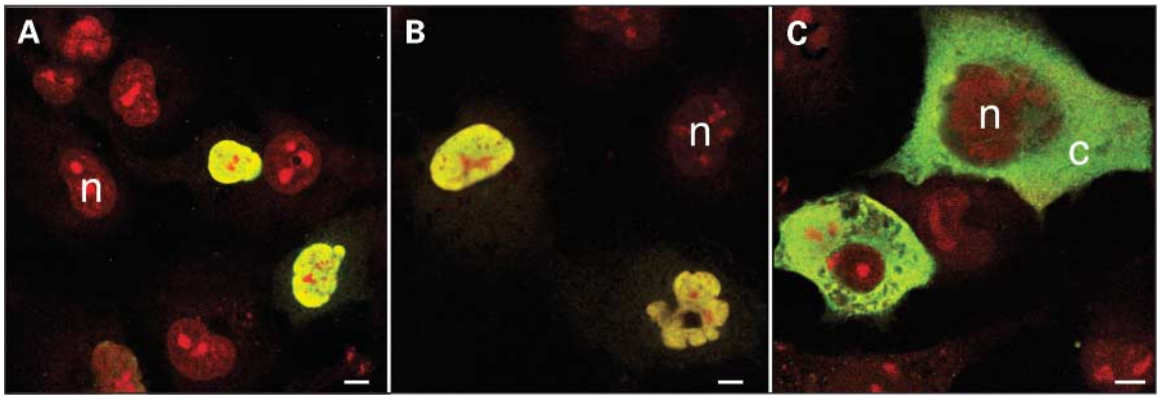

Figure 4. Cellular localization of normal and mutant RAX proteins. Plasmids containing FLAG-tagged *RAX*, R192Q *RAX* and Q147X *RAX* cDNAs were transiently transfected into Cos-7 cells. After a day in culture, the cells were fixed and probed with anti-FLAG antibody and AlexaFluor 488 goat anti-mouse antibody (green) and propidium iodide (red). (**A**) In transfected cells, wild-type RAX is localized to the nucleus, with colocalization of the two dyes appearing yellow. (**B**) The R192Q RAX protein also localizes to the nucleus. (**C**) The Q147X mutation causes the altered RAX protein to be excluded from the nucleus in most cells (n = nucleus, c = cytoplasm). Scale bars in each panel represent 10 μm.

promoter exceeds the capacity of the nonsense-mediated decay machinery. Our results show that the R192Q mutation causes a severe reduction in the DNA-binding ability of the mutant RAX protein without affecting subcellular targeting.

Multiple lines of evidence suggest that genes in the *Rx/Rax/RAX* family function as transcriptional activators of retinal cell fate, and are able to bind the PCE I site, possibly in conjuction with Pax6 and other homeodomain proteins, to stimulate transcription (29,39,40). A complete loss of Rax function in mice blocks the earliest stages of ocular development, but also leads to organogenic defects in the ventral forebrain that presumably cause the lethal phenotype of the null mice (18). Based on this mutant phenotype in mice, it is probable that the limited remaining DNA-binding activity of the R192Q RAX protein is sufficient to allow for both the overall viability and partial ocular development observed in the proband.

The normal eye formation of the proband's family members and mice heterozygous for *RAX* gene mutations suggests that 50% or greater of normal RAX protein activity is sufficient to drive proper ocular development. Reductions below 50% of normal RAX activity are likely to have a graded effect, with the most severe phenotypes associated with significant functional defects in the RAX protein. As an example, the *eyeless* mutation in ZRDCT mice is caused in part by an M10L mutation in the *Rax* gene. This mutation affects the overall level of Rax protein synthesis by disrupting a highly conserved, alternative translational start site for the Rax protein (21). Collectively, the defects associated with loss or reduction of Rax activity would suggest that a threshold limit of functional RAX protein is necessary to stimulate and/or maintain ocular development. In the proband, the 10-fold reduction in DNA-binding activity from the R192Q RAX protein, when coupled with the presumed loss-of-function Q147X allele, is apparently not sufficient to reach or maintain that threshold.

