Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate *Rax* expression

Hiroki Danno*, Tatsuo Michiue†, Keisuke Hitachi*, Akira Yukita*‡, Shoichi Ishiura*, and Makoto Asashima*†§¶

*Department of Life Sciences (Biology), Graduate School of Arts and Sciences, University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan; †Organ Development Research Laboratory, National Institute of Advanced Industrial Science and Technology, Tsukuba Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan; and §ICORP Organ Regeneration Project, Japan Science and Technology Agency, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan

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The neural-related genes *Sox2*, *Pax6*, *Otx2*, and *Rax* have been associated with severe ocular malformations such as anophthalmia and microphthalmia, but it remains unclear as to how these genes are linked functionally. We analyzed the upstream signaling of *Xenopus Rax* (also known as *Rx1*) and identified the Otx2 and Sox2 proteins as direct upstream regulators of *Rax*. We revealed that endogenous Otx2 and Sox2 proteins bound to the conserved noncoding sequence (CNS1) located ≈2 kb upstream of the *Rax* promoter. This sequence is conserved among vertebrates and is required for potent transcriptional activity. Reporter assays showed that Otx2 and Sox2 synergistically activated transcription via CNS1. Furthermore, the Otx2 and Sox2 proteins physically interacted with each other, and this interaction was affected by the *Sox2*-missense mutations identified in these ocular disorders. These results demonstrate that the direct interaction and interdependence between the Otx2 and Sox2 proteins coordinate *Rax* expression in eye development, providing molecular linkages among the genes responsible for ocular malformation.

anophthalmia | comparative genomics | microphthalmia | rx1 | *Xenopus*

Severe forms of ocular malformation, such as anophthalmia (absence of the eye) and microphthalmia (very small eye), appear in the human population at a frequency of ≈1 per 5,000–10,000 persons (1). These malformations of the eye are caused by genetic and molecular disruption of the development of the anterior neuroectoderm and forebrain, which contribute to the nascent eye. The vertebrate eye develops from a part of the forebrain that is called the optic vesicle. Progress has been made in understanding the molecular and cellular mechanisms underlying the formation of the optic vesicle, revealing the involvement of several transcription factors in its development (2). Recent advances in human genetics have identified the causative genes for ocular malformations, which include *Sox2*, *Pax6*, *Otx2*, and *Rax* (3–7). These genes all encode transcription factors that are highly conserved among vertebrates.

Sox2 encodes a high-mobility group (HMG) domain-containing transcription factor, which is a member of the SOXB1 subfamily in the larger family of SOX proteins. Heterozygous mutations in *Sox2* have been reported as the cause of 10–20% of cases of anophthalmia and severe microphthalmia (3, 8). The requirement for *Sox2* during eye development has been confirmed by the generation of a gene-dosage allelic series of *Sox2* mutations in the mouse (9). The Sox2 protein is thought to exert its function in cooperation with other transcription factors (10). During lens development, Sox2 interacts with Pax6 to bind cooperatively to DNA, thereby regulating δ-*crystallin* expression (11). *Pax6*, which is an essential eye regulator gene, was the first causative gene for anophthalmia to be identified (6, 12).

A candidate gene approach subsequently identified *Otx2* as another causative gene for anophthalmia and microphthalmia (4). *Otx2* encodes a bicoid-type homeodomain transcription factor and is a vertebrate homolog of *otd*, which was identified in the fruit fly as being required for the formation of the anterior neural structure (13, 14). The requirements of *Otx2* for the formation of the brain and eye in vertebrates have been investigated by generating targeted mutant mice (15). Murine *Otx2* is expressed in the visceral endoderm and anterior neuroectoderm, which eventually develop into the eye and brain. *Otx2*-null mice show a severe head defect, which is accompanied by abnormal development of the visceral endoderm that comes in contact with the neuroectoderm and directs its fate. A study using chimerae has demonstrated that *Otx2* in the visceral endoderm is required for induction of the forebrain and midbrain and that *Otx2* in the anterior neuroectoderm is required for its regional specification (16).

Rax, which is a paired-type homeobox gene, is another causative gene for anophthalmia (5, 17, 18). *Rax*-null mice lack eyes (18). The Rax protein directly or indirectly regulates the expression of downstream genes, including *Xhmgb3*, *IRBP*, *arrestin*, *Xhairy2*, *Zic2*, and *XOptx2* (19–21). These genes are involved in the specification of the eye field and the proliferation of retinal progenitor cells. Furthermore, previous studies have shown that the 5′-upstream regions of *Rax* in *Xenopus laevis* and *Xenopus tropicalis* contain *cis*-regulatory elements that direct *Rax* expression in the developing eye (22, 23). However, the *trans*-acting factors that bind directly to the *cis*-regulatory elements to regulate *Rax* expression remain unknown.

Initially, we explored the upstream regulation of *Rax* expression in the African clawed frog (*X. laevis*). The present work provides evidence for two direct linkages between the causative genes for ocular malformation: (*i*) the transcription of *Rax* is regulated directly by the Otx2 and Sox2 proteins; and (*ii*) the Otx2 protein interacts directly with the Sox2 protein, and their interdependence coordinates transcriptional activation.

