Potential Novel Mechanism for Axenfeld-Rieger Syndrome: Deletion of a Distant Region Containing Regulatory Elements of PITX2

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PURPOSE. Mutations in PITX2 are associated with Axenfeld-Rieger syndrome (ARS), which involves ocular, dental, and umbilical abnormalities. Identification of cis-regulatory elements of PITX2 is important to better understand the mechanisms of disease.

METHODS. Conserved noncoding elements surrounding PITX2/pitx2 were identified and examined through transgenic analysis in zebrafish; expression pattern was studied by in situ hybridization. Patient samples were screened for deletion/duplication of the PITX2 upstream region using arrays and probes.

RESULTS. Zebrafish pitx2 demonstrates conserved expression during ocular and craniofacial development. Thirteen conserved noncoding sequences positioned within a gene desert as far as 1.1 Mb upstream of the human PITX2 gene were identified; 11 have enhancer activities consistent with pitx2 expression. Ten elements mediated expression in the developing brain, four regions were active during eye formation, and two sequences were associated with craniofacial expression. One region, CE4, located approximately 111 kb upstream of PITX2, directed a complex pattern including expression in the developing eye and craniofacial region, the classic sites affected in ARS. Screening of ARS patients identified an approximately 7600-kb deletion that began 106 to 108 kb upstream of the PITX2 gene, leaving PITX2 intact while removing regulatory elements CE4 to CE13.

CONCLUSIONS. These data suggest the presence of a complex distant regulatory matrix within the gene desert located upstream of PITX2 with an essential role in its activity and provides a possible mechanism for the previous reports of ARS in patients with balanced translocations involving the 4q25 region upstream of PITX2 and the current patient with an upstream deletion. (Invest Ophthalmol Vis Sci. 2011;52:1450–1459) DOI:10.1167/iovs.10-6060

Axenfeld-Rieger syndrome (ARS) is a relatively rare developmental disorder characterized by maxillary hypoplasia, anterior segment defects of the eye (with glaucoma in ~50%), and dental and umbilical abnormalities. The PITX2 homeodomain-containing transcription factor gene plays a major role in this condition, explaining approximately 40% of classic ARS.1–5 In addition, PITX2 mutations were shown to cause isolated ocular conditions.6–8 to be associated with additional brain anomalies.9,10 and possibly to contribute to other phenotypes such as omphalocele and VATER-like association.11

The function of Pitx2 is conserved in vertebrates. Total or conditional knockouts of Pitx2 result in severe developmental phenotypes in mice that include craniofacial, ocular, dental, brain, heart, lung, and other systemic defects consistent with the expression sites of Pitx2. With respect to Axenfeld-Rieger syndrome, Pitx2-deficient mice exhibit arrest of eye and tooth organogenesis, defective body wall closure and brain abnormalities.12–17 The defects observed in mice are associated with a complete loss of Pitx2 activity in the corresponding structures and, therefore, are noticeably more severe that what is reported in humans with heterozygous PITX2 mutations, whereas heterozygous mice are generally described as normal. This suggests a variable requirement of PITX2/Pitx2 for normal human/mouse embryogenesis, with human development more sensitive to correct PITX2 dosage.

Human PITX2 mutations mainly result in a complete or partial loss of function, with mutations retaining some wild-type activity producing milder phenotypes.18–20 Some mutations identified in patients with Axenfeld-Rieger syndrome displayed very minor defects in DNA-binding and transactivation activities, again suggesting that normal human development is highly susceptible to alterations of PITX2 function.5 In addition, several human mutations associated with gain-of-function have been reported,21 consistent with the dramatic ocular and limb phenotypes seen in mice with Pitx2 overexpression during eye and forelimb development.22,23 Therefore, correct dosage of PITX2 is critical for normal development, which further underscores the importance of precise transcriptional regulation of its expression.