The low frequency with which we are able to identify *RAX* gene mutations in our anophthalmia/microphthalmia patient population (2.4% of anophthalmia patients; 1.3% of all patients) suggests that other genetic or epigenetic events are the primary cause(s) for these conditions. In analyzing other genes that have been screened for mutations in the MAC patient population, a similar low frequency of mutations is observed. The most prevalent among these gene mutations is the *SOX2* gene, with mutations present in 11.4% of anophthalmia patients and 3.9% of patients with ocular anomalies within the MAC spectrum (4). Other important genetic factors include the *CHX10* gene (1.7% of non-syndromic microphthalmia; 0.4% of ocular anomalies) (2,10), the *PAX6* gene (80–90% of aniridia, but only 1.6% of anophthalmia) (2,5–7), the *PAX2* gene (1.0% of colobomas) (41), the *SHH* gene (0.9% of ocular anomalies) (11), and potentially the *SIX3* gene (although 0% of ocular anomalies screened) (2). Thus, multiple genetic loci are implicated in the MAC spectrum, with the potential for several other genes to be identified as additional low-frequency causes or mutations in an as yet unidentified gene that could account for the majority of MAC cases.

Given that no gross morphological deficits are observed in the central nervous system of the proband compared with those seen in the development of ocular tissue, we conclude that the levels of RAX protein are less crucial for initiation and proliferation of the ventral forebrain than for formation of the eye. This conclusion is consistent with our recent findings in the mouse *Rax* deletion model, where markers of ventral forebrain are still active in the mutant, despite the lack of optic vesicle formation (E.A. Kozhemyakina and P.H Mathers, unpublished data). Alternatively, the establishment of optic vesicles in the patient could act to promote the development of the ventral forebrain through the release of important growth factors, which would be missing in the *Rax* deletion model. Conditional deletion of the mouse *Rax* gene in the optic vesicle and ventral forebrain separately may help to answer these questions. Based on our current identification of *RAX* gene mutations in a patient with anophthalmia and sclerocornea and published reports of *Rax* mutations leading to eyeless phenotypes in mouse, zebrafish and medaka fish, we conclude that the *RAX*/*Rax* gene family is crucial for the proper establishment of retinal cell fate and is required for optic vesicle formation in a wide range of vertebrates.

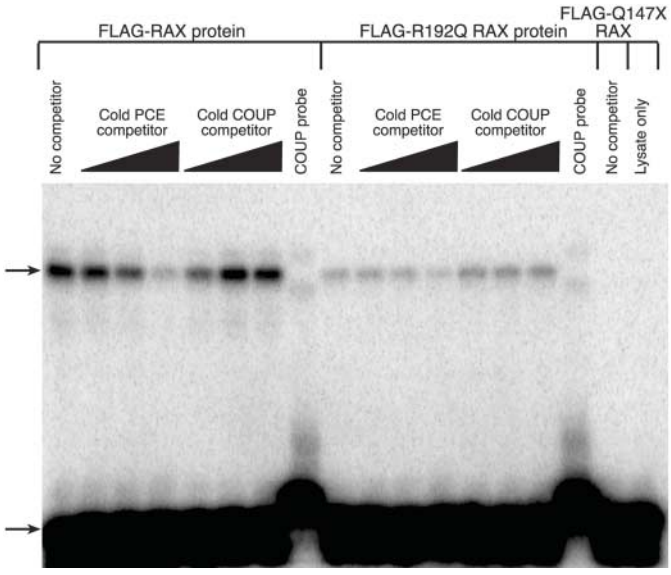

Figure 5. Electrophoretic mobility shift assay with normal and mutant RAX proteins. Lysates containing FLAG-tagged RAX, R192Q RAX and Q147X RAX proteins were incubated with radioactively labeled oligonucleotides containing a RAX-binding site (PCE I) or an unrelated binding site (COUP-TF). The specificity of RAX binding was determined by competing the interaction with excess unlabeled oligonucleotides at 4-, 100- and 1000-fold molar excess (designated by increasing size of the triangle under the appropriate competitor). The upper arrow denotes the position of the protein-retarded probe, while the lower arrow represents unbound probe. Note that equal concentrations of lysate were loaded per lane, with equal concentrations of RAX proteins confirmed by western blot analysis using an anti-FLAG antibody (data not shown).