Results

Conserved Noncoding Sequence 1 (CNS1) Has *cis*-Regulatory Activity. Initially, we compared three upstream sequences of frog *Rax* (*X. laevis*, AY250711 and RaxG4; *X. tropicalis*, XtRaxG) by pairwise alignment and dot-matrix analyses (Fig. 1*A*). Although the total

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‡Present address: Department of Oral Histology, Matsumoto Dental University, 1780 Gobara, Hirooka, Shiojiri, Nagano 399-0781, Japan.

¶To whom correspondence should be addressed. E-mail: asashi@bio.c.u-tokyo.ac.jp.

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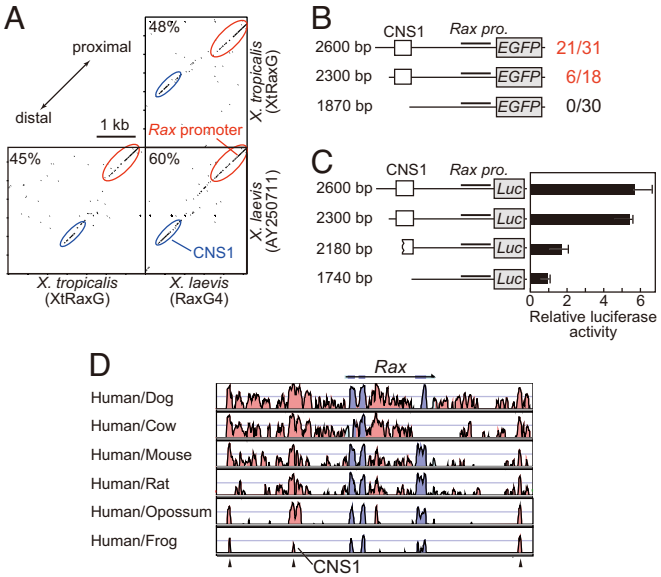

Fig. 1. Conservation and *cis*-regulatory activity of the sequences upstream of *Rax*. (*A*) Comparison of three upstream sequences of frog *Rax* (*X. laevis*, AY250711 and RaxG4; *X. tropicalis*, XtRaxG). A proximal region that contains a minimal promoter and transcriptional start site (red), as well as a distal noncoding region (blue), are highly conserved among the three clones. The distal region is termed the conserved noncoding sequence 1 (CNS1). The percentages indicate sequence similarities. (*B*) Transgenesis in *X. laevis*. Two constructs contain CNS1 and up-regulate the *EGFP* reporter. The number of transgenic embryos that express EGFP in the optic vesicles of normally developing embryos obtained in this assay is indicated on the right. Black lines indicate the *Rax* promoter. (*C*) Luciferase reporter activities of sequences upstream of *Rax* in *X. laevis* embryos. (*D*) VISTA view of the occurrence of the conserved sequence domain in the genomic region that encompasses the *Rax* gene. Colored peaks (purple, coding; pink, noncoding) indicate regions of at least 100 bp and 60% similarity. There are three conserved noncoding regions (arrowheads) in a region of ≈20 kb, and the most proximal one is CNS1.

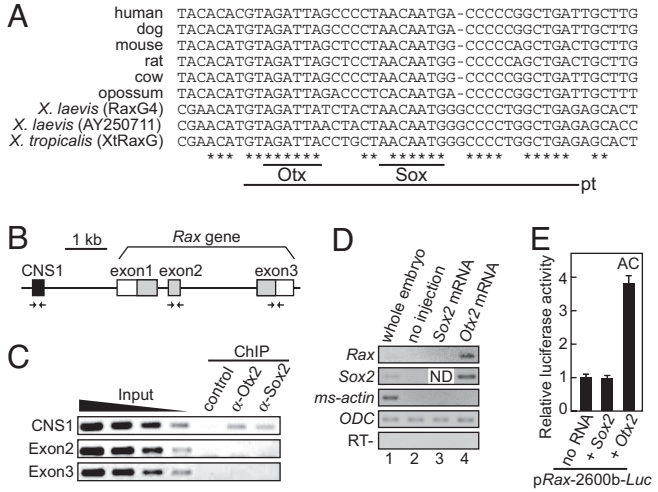

Fig. 2. Otx2 and Sox2 are upstream regulators of *Rax*. (*A*) Multiple DNA sequence alignment of vertebrate CNS1. CNS1 contains a specially conserved 35-nucleotide sequence (pentatriacontamer, pt) that contains consensus binding sites for Otx and Sox. (*B*) Genomic structure of the *Xenopus Rax* locus. Arrows indicate the primers used in the ChIP assays. CNS1, the coding region and untranslated region of *Rax* are indicated as black, gray, and white boxes, respectively. (*C*) The ChIP assay demonstrates that endogenous Otx2 and Sox2 proteins bind to CNS1 *in vivo*. (*D*) RT-PCR analysis showing that overexpression of Otx2, but not of Sox2, induces *Rax* in *Xenopus* animal cap cells. (*E*) Luciferase assays using *Xenopus* animal cap (AC) cells shows that overexpression of Otx2 induces transcriptional activation of p*Rax*-2600b-*Luc*, whereas Sox2 overexpression does not. (*D* and *E*) *Sox2* or *Otx2* mRNA (100 pg) was injected.