Regulation of Pitx2 expression has been studied by several groups, but only a few cis-regulatory elements have been identified and examined in animal models. In mouse, Pitx2 appears to be a part of the Wnt/b-catenin/TCF/LEF and cAMP-CREB signaling pathways.24,25 Transcriptional activation of Pitx2 during eye development has been shown to be dependent on retinoic acid signaling26–28; the nature of this interaction, direct or indirect, has not yet been established. A few studies...
reported in vivo identification of Pitx2 enhancer elements, which facilitated upstream factor analysis. Asymmetric Pitx2 expression in the developing visceral organs was shown to require an intronic enhancer located in the proximity of the last exon,\textsuperscript{29,30} whereas a 7-kb fragment located 4.2 kb downstream of the last exon of Pitx2 was found to direct reporter expression in the oral ectoderm and Rathke’s pouch.\textsuperscript{31} Finally, several regulatory elements associated with pitx2 expression in the stomodeum territory have been isolated in ascidians; the cis-sequences were either intronic or located within 2.5 kb of the 5’ or 3’ end of the gene.\textsuperscript{32}

\textit{PITX2} is flanked by glutamyl aminopeptidase (aminopeptidase A) (\textit{ENPEP}) located approximately 54 kb downstream and chromosome 4 open reading frame 32 (\textit{C-korf32}) expressed sequence positioned approximately 1.5 Mb upstream of \textit{PITX2}; thus, the 1.5-Mb sequence upstream of the gene is considered a gene desert. Gene deserts often contain conserved sequences involved in transcriptional regulation of nearby genes.\textsuperscript{33} The presence of \textit{PITX2} regulatory elements in this region were consistent with the previous reports of patients with Axenfeld-Rieger syndrome with translocation breakpoints that occurred within the distant upstream region and did not disrupt the coding region of \textit{PITX2}.\textsuperscript{1,2,54,55}

We describe identification of several novel regulatory sequences in the \textit{PITX2}/\textit{pitx2} region, including several distant elements located within the gene desert upstream of \textit{PITX2}. The activities of these elements appear to be consistent with \textit{PITX2}/\textit{pitx2} expression, suggesting that they play a role in this gene’s function. In addition, we report the identification of a de novo deletion located 106 to 108 kb upstream of \textit{PITX2} in a patient with Axenfeld-Rieger syndrome, thus providing additional support for the essential role of the identified regulatory elements in \textit{PITX2} function.

\section*{Methods}

\subsection*{Animals}

Zebrafish (\textit{Danio rerio}) maintenance and developmental staging were performed as previously described.\textsuperscript{56} All experiments were conducted in accordance with the guidelines set forth by the animal care and use committees at the Medical College of Wisconsin and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

\subsection*{In Situ Hybridization}

To prepare probes for in situ hybridization, a plasmid containing the final \textit{pitx2} exon, which is common to both isoforms \textit{pitx2a} and \textit{pitx2c}, and a pG1 plasmid-containing GFP sequence (obtained from Chi-Bin Chien, University of Utah) were used. The \textit{pitx2} (1345 bp) and \textit{GFP} (750 bp) digoxigenin-labeled antisense riboprobe probes were synthesized from the corresponding plasmids and used in situ hybridization, as previously described.\textsuperscript{56} Sections measuring 5 to 10 mm were cut on a cryostat (Richard Allan Scientific Microm HM 550; Thermo Fisher Scientific Inc., Waltham, MA) and mounted on gelatin-coated glass slides. The samples were examined with a microscope (AxioImager [Z1]; Carl Zeiss Microimaging, Inc., Thornwood, NY), and photographs were taken with a digital color camera (AxioCam MRc5; Carl Zeiss Microimaging, Inc.).

\subsection*{Sequence Analyses and Construction of Reporter Plasmids}

The \textit{PITX2} genomic region and 1650 kb of surrounding sequence were analyzed for conserved noncoding elements using the multiple species alignment tool at UCSC Genome Browser (http://genome.ucsc.edu) and BLAST (http://blast.ncbi.nlm.nih.gov) with a focus on regions conserved between human and zebrafish (July 2007 zebrafish and March 2006 human genome assemblies were initially used and then verified using NW_001838915.1, \textit{Homo sapiens} chromosome 4 genomic reference (HuRef) and NW_001877464.1, \textit{D. rerio} chromosome 1 genomic reference assembly (D. rerio Zv7_scaffold1394)).

