Erratum

**CBF1 controls the retinotectal topographical map along the anteroposterior axis through multiple mechanisms**


An error in this article was not corrected before going to press.

The legend to Fig. 3 should read ‘CBF1/RCAS-electroporated retinae’ and not ‘CBF1/RCAS-electroporated reginae’.

We apologise to readers and the authors for this mistake.
CBF1 controls the retinotectal topographical map along the anteroposterior axis through multiple mechanisms

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Summary

Chick brain factor 1 (CBF1), a nasal retina-specific winged-helix transcription factor, is known to prescribe the nasal specificity that leads to the formation of the precise retinotectal map, especially along the anteroposterior (AP) axis. However, its downstream topographic genes and the molecular mechanisms by which CBF1 controls the expression of them have not been elucidated. We show that misexpression of CBF1 represses the expression of EphA3 and CBF2, and induces that of SOHo1, GH6, ephrin A2 and ephrin A5. CBF1 controls ephrin A5 by a DNA binding-dependent mechanism, ephrin A2 by a DNA binding-independent mechanism, and CBF2, SOHo1, GH6 and EphA3 by dual mechanisms. BMP2 expression begins double-gradiently in the retina from E5 in a complementary pattern to Vventroptin expression. Vventroptin antagonizes BMP2 as well as BMP4. CBF1 interferes in BMP2 signaling and thereby induces expression of ephrin A2. Our data suggest that CBF1 is located at the top of the gene cascade for the regional specification along the nasotemporal (NT) axis in the retina and distinct BMP signals play pivotal roles in the topographic projection along both axes.

Key words: Retina, Chick, Retinotectal projection, CBF1, BMP2

Introduction

One of the most common characteristics of axonal connection patterns in the nervous system is their organization into topographic maps, in which the spatial order of neurons at the origin is reflected in the order of their axon terminals in the target. The projection from the retina to the tectum, or its mammalian homolog, the superior colliculus (SC), has been a good model system for studies of topographic map formation. In this system, retinal ganglion cell axons from the anterior (nasal) retina project to the posterior region of the tectum (or SC), while axons from the posterior (temporal) retina project to the anterior tectum. Along the dorsoventral (DV) axis of the retina, the dorsal and ventral retinal axons are connected to the ventral (lateral) and dorsal (medial) tectum (SC), respectively. Recent studies have implicated the Eph family of receptor tyrosine kinases and their ligands, ephrins, as the topographic molecules in the retinotectal system.

The Eph family of receptors are classified into two subfamilies, EphA and EphB, according to their preference for either glycosyl-phosphatidyl-inositol (GPI)-anchored ephrin A ligands or transmembrane ephrin B ligands (Eph Nomenclature Committee, 1997). In chick, the EphA3 receptor is expressed in a temporal high/nasal low gradient in the retinal ganglion cells (Cheng and Flanagan, 1994), and ephrin A2 and ephrin A5 are expressed in posterior high/anterior low gradients in the tectum (Cheng and Flanagan, 1994; Drescher et al., 1995; Feldheim et al., 1998; Frisén et al., 1998). Several lines of evidence suggest that ephrin A ligands are repulsive to EphA-expressing axons (Cheng and Flanagan, 1994; Drescher et al., 1995; Feldheim et al., 1998; Frisén et al., 1998), and thereby elongation of retinal axons stop at precise locations along the AP axis on the tectum (SC) (Frisén et al., 1998; Brown et al., 2000; Feldheim et al., 2000).

In addition to EphA3, four EphA receptors (EphA4, EphA5, EphA6 and EphA7) are uniformly expressed in the chick retina (Monschau et al., 1997; Connor et al., 1998). On the other hand, ephrin A2 and ephrin A5 are expressed in nasal high/temporal low gradients in the retina (Marcus et al., 1996; Connor et al., 1998; Hornberger et al., 1999). Overexpression of ephrin As in temporal axons leads to errors in the topographic targeting of temporal axons (Dütting et al., 1999; Hornberger et al., 1999), suggesting a role for retinal ephrin As in the formation of topographic projections. Thus, EphAs uniformly expressed in the retina are also thought to be involved in the topographic projection along the AP axis, together with ephrin As gradiently expressed in the retina.