sequence of ≈3 kb showed low similarity among the three clones, two restricted regions showed high sequence similarity. One of these regions contained a potential TATA motif for the *Rax* gene promoter, whereas the other, located 2 kb upstream of the promoter, neither contained a promoter nor represented a gene; we named this latter region CNS1. Although CNS1 is a noncoding region, it is specifically conserved among frog genomes, which suggests that it has *cis*-regulatory activity. Transgenic analyses demonstrated that regions upstream of *X. laevis Rax* drove EGFP expression in optic vesicles where endogenous *Rax* mRNA is expressed and that loss of CNS1 abolished this expression [Fig. 1*B* and supporting information (SI) Fig. S1*A*]. Similarly, the upstream sequences that contained CNS1 showed strong transcriptional activities in the luciferase assay, whereas upstream sequences that partially or completely lacked CNS1 showed markedly lower activities (Fig. 1*C*). These two reporter assays showed that CNS1 exerts a *cis*-regulatory activity in *X. laevis* embryos.

CNS1 Is Conserved Among Vertebrates and Contains both Otx- and Sox-Binding Sites. Because CNS1 is conserved and has transcriptional activity in frogs and as Rax is a highly conserved transcriptional factor among vertebrates, we investigated whether CNS1 is conserved exclusively among frogs or more broadly among vertebrates by using the VISTA Browser (24). We plotted sequence similarities over a 20-kb genomic region that contained *Rax*, with the human genome as the base sequence (Fig. 1*D*). Thus, we identified three conserved noncoding sequences in the *Rax* regions of the human, dog, cow, mouse, rat, opossum, and frog genomes (Fig. 1*D*, arrowhead). The most-proximal sequence was CNS1, which is described above as a sequence that is conserved among frog genomes.

Phylogenetic footprinting using vertebrate CNS1 sequences identified conserved 35-nucleotide regions (pentatriacontamer, pt) in the CNS1 domain of *Rax*, including putative Otx- and Sox-binding sites (Fig. 2*A*). Given the causative role of *Rax* in anophthalmia, we hypothesized that the anophthalmia-associated proteins Otx2 and Sox2 would bind to these sites. In *Xenopus* at the late neurula stage, we observed that both genes were coexpressed with *Rax* in the optic vesicle (Fig. S1*B*). Electrophoretic mobility shift assays (EMSAs) confirmed that the *Xenopus* Otx2 and Sox2 proteins bound to these putative binding sites *in vitro* (Fig. S2 *A–D*). To investigate whether endogenous Otx2 and Sox2 proteins bind to *Rax* CNS1 in the optic vesicle, we performed chromatin immunoprecipitation (ChIP) assays (Fig. 2 *B* and *C*). Genomic fragments bound by the Otx2 or Sox2 protein were immunoprecipitated with specific antibodies and analyzed by PCR with primer sets designed to amplify the CNS1 region. As a result, *Rax* CNS1 was immunoprecipitated by the anti-Otx2 and anti-Sox2 antibodies but not by nonspecific IgG (normal rabbit IgG) or when using primers that amplify a part of *Rax* exon 2 or exon 3. We also confirmed the binding capabilities of overexpressed myc-Otx2 and myc-Sox2 proteins to *Rax* CNS1 using the ChIP assay with the anti-myc antibody (Fig. S2 *E* and *F*). Taken together, these experiments demonstrate specific binding of the Otx2 and Sox2 proteins to *Rax* CNS1.

Overexpression of Otx2, but Not of Sox2, Induces *Rax* Expression in *Xenopus* Animal Cap Cells. Based on the results of the *in vitro* and *in vivo* DNA-binding assays, Otx2 and Sox2 were identified as candidates for upstream proteins that regulate the transcription of *Rax*. We then used RT-PCR to examine whether overexpression of Otx2 or Sox2 causes up-regulation of *Rax* in *Xenopus* animal cap cells. Animal cap cells, which are part of the