To generate the \textit{Tg}(2-6\textit{pitx2:GFP}) promoter construct, a 2067 bp \textit{pitx2c} zebrashif promoter (that included conserved element [CE1]) was amplified using forward (GAGCAGTAAATGCAAAGC) and reverse (AGACAAGCTTTACCGAG) primers and inserted upstream of the GFP coding region into promoterless pG1 plasmid (obtained from Chi-Bin Chien, University of Utah). An additional promoter construct, \textit{Tg}(1-3\textit{pitx2:GFP}), was generated that contained 1.9 kb \textit{pitx2c} promoter sequence but lacked CE1 using the same reverse primer and an alternative forward primer, GAGGCCACATCTAAACACC. The \textit{Tg}(2-6\textit{pitx2:GFP}) promoter plasmid was then used to generate additional constructs containing the CE2 to CE13 noncoding conserved sequences, \textit{Tg}(2-6\textit{pitx2:CE2-CE13:GFP}) and \textit{Tg}(2-6\textit{pitx2:CE13:GFP}). The CE2 to CE13 zebrashif regions (Supplementary Fig. S1, http://www.iosv.org/lookup/suppl/doi:10.1167/iosv.10-0606/DCSupplemental) were PCR amplified using \textit{PfuUltra} high-fidelity DNA polymerase (Strategene, La Jolla, CA) and standard conditions, cloned into pCRII-TOPO vector (Invitrogen), and subcloned downstream of the GFP coding region in the \textit{Tg}(2-6\textit{pitx2:GFP}) promoter plasmid. All constructs were named consistent with ZFIN nomenclature (http://zfin.org/zf_info).

\subsection*{Generation and Analyses of Transgenic Fish}

Reporter plasmid DNA was prepared using a midki (Qiagen, Valencia, CA) and suspended at 25 ng/µl in injection buffer [0.1% (wt/vol) phenol red (Sigma-Aldrich, St. Louis, MO) in 0.3× Danieau buffer (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO\textsubscript{4}, 1.8 mM Ca(NO\textsubscript{3})\textsubscript{2}, and 1.5 mM HEPES, pH 7.6)]. Microinjections of plasmid DNA into zebrashif embryos were performed immediately after fertilization at the one- to two-cell stage. Microinjections were performed with an injector (MM33 Micromanipulator [Stoelting Co., Wood Dale, IL] or Nanoject II [Drummond Scientific, Broomall, PA]). Embryos were incubated at 28.5°C and maintained in 0.2 mM 1-phenyl-2-thiourea to inhibit pigmentation and were anesthetized with 0.05% tricaine before imaging. Developing embryos were inspected for GFP expression until 5 days after fertilization, and the cis-regulatory activity of each construct was documented; at least 100 transient transgenics were examined for each construct. Fluorescence microscopes (SMZ 1500 [Nikon Instruments, Inc., Melville, NY] and AxioImager [Carl Zeiss Microimaging, Inc.]) and in situ hybridization with GFP-specific probe were used in this process. To generate permanent lines, approximately 200 embryos were injected with linearized plasmid DNA and raised to maturity, and their progeny were examined for germline transmission of the transgene using the same techniques as described. Transgenic fish were raised and inbred to establish a permanent line.

\subsection*{Human Sample Analysis}

This human study research adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board of the Children’s Hospital of Wisconsin, with informed consent obtained for every subject. Eight patients were screened for deletions or duplications of the \textit{PITX2} upstream region: three patients had classic Axenfeld-Rieger syndrome, three had syndromic Axenfeld-Rieger anomaly without dental or umbilical features, and two had isolated Axenfeld-Rieger syndrome. Previous screening of the \textit{PITX2} coding sequence did not identify a causative mutation in these patients. The screening for the coding sequence mutations was performed by direct bidirectional DNA sequencing of PCR products encompassing all coding exons of \textit{PITX2A}, \textit{PITX2B}, and \textit{PITX2C} isoforms using previously described gene-specific primers.\textsuperscript{11} Six patients with classic Axenfeld-Rieger syndrome and previously identified \textit{PITX2} mutations (four patients) or whole-gene deletion (two patients) were excluded from this analysis. Patients were screened using Affymetrix Genome-Wide Human SNP Array 6.0, as previously described\textsuperscript{57}, and/or TaqMan assays
Identification of Conserved Noncoding Elements in Human and Zebrafish PITX2/pitx2