The achievement of graded distributions of topographic molecules along the AP and DV axes in the retina and tectum during development is a crucial step in the formation of the topographic map. From the early developmental stages of the retina and tectum, a number of molecules that belong to morphogens and transcription factors show asymmetrical expression patterns along the two axes. Studies on some of these molecules have demonstrated that they regulate the graded distributions of topographic molecules, which finally lead to the topographic projection, along the two axes in the retina and optic tectum. We previously found that two winged-helix (WH) transcription factors, chick brain factor 1 and 2
(CBF1 and CBF2), are expressed specifically in the nasal and temporal regions of the developing chick retina, respectively (Yuasa et al., 1996). We further demonstrated that ectopic misexpression of CBF1 or CBF2 reversed the topographic map in the retinotectal system along the AP axis (Yuasa et al., 1996). Although CBF1 and CBF2 are supposed to determine the regional specificity along the NT axis in the retina through the regulation of expression of their downstream target genes, their modes of action remain unknown. In addition to CBF1, two homeobox transcription factors, SOHo1 and GH6, are also expressed specifically in the nasal region of the developing retina (Schulte and Cepko, 2000). Misexpression of these molecules in the retina results in projection errors of retinal axons along the AP axis, due to the repression of EphA3 expression (Schulte and Cepko, 2000). As CBF1 and CBF2 are expressed in the retina before the expression of SOHo1 and GH6 starts, there is the possibility that these homeobox transcription factors are downstream target genes of CBF1 or CBF2. However, the relationship between CBFs and these homeobox proteins has not been fully investigated.

We recently identified a novel secreted molecule, Ventroptin, which is an antagonist of bone morphogenetic protein 4 (BMP4) in the retina, and demonstrated that Ventroptin is implicated in the retinotectal topographic projection along both the DV and AP axes (Sakuta et al., 2001). At the early developmental stages of the eye, Ventroptin is specifically expressed in the ventral retina, in a complementary pattern to that of the dorsal-specific expression of BMP4. The counteraction between BMP4 and Ventroptin governs the regional specification along the DV axis by regulating the distributions of downstream target genes, such as Tbx5 and Vax (Sakuta et al., 2001). At the later stages (E6 onward), Ventroptin begins to be expressed in a nasal high/temporal low gradient, in addition to a ventral high/dorsal low gradient. This is the first demonstration of a molecule with a double-gradient pattern of expression in the retina. Asymmetrical expression of Ventroptin along the NT axis regulates the graded expression of ephrin A2 along this axis, but not of ephrin A5 or EphA3 in the retina, which is associated with the retinotectal mapping along the AP axis (Sakuta et al., 2001). As expression of BMP4 declines in the retina from E5, a member of the TGF family other than BMP4 is supposed to appear in a temporal high/ nasal low gradient along the NT axis with a complementary pattern to the Ventroptin expression (Sakuta et al., 2001).

In the present study, to gain insight into the downstream target genes of CBF1, we employed, for the first time, electroperoration of a retroviral vector carrying the CBF1 gene into the optic vesicle, and examined effects of the misexpression of CBF1 on the expression of topographic molecules and other asymmetrically distributed molecules. The in ovo electroperoration of retrovirus vector at Hamburger-Hamilton (HH) stage 8 (Hamburger and Hamilton, 1951) allows the immediate (at stage 10) and sustained expression of a transgene. We show that ectopic expression of CBF1 in the temporal retina represses expression of EphA3 and CBF2, and induces that of SOHo1, GH6, ephrin A2 and ephrin A5. The mode of action of CBF1 on these molecules has been revealed to be classified into three distinct categories. In addition, we show that a TGFβ family member, BMP2, begins to be expressed in a pattern complementary to that of Ventroptin, with a double-gradient along the NT and DV axes from E5 onward. Misexpression of BMP2 in the developing retina represses expression of Ventroptin and ephrin A2. Moreover, we demonstrate that CBF1 perturbs BMP signaling through a DNA binding-independent mechanism, which resultanty leads to the induction of ephrin A2 expression. Based on these results, we suggest that CBF1 plays pivotal roles in the determination and maintenance of the NT specificity in the retina by integratively regulating the expression of asymmetrically distributed molecules through multiple mechanisms.