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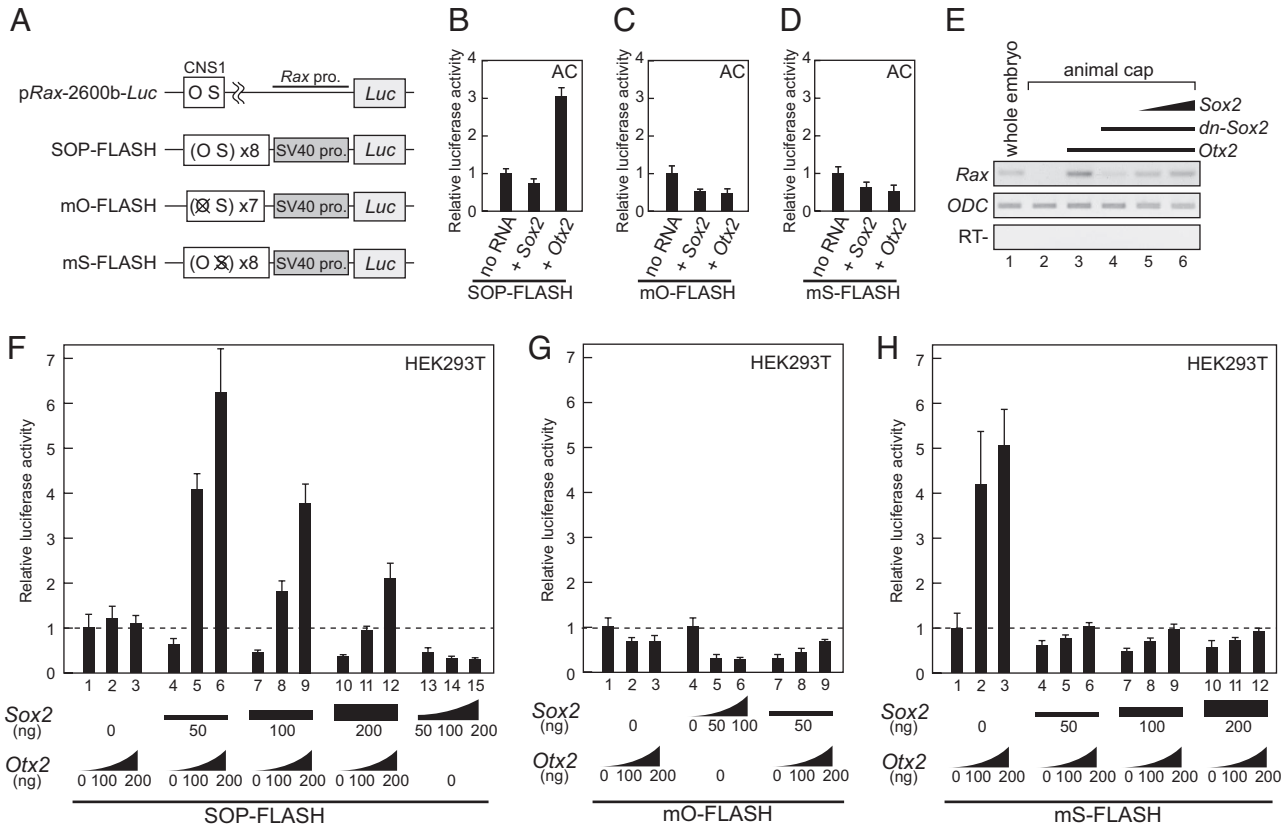

Fig. 3. Synergistic actions of Otx2 and Sox2 on transcription via CNS1. (*A*) Diagram of luciferase reporter constructs used in the present work. (*B–D*) Luciferase assays using *Xenopus* animal cap (AC) cells. Transcription from SOP-FLASH (*B*) is induced by the injection of *Otx2* mRNA (100 pg) but not by the injection of *Sox2* mRNA (100 pg). Overexpression of Otx2 does not induce transcription from either mO-FLASH (*C*) or mS-FLASH (*D*). (*E*) RT-PCR analysis shows that dominant-negative Sox2 (dn-Sox2) represses Otx2-induced *Rax* expression in animal cap cells. The amounts of mRNA injected were: *Otx2*, 100 pg; *dn-Sox2*, 1,000 pg; and *Sox2*, 10 or 30 pg. (*F–H*) Luciferase assays using HEK293T cells. (*F*) Synergistic effect of Otx2 and Sox2 on transcription from SOP-FLASH. (*G*) Transcription from mO-FLASH is not induced by the combination of Otx2 and Sox2 in HEK293T cells. (*H*) From mS-FLASH, transcription is induced by transfection with *Otx2* alone in HEK293T cells. This increase is attenuated rather than enhanced by the addition of *Sox2*.

undifferentiated ectodermal tissue of the *Xenopus* blastula, are competent to respond to an inductive signal or overexpressed genes. RT-PCR analysis revealed that overexpression of Otx2 increased the level of *Rax* mRNA in animal cap cells, whereas overexpression of Sox2 had no effect (Fig. 2*D*, topmost row). In addition, *Sox2* expression was induced in animal cap cells by Otx2 overexpression (Fig. 2*D*, second row from top).

We also used the luciferase assay to examine the response patterns of the sequences upstream of *Rax* to the overexpression of Sox2 and Otx2 in animal cap cells. In this assay, we used the reporter construct p*Rax*-2600b-*Luc*, which contains all of the sequences required to drive appropriate expression of a reporter gene in transgenic embryos (Fig. S1*A*). Overexpression of Otx2 increased luciferase activity 3.7-fold, whereas overexpression of Sox2 again had no effect (Fig. 2*E*). These results are in accordance with the RT-PCR results, in that they implicate Otx2 as a positive regulator of *Rax*. However, it should be noted that Otx2 overexpression was accompanied by increased expression of *Sox2*.