Human PITX2 and zebrafish pitx2 genes are located at chromosomes 4q25 and 14, respectively. To identify regulatory sequences responsible for the expression patterns of PITX2/pitx2 during development, we scanned a 1.6-Mb sequence around the human PITX2 gene for noncoding genomic elements conserved between human and zebrafish genomes. Thirteen major conserved elements, CE1 to CE13, were identified that ranged in size from approximately 50 to 300 bp, demonstrated approximately 80% to 90% sequence identity at the nucleotide level, and were positioned in the same order between human and zebrafish genomes (Fig. 1; Supplementary Table S1 and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental). In addition to this, BLAST comparison of the CE1 to CE13 sequences against mouse, chicken, and fugu genomes revealed similarly strong conservation of all elements: 94% to 100% nucleotide identity (mouse), 71% to 98% (chicken; except for the CE1 region, which showed no significant homology), and 50% to 92% (fugu). All conserved elements are located upstream of the PITX2/pitx2 isoform; with respect to the PITX2A/pitx2a isoform, CE1 and CE2 are positioned internally whereas CE3 to CE13 are upstream of the gene at a distance of approximately 2.4 to 1111 kb (human) or approximately 1 to 260 kb (zebrafish) (Fig. 1; Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental).

The probable association of these CEs with PITX2/pitx2 function and not another gene in the region is supported by the fact that PITX2/pitx2 is flanked by different genes in the human and zebrafish genomes. The human PITX2 gene is bordered by chromosome 4 open reading frame 32 (C4ORF32) at the 5' end and glutamyl aminopeptidase (aminopeptidase A) (ENPEP) at the 3' end (GRCh37/hg19; UCSC genome browser http://genome.ucsc.edu). Analysis of the zebrafish genome using cross-species megabLAST identified no significant homology for the human C4ORF32 and several regions of low homology (corresponding to only ~3%-11% of the human sequence) for the ENPEP gene involving zebrafish chromosomes 21, 25, and 13. The zebrafish pitx2 gene is flanked by the hypothetical protein LOC402939 and the elovl family member 6, elongation of long chain fatty acids (elovl6) genes (Zv8danRer6; UCSC genome browser http://genome.ucsc.edu). Cross-species megablast comparison identified that zebrafish LOC402939 is highly homologous to several human genomic regions, including ADH4 and AHDS located 11.5- and 11.6-Mb up.

FIGURE 1. Schematic drawing of the genomic organization/isosforms of the (A) human PITX2 and (B) zebrafish pitx2 genes. PITX2/pitx2 exons are indicated as numbered boxes. Conserved elements are shown as black boxes/lines and are marked CE1, CE2, CE3, and CE4-CE13. Exonic sizes are indicated at the top intronic sizes are shown at the bottom of the numbered boxes. Distance to the distant element CE4 from the first exon and size of the genomic region containing CE4 to CE13 elements are indicated. The edges of the brackets encompassing the corresponding regions (see Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental, for more details). Position of the pitx2 probe used in situ hybridization experiments is indicated.
stream of PITX2, whereas zebrafish elovl6 is homologous to the human ELOVL6 sequence located 567,786-bp downstream of PITX2 (Supplementary Table S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6060/-/DCSupplemental). Therefore, even though the ADH4/5-PITX2-ELOVL6 order has been maintained in both zebrafish and human genomes, the human genome apparently acquired additional genes upstream and downstream of PITX2, suggesting that the CEs are not involved in regulating the flanking genes.

Finally, the identified conserved sequences did not appear to be part of any zebrafish or human transcript based on database records. In addition to this, RT-PCR analysis using CE-specific primers and RNA extracted from zebrafish embryos and human cell lines failed to amplify any transcripts (data not shown). Therefore, we propose that the high conservation of these regions is likely to be due to their function in transcriptional regulation of pitx2/PITX2.