MATERIALS AND METHODS

Clinical samples

All work performed with human subjects received prior informed consent and/or assent, in compliance with the Institutional Review Boards of West Virginia University and Albert Einstein Medical Center. Buccal mucosa or saliva samples of individuals with anophthalmia and/or microphthalmia were obtained through the West Virginia University Department of Ophthalmology and the International Children's Anophthalmia Network (ican). Affected individuals were evaluated by their local ophthalmologist. Samples from 75 patients were screened for mutations in the human *RAX* gene. Of these patients, 26 of them were bilaterally affected with anophthalmia, 11 had microphthalmia in both eyes, seven were anophthalmic on one side and microphthalmic on the other, eight were anophthalmic on one side, and 23 were microphthalmic in only one eye. Saliva was collected from a group of 55 normal, ethnically matched, individuals as controls.

The proband is a 12-year-old male born to non-consanguinous parents. Shortly after birth the patient's eyes were noted to be abnormal. Otherwise, the head was of normal shape and size. On the right side, the eyelids were small and fused at the medial aspect (ankyloblepharon). There was no visible or palpable globe on the right side, although a conjunctival sac could be visualized. Ultrasound of the right orbit showed a very small, cystic remnant of a globe. The bony orbit was small on the right side. On the left side the eyelids were small, but normal in shape and contour. The globe was reported as small with sclerocornea, although axial length at 2 years measured 22 mm on CT scan. Ocular ultrasound showed persistent fetal vasculature (PHPV) (42) with a total retinal detachment. In addition to these ocular findings, EEG at 7 years of age showed abnormal slowing of background activity consistent with underlying cortical abnormality, and the patient was diagnosed as autistic. However, an MRI of the brain was completely within normal limits.

Isolation of human *RAX* genomic clones

Three independent human *RAX* clones were isolated from a λ phage genomic DNA library (Stratagene, La Jolla, CA, USA), using a human *RAX* 5′-RACE cDNA clone as probe (18). These overlapping clones represent approximately 22 kb from the *RAX* locus. Coding regions within the *RAX* locus were identified by cross-hybridization with mouse *Rax* cDNA clones (18), and fragments containing these coding regions were subcloned and sequenced. Alignment to the mouse *Rax* gene sequence was used to determine intron–exon junctions. During this research project, the human genome sequence became available, confirming our sequencing results.

Mutation screening

DNA from buccal mucosa samples was purified using the MasterAmp™ Buccal Swab DNA Extraction kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's protocol. DNA from saliva samples was purified using a Blood Amp kit (Qiagen Inc., Valencia, CA, USA). The RAX coding region and flanking intronic sequences were PCR-amplified with a 50 : 1 mixture of *Taq* DNA polymerase (Sigma, St Louis, MO, USA or Invitrogen, Carlsbad, CA, USA) and Deep Vent DNA polymerase (New England Biolabs, Boston, MA, USA) for 40–45 cycles from genomic DNA in separate reactions for each of the three exons. The primers for exon 1 were 5′-GGGCGCCCGAACGGCCTC and 5′-GCCTCTCCTCTCCGTCTCC. Primers for amplifying exon 2 were 5′-GGAGTGCATCTGACCCTCC and 5′-TGGCTGCAATTTGGGCCTCG. Primers for amplifying exon 3 were 5′-GAGCTGAACCGGCTCAGG and 5′-GGATCCCAAGACGTTCCCC. These same primers were also used for sequencing reactions, with the added internal primers 5′-AGCTGGCAGGCAGGCTCT and 5′-GCTGGAGTCCTGGCTCG used for exon 3 because of its length. Each fragment was gel-purified and used for direct sequencing using the SequiTherm EXCEL II DNA sequencing kit (Epicentre Technologies, Madison, WI, USA). Samples were analyzed by manual DNA sequencing. Anomalous bands were confirmed by resequencing both strands of the region in question. Mutations were also verified by restriction enzyme digestion. The Q147X mutation causes the loss of a Pvu II site, while the R192Q mutation leads to the introduction of a novel MspA1 I site. For the Q147X mutation, *RAX* exon 2 was amplified using 5′-TTTTGGGGAGTGCATCTGAC-3′ and 5′-CTGTGCCTCTCCCTT-GAGAC-3′ followed by purification and subsequent *Pvu*II-digestion. For the R192Q mutation, *RAX* exon 3 was amplified using 5′-CCTCCGCTGCTGCCCGA-3′ and 5′-AGCTGGCAGGCAGGCTCT-3′ followed by purification and subsequent MspA1 I-digestion. In addition to these mutations, prevalent

polymorphisms were identified in exon 1 at amino acid position 44 (D44E) and in exon 3 at amino acid position 294 (Q294Q).