Otx2-Dependent Transactivation in *Xenopus* Animal Cap Cells Requires Both the Otx- and Sox-Binding Sites of CNS1. For further luciferase assays, we generated a SOP-FLASH vector that contained multiple Otx- and Sox-binding sites derived from CNS1 (Fig. 3*A*). Microinjection of *Otx2* mRNA, but not *Sox2* mRNA, increased by 3-fold the transcription from SOP-FLASH in animal cap cells (Fig. 3*B*). Therefore, the responses of SOP-FLASH and p*Rax*-2600b-*Luc* to overexpression of Otx2 and Sox2 were similar (Fig. 2*E*). To examine whether the sequence of CNS1 is responsible for Otx2-dependent transactivation, we introduced point mutations into the Otx- and Sox-binding sites of SOP-FLASH to generate mO-FLASH and mS-FLASH, respectively (Fig. 3*A*). Overexpression of Otx2 did not cause an increase in transcription from mO-FLASH (Fig. 3*C*), which indicates that transactivation by Otx2 requires an Otx-binding site on CNS1. Surprisingly, Otx2 overexpression did not drive transcription from mS-FLASH (Fig. 3*D*). A competitive EMSA confirmed that a substitution at the Sox-binding site of CNS1 did not affect the binding of Otx2 to a neighboring Otx-binding site (Fig. S2 *G* and *H*). The observation that a Sox-binding site affects transactivation without exogenous Sox2 protein suggests that Otx2-dependent transactivation requires the binding of endogenous Sox2 protein to the Sox-binding site in animal cap cells.

Both Otx2 and Sox2 Are Required for Transcriptional Activation Through CNS1 in *Xenopus* Animal Cap Cells and HEK293T Cells. As mentioned above, Otx2 overexpression induced the up-regulation of *Sox2* in animal cap cells (Fig. 2*D*). It is possible that the induced Sox2 protein collaborates with Otx2 protein in transactivation via CNS1. To test whether Sox2 is required for *Rax* up-regulation induced by Otx2, we performed loss-of-function experiments by using a dominant-negative construct of Sox2 (dn-Sox2), which lacks most of the HMG domain (25). RT-PCR analysis showed that Otx2-induced *Rax* expression in animal cap cells was repressed by coinjection of *dn-Sox2* mRNA

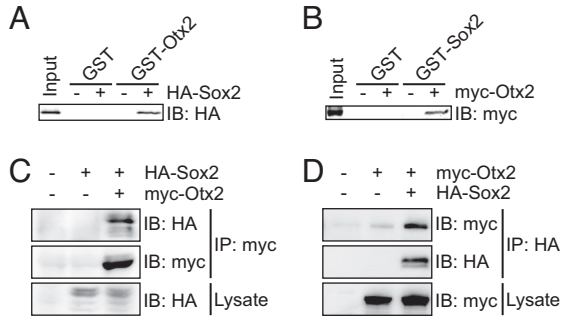

Fig. 4. Physical interactions between Otx2 and Sox2 proteins *in vitro* and *in vivo*. (*A*) GST pulldown by immobilized GST-Otx2 of *in vitro*-translated HA-Sox2 protein. (*B*) GST pulldown by immobilized GST-Sox2 of *in vitro*-translated myc-Otx2 protein. (*C* and *D*) Coimmunoprecipitation assays demonstrating *in vivo* interactions between the Otx2 and Sox2 proteins. (*C*) HA-Sox2 is coimmunoprecipitated with myc-Otx2 by the anti-myc antibody (9E10). (*D*) Myc-Otx2 is coimmunoprecipitated with HA-Sox2 by the anti-HA antibody (Y-11). Full scans of the Western blotting data are presented in Fig. S7.

(Fig. 3*E*). This repression was rescued by the additions of wild-type *Sox2* mRNA, which suggests that Sox2 is required for Otx2-induced *Rax* expression *in vivo*. To elucidate the underlying mechanisms in more detail, we used the HEK293T cell line, which is derived from a human embryonic kidney and does not express either Sox2 or Otx2 endogenously. In addition, Sox2 expression was not induced by overexpression of Otx2 in these cells (Fig. S3). Similar to the results obtained for the *Xenopus* animal cap cells, overexpression of Sox2 in HEK293T cells did not induce transcription from SOP-FLASH (Fig. 3*F*, lanes 1 and 13–15). However, overexpression of Otx2 alone did not increase transcription in these cells (Fig. 3*F*, lanes 1–3), in contrast to the earlier animal cap experiments, in which Otx2 overexpression induced transcription from SOP-FLASH (Fig. 3*B*). Surprisingly, cotransfection of *Otx2* and *Sox2* markedly increased transcription (Fig. 3*F*, lanes 4–6), and this synergistic transactivation was dose-dependent (Fig. 3*F*, lanes 4–12). Although an appropriate dose of *Sox2* (50 ng) was required for synergistic transactivation with *Otx2*, an excess of *Sox2* (100 or 200 ng) attenuated the increase in transcription. To confirm that the observed synergism between Otx2 and Sox2 requires the binding of these proteins to the CNS1 region, we performed additional luciferase assays by using Otx2 that was mutated at a critical lysine residue (K50) in the homeodomain as the expressed protein (26) and mO-FLASH or mS-FLASH as the reporter. K50-mutated Otx2 did not activate transcription in a cooperative manner with Sox2 (Fig. S4*A*), whereas mO-FLASH expression was not associated with synergistic transactivation under any condition tested (Fig. 3*G*). Experiments with mS-FLASH showed that Otx2 expression alone increased luciferase activity and that the addition of Sox2 induced no further increase; indeed, luciferase activity was strongly suppressed in the latter case (Fig. 3*H*). Possible explanations for the differences in responsiveness between SOP-FLASH and mS-FLASH are addressed in *Discussion*.