**Conserved Elements Mediate Expression during Brain, Eye, and Craniofacial Development**

To test the identified conserved noncoding elements for enhancer activity, we developed a series of reporter constructs based on the pG1 promoterless plasmid containing the coding region of green fluorescent protein (GFP). The Tg(-2.6pitx2:GFP) construct contains 2.6 kb zebrafish pitx2c promoter sequence (that includes the CE1 region), the Tg(-1.9pitx2:GFP) construct contains a 1.9-kb pitx2 promoter region lacking the CE1 conserved element, and Tg(-2.6pitx2-CE2:GFP) through Tg(-2.6pitx2-CE13:GFP) plasmids contain 2.6 kb promoter along with the CE2 through CE13 elements, respectively, inserted downstream of GFP. These constructs were tested for cis-regulatory activity by transient transgene expression in zebrafish embryos. Zebrafish embryos injected with promoterless pG1 plasmid displayed either weak sporadic GFP fluorescence or none at all.
(data not shown). In contrast, transient transgenic embryos generated with the various pitx2 reporter constructs demonstrated strong and specific expression patterns that were largely consistent with endogenous expression of pitx2. Specifically, embryos injected with Tg(-2.6pitx2:GFP) reporter exhibited robust expression in the developing trunk muscles but lacked fluorescence in the head region (Fig. 3A). Embryos injected with the Tg(-1.9pitx2:GFP) construct lacking CE1 displayed expression in the developing muscles similar to the Tg(-2.6pitx2:GFP) construct but with reduced overall signal intensity (data not shown). Expression patterns in transient transgenic fish for reporters containing conserved elements Tg(-2.6pitx2-CE2:GFP) through Tg(-2.6pitx2-CE13:GFP), were as follows: CE2-, CE-3, CE6-, and CE12-containing plasmids showed strong and consistent expression in the developing midbrain or diencephalic neurons (Figs. 3B–G, 3J); the CE9 region demonstrated a dynamic pattern with strong brain expression in 48-hpf embryos that weakened at 80 hpf when heart expression became evident (Figs. 3H, 3I); CE13 was also associated with heart expression in addition to strong craniofacial and some brain expression (Figs. 3K, 3L); CE5, CE7, and CE10 directed expression in the periocular mesenchyme (Figs. 3M–O) with brain expression also observed for CE5 and CE7 (data not shown). When these three ocular elements (CE5, CE7, CE10) were consolidated in the same reporter, stronger ocular expression was observed, suggesting an additive effect (Fig. 3P). Weak transgene expression during brain development was observed for the CE11-containing reporter, and no specific activity was detected for the CE8 region (data not shown). Finally, CE4 was associated with the most complex pattern of expression, including the developing eye, brain, and craniofacial region (Fig. 4).

Since Tg(-2.6pitx2:CE4:GFP) transgenic animals demonstrated a complex pattern of expression that involved the developing brain, jaw, and eye consistent with the multiple sites affected in Axenfeld-Rieger syndrome patients, a permanent transgenic line carrying this transgene was generated to further study the expression mediated by the CE4 conserved region (Fig. 4). In situ hybridization was performed to better visualize the expression and to confirm specificity. In the 20–24-hpf Tg(-2.6pitx2-CE4:GFP) permanent transgenic embryos, strong GFP expression was detected in the periorcular mesenchymal cells migrating into the anterior segment of the eye (Figs. 4A, 4B) and in the developing somites and brain. At 48 hpf, some expression around the oral cavity could also be observed (Figs. 4C, 4D, 4F). At 72 to 120 hpf, expression remained in the developing brain, jaw, pharyngeal arches, and anterior segment of the eye (iridocorneal angle) (Figs. 4E, 4G, 4H). Overall, the observed transgene expression demonstrated significant overlap with the endogenous pitx2 pattern; some specific sites were not present or demonstrated noticeably weaker expression (oral cavity, for example), and ectopic expression was detected in some tissues (strong hindbrain and retinal expression in some embryos; data not shown).

These data support the possibility that the identified conserved elements are involved in regulation of pitx2/PITX2 expression and thus play an important role in pitx2/PITX2 function.