Cell culture and DNA transfection

Cos-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). PCR-mediated site-directed mutagenesis was used to create the Q147X and R192Q mutations in a *RAX* cDNA clone (provided by Dr T. Shinohara). These modified and wild-type *RAX* genes were subcloned into the pCMV-Tag2B plasmid vector (Stratagene, La Jolla, CA, USA), which introduces a FLAG epitope tag at the amino-terminal end of the protein. Cos-7 cells grown on coverslips were transfected with these mutated and wild-type FLAG-*RAX* plasmids. Transient transfections were performed with Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Immunofluorescence

Twenty-four hours after transfection, Cos-7 cells were fixed in 4% paraformaldehyde for 25 min, washed with phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA, USA), equilibrated in 2 × SSC (Invitrogen, Carlsbad, CA, USA), treated with 100 µg/ml RNase A for 30 min and washed three times with 2 × SSC. After permeabilization with 0.15% saponin (w/v) for 1 h, cells were washed with PBS and incubated in blocking solution containing 1% (w/v) bovine serum albumin, 1% (w/v) goat serum for 1 h followed by incubation with 20 µg/ml anti-FLAG M2 antibody (Stratagene, La Jolla, CA, USA) overnight at 4°C. After washing three times with PBS + 0.1% Tween 20, cells were treated with 20 µg/ml AlexaFluor 488 goat anti-mouse antibody (Molecular Probes, Eugene, OR, USA) for 2 h. Cells were washed three times in PBS + 0.1% Tween 20, equilibrated with 2 × SSC and treated with 500 nM propidium iodide (Molecular Probes, Eugene, OR, USA), for 5 min. After washing three times in 2 × SSC, cells were examined using a Zeiss (Jena, Germany) LSM510 Meta confocal microscope with a 63× objective and 488 nm line for excitation.

Electrophoretic mobility shift assays

Mutant and wild-type RAX proteins containing an amino-terminal FLAG-tag were synthesized in the TNT coupled reticulocyte lysate system (Promega Inc., Madison, WI, USA). RAX-containing lysates were used in electrophoretic mobility shift assays with PCE I homeodomain recognition site (29,34). Approximately equal loading of RAX-containing lysates for DNA-binding assay was confirmed by western blotting of lysates probed with anti-FLAG antibody (Stratagene, La Jolla, CA, USA). Oligonucleotides of the PCE1 binding site (5′-CAGAAGCTTTCAATTAGCTATT-3′) and (5′-CTGAATAGCTAATTGAAAGCTT-3′) and the COUP-TF binding site (5′-CAGCTTCTATGGTGTCAAAGGTCAAACTTCTG-3′) and (5′-CTGCAGAAGTTTGACCTTTGACACCATAGAAG-3′) were used as specific and non-specific RAX binding site probes, respectively. Oligonucleotides were annealed and end-labeled with [α-^{32}P] dCTP (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA) using the large (Klenow) fragment of DNA Polymerase I (Invitrogen, Carlsbad, CA, USA). Labeled oligonucleotides (2×10^4 cpm per reaction) were incubated with 3 µl of TNT coupled reticulocyte lysate in 20 µl volume containing 10 mM HEPES (pH 7.9), 75 mM KCl, 2.5 mM $MgCl_2$, 0.1 EDTA, 1 mM DTT, 3% Ficoll, 1 µg of poly(dI) : poly(dC) (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA), 1 µg BSA for 1 h at 4°C. Samples were electrophoresed at 100V in a 5% precast TBE polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) at 4°C. Specificity of the mobility shift observed with RAX binding to the PCE I site was confirmed by competing the shifted band away with increasing concentrations of unlabeled oligonucleotide in the binding assay. Unlabeled oligonucleotides were added at a 4-, 100- and 1000-fold higher concentrations above the radioactive probe. The gel was dried, exposed to a PhosphoImager plate (Molecular Dynamics) and quantified.

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