Physical Interactions Between the Otx2 and Sox2 Proteins *in Vitro* and *in Vivo*. The luciferase assays showed that Otx2 and Sox2 activated transcription interdependently. In addition, the six-nucleotide gap between the Sox-binding and Otx-binding sites on CNS1 is very short and is conserved among vertebrates (i.e., a six-nucleotide gap rather than a specific six-nucleotide motif) (Fig. 2*A*). These observations prompted us to look for a physical interaction between the Otx2 and Sox2 proteins. GST pulldown assays with tagged proteins demonstrated that Otx2 and Sox2 physically bound to each other *in vitro* (Fig. 4*A* and *B*). Pulldown assays using deletion mutants of these proteins demonstrated the DNA-binding domains of Otx2 and Sox2, the HMG domain of Sox2, and the homeodomain of Otx2 were essential for this binding (Fig. S5 *A–D*). These domains are all remarkably conserved among vertebrates (Fig. S5 *J* and *K*), suggesting similar conservation of the interaction between Otx2 and Sox2. Assays using additional deletion constructs further implicated helices 2 and 3 of the Sox2 HMG domain and the N- and C-flanking amino acids of the Otx2 homeodomain in the modulation of this interaction (Fig. S5 *E–I*). To examine these interactions *in vivo*, we performed coimmunoprecipitation assays using the two-step lysis method, which was developed for the detection of nuclear complexes (27). Proteins were collected from HEK293T cells that transiently expressed tagged Sox2 and/or Otx2. HA-Sox2 expressed alone was not immunoprecipitated by the anti-myc antibody. However, when HA-Sox2 and myc-Otx2 were coexpressed, they were coimmunoprecipitated by the anti-myc antibody (Fig. 4*C*). Reverse immunoprecipitation experiments confirmed this result (Fig. 4*D*). Immunocytochemistry showed that the two proteins colocalized to the nucleus (Fig. S5 *L* and *M*). Collectively, these results demonstrate that Otx2 and Sox2 interact directly with each other both *in vitro* and *in vivo*.

Missense Mutations Identified in the Sox2 HMG Domain Affect Sox2 Activity Associated with Otx2. In severe ocular malformations, the majority of the mutations identified in the *Sox2* locus are frameshift or nonsense mutations and are expected to produce a truncated Sox2 protein (7). However, missense mutations that lead to amino acid changes have been found in three cases (8, 28, 29). Intriguingly, two of these three missense mutations are predicted to alter the conserved residues in helices 2 and 3 of the Sox2 HMG domain (R74P and L97P, respectively), which in the pulldown assays of the present work were revealed to have a role in the interaction with the Otx2 protein (Fig. S5 *E* and *F*).

To characterize the mutated Sox2 proteins in the cases of missense mutations in *Sox2*, we introduced point mutations into the corresponding residues of the *Xenopus* Sox2 protein to generate R74P-Sox2 and L97P-Sox2 (Fig. 5*A*). The helices and tail of the HMG domain come in contact with the minor groove of the double-stranded DNA, and the determined 3D structure of the HMG domain shows that the side chains of the 74th arginine and the 97th lysine protrude opposite to the DNA and are not directly in contact with the DNA (Fig. 5*B*) (30). Initially, we performed luciferase assays to examine whether these mutated proteins could activate transcription in cooperation with Otx2 (Fig. 5*C*). Although Otx2 could induce transcription synergistically with wild-type Sox2, Otx2 could not increase luciferase activity in combination with L97P-Sox2. In addition, R74P-Sox2 drove transcription in combination with Otx2, albeit to a much lesser extent than the wild-type Sox2. We then tested the DNA-binding abilities of these mutated Sox2 proteins by EMSA (Fig. S2*I*). Wild-type Sox2 protein potently bound to the CNS1 fragment, whereas the R74P-Sox2 and L97P-Sox2 proteins did not bind to CNS1. Finally, we examined the binding activities of the mutated Sox2 proteins to Otx2 protein in a GST pulldown assay (Fig. 5 *D* and *E*). Whereas the wild-type Sox2 protein was efficiently pulled down by GST-Otx2, the R74P-Sox2 and L97P-Sox2 proteins were pulled down to markedly lesser extents. These results suggest that the missense mutations in the *Sox2* gene are associated with reductions in the transactivational activity on *Rax* CNS1 and a loss of ability to bind to both DNA and the Otx2 protein.