**Mutation Search Identifies a Deletion in a Patient with Axenfeld-Rieger Syndrome Located 106 to 108 kb Upstream of PITX2, Thus Removing Distant Conserved Elements CE4 to CE13**

DNA samples from eight patients with Axenfeld-Rieger syndrome (three patients), syndromic Axenfeld-Rieger anomaly without dental or umbilical findings (three patients), and isolated Axenfeld-Rieger anomaly (two patients) were screened for deletions and duplications of the upstream sequence. Copy number variation analysis was performed (Affymetrix Genome-Wide Human SNP Array 6.0 and/or TaqMan assays) for the PITX2 region. Patient 1, affected with Axenfeld-Rieger syndrome (Fig. 5), was found to carry a 7645-kb deletion involving the 4q25-q26 region (Fig. 6A); the proximal end of the deletion is located between probes at approximately 105.6 kb (diploid).

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**Figure 3.** Reporter expression mediated by conserved elements identified in the PITX2 region. GFP expression associated with pitx2 promoter region only (A), and pitx2 promoter region with additional elements CE2 (B–D), CE3 (E, F), CE6 (G), CE9 (H, I), CE12 (J), CE13 (K, L), CE5 (M), CE7 (N), CE10 (O), and a combination of CE5, CE7, and CE10 (P) in 36- to 80-hpf embryos. Note the strong expression in the developing brain in 36-hpf (B) and 48-hpf embryos for CE2 (C, D) and 48-hpf embryos for CE3 (E, F), CE6 (G), CE9 (H), and CE12 (J). For CE9, expression in the developing brain weakened by 80 hpf, whereas robust expression in the heart region became evident (I). CE13 was associated primarily with expression during heart and craniofacial development in 24-hpf (K) and 48-hpf (L) embryos. CE5, CE7, and CE10 regions appeared to be associated with ocular expression that could be easily observed in 48-hpf embryos (M–P). b, brain; di, diencephalon; h, heart; mb, midbrain; oc, oral cavity; pm, periocular mesenchyme; sm, skeletal muscles (trunk).
state) and approximately 108.1 kb (haploid state) upstream of PITX2, and its distal end is positioned between probes at approximately 7753 kb (haploid state) and approximately 7759 kb (diploid state) upstream of PITX2; 4768 of 4777 (99.8%) probes (Affymetrix Array 6.0) in this region showed a copy number of 1 (Figs. 6A, 6B). Analysis of 22 unaffected persons included in our study and genomic variation reported in the Database of Genomic Variants (http://projects.tcag.ca/variation?) revealed an absence of similar deletions in controls.

This deletion, in addition to the removal of the PITX2 distant upstream region, results in deletion of one copy of the following genes C4ORF32, C4ORF16, TIFA, ALPK1, NEUROG2, LOC91431, C4ORF21, LARP7, ARSJ, UGT8, NDST4, TRAM1L1, NDST3, and PRSS12 but leaves PITX2 intact (Figs. 6A, 6B). Quantitative PCR data obtained with TaqMan probes located within the PITX2 gene and the upstream region were consistent with the Affymetrix array results. Results for PITX2_CNV probes 1, 2, and 3, which correspond to sequences within PITX2 exons including the most 5’ exon 1, were consistent with the diploid state, whereas data obtained for PITX2_CNV probes 4, 5, and 6, corresponding to the distant 5’ region upstream of PITX2 (~110, 284, and 649 kb from the gene), confirmed the haploid state in patient 1 (Fig. 6C). Analysis of parental samples showed a normal diploid state for all six probes (Fig. 6C), consistent with their unaffected status.

The phenotype of the patient is consistent with Axenfeld-Rieger syndrome associated with PITX2 haploinsufficiency. Patient 1 is a Caucasian (Finland) male referred at age 7 months with bilateral iris hypoplasia and iridocorneal adhesions, left corectopia, maxillary hypoplasia, broad nasal bridge, redundant periumbilical skin, and Meckel’s diverticulum. Height was in the low-normal range at −1.5 SD. Chromosomal analysis showed a normal 46,XY karyotype with no evidence of translocations (400 band resolution,

**Figure 4.** Reporter expression in Tg(-2.6pitx2-CE4:GFP) permanent transgenic line. (A–E) GFP fluorescence images. (F–H) Images of in situ hybridization performed using Tg(-2.6pitx2-CE4:GFP) embryos and GFP antisense riboprobe. Developmental stages are indicated in the lower right of every image. Note the strong expression in the periocular mesenchymal (pm) cells of neural crest origin that migrated to the anterior segment region (A, B) and expression in the developing brain (A–G) and around the oral cavity (C–G), pharyngeal arches (E), and the anterior segment of the eye (G, H). ac, anterior segment of the eye; b, brain; di, diencephalon; e, eye; mb, midbrain; oc, oral cavity; pm, periocular mesenchyme; re, retina.

**Figure 5.** Images of patient 1 with Axenfeld-Rieger syndrome. (A) Facial photograph showing maxillary hypoplasia, thin upper lip, and broad nasal bridge. (B) Left eye with corectopia. (C) Right eye with posterior embryotoxon. (D) Dental anomalies, including maxillary hypodontia. (E) Redundant periumbilical skin.
Giemsa staining). In follow-up at 6 years of age, ocular, dental, and umbilical anomalies were again noted. Specifically, posterior embryotoxon was seen in the right eye, and amblyopia and iridocorneal adhesions were noted in the left eye. Intraocular pressure was normal (18 mm Hg bilaterally), there were no signs of glaucoma, and lenses, papillae, and maculae were normal. Dental anomalies included several missing permanent teeth (especially in the maxilla), general teething delay, and retrognathia of the maxilla and mandible (more pronounced in the maxilla). He has mild mental retardation (IQ scores not available), short stature (102.5 cm at 5 years 5 months [−1.9 SD]), and mildly delayed bone age. Patient 1 is the only child born to his parents. Both parents are unaffected; his mother has posterior embryotoxon (reported in healthy persons, including in 30% of one isolated Finnish population) with no other signs of anterior segment dysgenesis and normal teeth/umbilicus.

**DISCUSSION**

The PITX family of transcriptional factors is involved in a number of human disorders and displays unique expression patterns during development that are conserved in vertebrates. Regions that are responsible for the specific expression of PITX factors remain largely unknown. The identification of regulatory sequences provides an opportunity to isolate upstream factors and to investigate the contribution of these regions and their interacting proteins to human disease.

**Figure 6.** Identification of 4q25–26 deletion in a patient with Axenfeld-Rieger syndrome. (A) Genome Browser view of the region of deletion in patient 1. The deleted region is indicated by a black rectangle, and the previously reported ARS translocations are indicated by wavy arrows. The shaded area is shown in more detail in B. (B) Enlargement of the region containing PITX2 and the upstream gene desert with regulatory elements. The shaded area is shown in more detail in C. (C) Copy number variation analysis with TaqMan probes within PITX2 (P1–P3) and upstream (P4–P6) regions.
the deletion of regulatory elements with the gene desert upstream of PITX2.

Furthermore, the occurrence of Axenfeld-Rieger syndrome in previously described families with balanced translocations with breakpoints upstream of the PITX2 gene\(^1\) supported the notion that removal of this upstream region from the PITX2 gene disrupts PITX2 expression and leads to disease. The CE4 to CE13 distant regulatory sequences described here are located upstream of the 4q25 breakpoints identified in three independent families with balanced translocations and Axenfeld-Rieger syndrome\(^1\), the breakpoints in all three families are located between CE3 and CE4. The t(4;16)(q26;q22) breakpoint was found in a mother and daughter exhibiting ocular and dental features diagnostic of Axenfeld-Rieger syndrome\(^1\). The t(4;11)(q27;q21) breakpoint was found in a patient with a de novo balanced translocation and all three cardinal features of Axenfeld-Rieger syndrome\(^1\) and additional regulatory elements within gene deserts were shown to have regulatory function due to the presence or absence of an expression domain of interest in the organisms included in the analysis. Absence of an expression domain in some species may be associated with an evolutionary loss of a corresponding enhancer as was demonstrated, for example, for the 3ox2 late lens enhancer in human and chicken genomes but with no counterpart in the mouse genome, consistent with the absence of 3ox2 expression in the mouse lens after the lens vesicle stage\(^1\).

To study regulatory regions of PITX2/pitx2 that demonstrate conservation across vertebrate species (zebrafish-human), we first performed careful analysis of the expression pattern of the zebrafish pitx2 gene. Although mouse Pitx2 is known to be expressed in the developing eye, pituitary, forebrain and midbrain, maxillary and mandibular epithelia, umbilicus, limb, heart, and some other internal organs, zebrafish pitx2 expression had been described only for earlier developmental stages (up to 28 hpf) and with a focus on left-right patterning\(^1\). During later stages of development, we detected pitx2 expression in the developing forebrain and midbrain, around the oral cavity, and in the pharyngeal arches, periocular mesenchyme, and developing anterior segment structures. This further demonstrates conservation of pitx2 expression patterns in vertebrates and correlates with the sites affected in patients with Axenfeld-Rieger syndrome.