Discussion

The transcription factor-encoding genes *Pax6*, *Sox2*, and *Otx2* have been identified as the causative genes for human ocular malformation. In the present work, we define the regulatory relationships between these genes (Fig. 5*F*). Previous studies

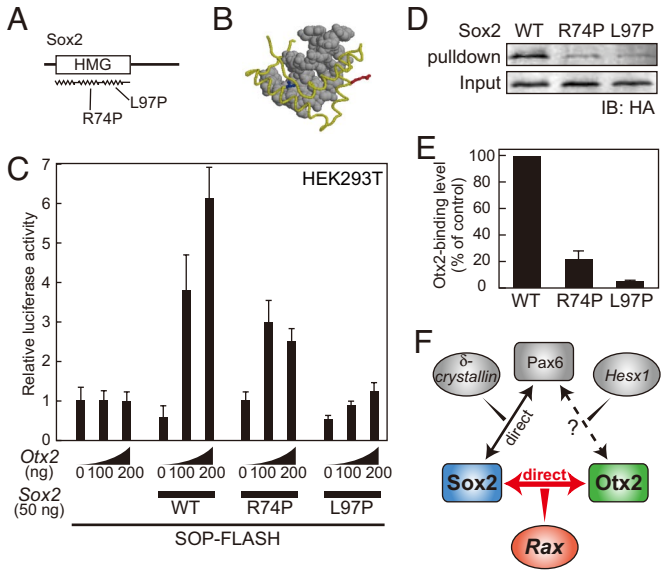

Fig. 5. Missense mutations in the Sox2 HMG domain affect the activities of Sox2 protein associated with Otx2. (*A*) Missense mutations identified in helices 2 and 3 of the human Sox2 HMG domain (R74P and L97P). (*B*) The 3D structure of the Sox2 HMG domain (yellow) binding to DNA (gray), based on a previous study (30). The side chains of the 74th arginine (blue) and 97th lysine (red) do not come in direct contact with the DNA. (*C*) Mutated Sox2 proteins do not induce transcription via CNS1 in cooperation with Otx2. (*D*) GST pulldown assay showing that the mutated Sox2 proteins lose Otx2-binding activity. HA-tagged Sox2 proteins were *in vitro*-synthesized and pulled down by GST-Otx2, followed by Western blotting with antibody against HA-tag. (*E*) Quantification of Otx2-binding levels by using the Odyssey infrared imaging system. Error bars indicate SD values. (*F*) Molecular relationships among ocular malformation-associated genes. The present work demonstrates that a direct interaction between the Otx2 and Sox2 proteins coordinately regulates *Rax* expression (colored). Upstream proteins and target genes are indicated as round rectangles and ovals, respectively. A full scan of the Western blotting data is shown in Fig. S7.

have shown that Pax6 and Sox2 form a complex to coregulate the expression of δ-*crystallin* (11). In addition, the findings that the Otx2-binding sites are required for activation of the chick *Hesx1* promoter and that overexpression of Pax6 in the chick or loss-of-function in mice causes repression or expansion of *Hesx1* expression, respectively (31), indicate that the Pax6 and Otx2 proteins coregulate *Hesx1* expression, although the nature of the interdependence and the form of the direct interaction between Pax6 and Otx2 remain unknown. The final combination of Otx2 and Sox2 was not addressed before the present work. We demonstrate that Otx2 and Sox2 interact directly with each other and synergistically activate *Rax* expression through CNS1. We propose that the genetic and molecular interactions among these three key transcription factors organize the developmental program of the vertebrate eye.

We have also discovered that Otx2 increases the level of *Sox2* mRNA in *Xenopus* animal cap cells. A previous study revealed that multiple *cis*-regulatory elements spatiotemporally control *Sox2* expression in neural development and are conserved among the chicken, mouse, and human (32). We found that the N-2 and N-3 elements contain Otx-binding sites (N-2, AGATTA; N-3, GGATTA) that are perfectly conserved among vertebrates, including the frog (data not shown). This observation raises the possibility that the Otx2 protein directly upregulates *Sox2* expression in *Xenopus* animal cap cells.

To understand the molecular mechanisms underlying transactivation via *Rax* CNS1 regulated by Otx2 and Sox2, we note the unexpected nature of the CNS1 response to Otx2 and Sox2. Our luciferase assays show that combined overexpression of Otx2 and Sox2 drives transactivation of SOP-FLASH (wild-type CNS1), whereas overexpression of Otx2 alone is sufficient for the transactivation of mS-FLASH (CNS1 mutated at the Sox-binding sites), as reported for other regulatory elements (33, 34). We attribute this difference to the existence of a Sox-binding site close to the Otx-binding site (Fig. S6). These results imply that a Sox-binding site possesses repressive activity. It is possible that, in the absence of Sox2, a repressive protein binds preferentially to the Sox-binding site of CNS1 and prevents Otx2 from binding to DNA or transactivating the basic transcription complex. In contrast, in the presence of Sox2, Sox2 may occupy the Sox2-binding sites in place of the repressive protein, thereby causing synergistic transactivation with Otx2. Further studies are needed to understand these intricate regulation mechanisms.