We then identified 13 genomic regions that are conserved between human and zebrafish genomes and are located in the approximately 1.6-Mb region surrounding the human PITX2 gene and tested them for their potential role in this gene’s expression. Eleven of these regions were found to be associated with specific expression patterns consistent with endogenous expression of pitx2 in the developing brain, eye, and craniofacial region. Nine of these enhancers, CE4 to CE13, were located at a significant distance from the PITX2 gene (111–1111 kb), within a so-called gene desert\(^1\). Conserved elements within gene deserts were shown to have regulatory activities consistent with expression of nearby genes and were, therefore, likely to be involved in their transcriptional activation\(^1\). The significance of gene deserts remains unclear because megabase deletions of some regions in mice resulted in the reported patient to have a deletion of this region. This statement is supported by the presence of the identified deletion in the affected patient but not in his unaffected parents, the absence of the deletion in healthy controls, the presence of the classic Axenfeld-Rieger syndrome features consistent with haploinsufficiency for PITX2 in the reported patient, an apparent presence of PITX2/pitx2 regulatory elements in the deleted region, and previous reports of balanced translocations in Axenfeld-Rieger syndrome patients that, similar to the deletion in patient 1, removed the PITX2 upstream region by displacing it to another chromosome without affecting the coding part of the gene.

In conclusion, PITX2 makes use of an extensive and conserved regulatory network to mediate its expression pattern. Eleven new conserved regulatory regions involved in different aspects of PITX2/pitx2 expression have been identified; nine are located within the upstream gene desert region. The existence of element(s) essential for PITX2 transcriptional activity in the remote upstream region of the gene provides a probable explanation for the occurrence of Axenfeld-Rieger syndrome in the patient reported here to have a deletion of this region. This statement is supported by the presence of the identified deletion in the affected patient but not in his unaffected parents, the absence of the deletion in healthy controls, the presence of the classic Axenfeld-Rieger syndrome features consistent with haploinsufficiency for PITX2 in the reported patient, an apparent presence of PITX2/pitx2 regulatory elements in the deleted region, and previous reports of balanced translocations in Axenfeld-Rieger syndrome patients that, similar to the deletion in patient 1, removed the PITX2 upstream region by displacing it to another chromosome without affecting the coding part of the gene.

The potential role of the identified regulatory elements/ gene desert region in PITX2 function is supported by our discovery of a novel deletion that removes CE4 to CE13 in a patient with Axenfeld-Rieger syndrome. Although there are a number of other genes in the region, only two have been previously associated with human phenotypes: heterozygous missense mutations in ANK2 are associated with autosomal dominant cardiac arrhythmia/Long QT syndrome (MIM 600919) in several families\(^1\) and a homozygous 4-lop deletion in PRRS12 has been associated with autosomal, recessive, nonsyndromic mental retardation (MIM 249500) in two Algerian families\(^1\). Microscopic and submicroscopic deletions of PITX2 are a common mechanism of Axenfeld-Rieger syndrome\(^5\), but a patient reported with a 4q26 deletion that did not affect 4q25 did not show signs of Axenfeld-Rieger syndrome\(^1\), suggesting that the additional deleted genes in patient 1 do not contribute to his Axenfeld-Rieger syndrome phenotype. Thus, we propose that its phenotype is caused by
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PITX2. At the same time, even though the deletion of the PITX2 upstream regulatory region in patient 1 represents a likely explanation of the observed phenotype, a mutation in a yet to be identified gene cannot be completely ruled out. Our finding suggests that analysis of the PITX2 distant upstream region, in addition to exonic sequencing/deletion analysis, must be performed in patients with Axenfeld-Rieger syndrome and may provide further support for this new mechanism. Discovery of regulatory elements is essential to the understanding of PITX2 developmental regulation and mechanisms of human disease.

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References