As reported, Sox2 protein has an inhibitory function (35); in mouse embryonic stem cells, elevation of Sox2 levels suppressed the expression levels of Sox2:Oct3/4 target genes. These investigators also showed that the suppression was mediated by the C-terminal region, not the DNA-binding domain, of the Sox2 protein. These findings are consistent with the results obtained in the present work. In the luciferase assay using HEK293T cells, whereas a low level of Sox2 expression (50 ng) caused synergistic activation of SOP-FLASH in a cooperative manner with Otx2, excess Sox2 (100 or 200 ng) inhibited the transcription induced by Otx2 and Sox2 (Fig. 3*F*). In addition, the transcription of mS-FLASH (mutated SOP-FLASH at Sox-binding sites), which was caused by Otx2 alone, was also suppressed by Sox2 (50, 100, or 200 ng; Fig. 3*H*). The fact that the mS-FLASH vector lacks Sox-binding sites suggests that this inhibitory function of Sox2 protein is not dependent on DNA binding but instead requires the C-terminal region of Sox2. Low levels of Sox2 expression (50 ng) sufficiently repressed Otx2-driven mS-FLASH activation, which suggests that transcription from SOP-FLASH is also inhibited by a low level of Sox2 expression (50 ng), whereas the interaction of Sox2 and Otx2 overrides this inhibition, inducing a high level of transactivation.

The stoichiometric association of Sox2 with Otx2 may explain why *Rax* is not expressed in the presumptive brain, in which *Otx2* and *Sox2* mRNA are coexpressed (Fig. S1*B*). It is possible that the ratio of Sox2 to Otx2 is in the range required for transcriptional activation of *Rax* in the optic vesicle, whereas this ratio is too high or too low in the presumptive brain, resulting in the loss of *Rax* expression. Of course, the involvement of other transcription factors in the regulation of *Rax* expression cannot be excluded. Furthermore, previous studies on Pax6–Sox2 cooperation have reported that differences in the sequences of *cis*-regulatory elements result in differences in the threshold protein levels required for the cooperative action to occur (36). The present work permits us to speculate that Otx2 and Sox2 coregulate the expression patterns of multiple target genes in various subdomains of the brain and eye and that the expression levels of the different target genes are controlled by the different stoichiometric ratios of the individual subdomains. Comprehensive identification of the target genes coregulated by Otx2 and Sox2 will allow us to compose a conceptual model for regionalization of the developing brain and eye.

We noted that a gap of six nucleotides between the Otx- and Sox-binding sites in CNS1 was conserved, even if the specific sequence was not conserved. Previous studies have reported that the distance between the Sox2- and Pax6-binding sites is conserved between two *cis*-regulatory elements and that insertions of a few base pairs between these sites ablate the cooperative action of Sox2 and Pax6 (11, 36), which raises the possibility that, in addition to the individual sequences of the binding sites, the gap between two binding sites contributes to the stoichiometric association and the interdependence of Otx2 and Sox2.

In conclusion, our analysis of the upstream region of the frog *Rax* gene reveals molecular linkages among three genes associted with human ocular malformation. The direct interaction and interdependence of the Otx2 and Sox2 proteins coordinate *Rax* expression through a conserved noncoding sequence. These findings of coordinated transcriptional regulation improve our understanding of eye development and ocular malformation in humans.

Materials and Methods

DNA Constructs. SOP-FLASH, mO-FLASH, and mS-FLASH were generated by inserting the multiple repeats of pt, mO-pt, and mS-pt, respectively, into the pGL3-Promoter vector (Promega). Details of the DNA construction are presented in *SI Materials and Methods*.

***Xenopus* Embryos.** Manipulation of *X. laevis* embryos and explants, whole-mount *in situ* hybridization (WISH), and transgenesis were performed as described (37, 38). DNA and mRNA were microinjected into four animal hemispheres of eight-cell-stage embryos. The expression vectors used for mRNA synthesis were: pCS2-XOtx2, pCS2-XSox2, pCS2-myc-XOtx2, pCS2-myc-XSox2, pCS2-dn-XSox2, and pCS2-nls-βgal.

RT-PCR and ChIP. For RT-PCR, total RNA was extracted from 15 animal cap explants or two whole embryos at the midneurula stage by using ISOGEN (Wako). ChIP assays were performed using anti-Otx2, anti-Sox2, or anti-myc antibodies. Details of the RT-PCR and ChIP assays are provided in *SI Materials and Methods*.

Luciferase Assay. The pRL-TK or pGL4.74 (Promega) plasmid was used as an internal control. *Xenopus* embryos and explants injected with the reporter vectors (firefly, 30 pg; *Renilla*, 10 pg) and the mRNA of *Otx2* and *Sox2* (100 pg) were harvested from stage-13 embryos. Cultured cells were transfected with the reporter vectors (firefly, 100 ng; *Renilla*, 10 ng) and the expression vectors pCS2-myc-XOtx2, pCS2-HA-XSox2, pCS2-HA-XSox2-R74P, pCS2-HA-XSox2-L97P, and pCS2-nls-βgal and were harvested 48 h after transfection. Reporter activities were measured by using the dual-luciferase reporter assay system (Promega). Each assay was performed in duplicate, and all results are shown as mean ± SD for at least three independent assays.

GST Pulldown Assays, Coimmunoprecipitation Assays, and Western Blotting. GST pulldown assays were performed according to standard procedures. Coimmunoprecipitation assays were performed according to the two-step lysis method (27). The details of these assays are provided in *SI Materials and Methods*.

Bioinformatics. The EMBOSS software was used for total sequence analyses (39). For comparative genomic analyses, the VISTA Browser (http://genome.lbl.gov/vista) was used (24). The 3D model of the Sox2 HMG domain was created by using Rasmol based on Protein Data Bank (PDB) ID code 1GT0.

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