### Materials and methods

#### Plasmids

To prepare the CBF1/RCAS construct, the coding region of CBF1 was amplified from a retroviral construct expressing CBF1 [CBF1/RCAS (B)] (Yuasa et al., 1996), subcloned once into a shuttle vector SLAX-NS containing the 5’-noncoding region of the Src gene (Suzuki et al., 2000), and then transferred into the RCAS-NS retrovirus vector (Suzuki et al., 2000).

To make CBF1eve/RCAS, the repression domain of the Drosophila Even-skipped protein (Han and Manley, 1993) from pCS2eve was first fused with CBF1 DNA binding domain (amino acid residues from 139 to 253). It was inserted into SLAX-NS myc, which was prepared with a shuttle vector SLAX-NS by inserting double myc-epitope tags at the Ncol-EcoRI site, and then subcloned into RCAS-NS retrovirus vector using NotI and SpeI sites. The CBF1AA mutant was generated by substitutions of asparagine 189 and histidine 193 with alanines by PCR, and cloned into RCAS-NS or pcDNA3.1(+)(Invitrogen). To prepare the BMP2/RCAS construct, the coding region of mouse BMP2 was once inserted into SLAX-NS, and then subcloned into RCAS-NS. Ventroptin/RCAS was described previously (Sakuta et al., 2001).

Mouse Smad1, Smad4 and Alk3 were cloned from a P0 mouse retina cDNA library by PCR and their sequences were verified by DNA sequence analysis. Smad1 and Smad4 were fused with double flag-epitope tags at the N terminus by PCR. A constitutively active mutant Alk3 (Alk3-CA) was generated by substitution of glutamine 233 with aspartic acid by PCR as described previously (Hoodless et al., 1996). They were subcloned into pcDNA3.1(+) vector.

#### In ovo electroporation

In ovo electroporation was performed as described previously (Sakuta et al., 2001). Retrovirus constructs for the electroporation were suspended at a concentration of 0.1-1.0 μg/μl in 10 mM Tris-HCl, 0.25 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0 containing 0.05%(w/v) Fast Green (Sigma). Embryos were electroporated at HH stage 8 and incubated in a humidified incubator. The embryos of normal size and morphology were used for assays.

#### In situ hybridization and riboprobes

Section in situ hybridization and whole-mount in situ hybridization were carried out as described previously (Suzuki et al., 2000). All the samples were treated in the same way throughout the study. Image acquisition and figure processing of the sections and whole-mount samples were performed as reported (Suzuki et al., 2000). We always compared electroporated experimental side with non-electroporated control side of one and the same embryo or section.

Templates used for digoxigenin-labeled RNA probes were as follows: the 516 bp fragment of chick CBF1 (nucleotide residues 1252-1761; GenBank Accession Number U47275), the 656 bp fragment of chick CBF2 (1191-1846; U47276), the 724 bp fragment of chick EphA3 (2267-2990; M68514), the 541 bp fragment of chick ephrin A2 (2267-2990; M68514), the 491 bp fragment of chick ephrin A5 (25-705; X90377), the 491 bp fragment of chick ephrin A6 (1-491; AF317286), the 782 bp fragment of chick SOHo1 (34-815; S69380),...
the 567 bp fragment of chick GH6 (4-570; AF227921) and the 1179 bp fragment of chick BMP2 (1-1179; AY237249). The template for the probe of Ventroptin has been described previously (Sakuta et al., 2001).

**Northern blot analysis**

Total RNA was prepared from one-third of the nasal or temporal E8 chick retina electroporated with CBF1/RCAS, with Trizol Reagent (Invitrogen) following the manufacturer’s protocol. Control RNA were prepared from the left eye of the same embryos. Northern blot analysis with 20 μg of total RNA was performed as described previously (Suzuki et al., 2000). Templates used for probe preparation were as follows: the same fragments of chick EphA3, ephrin A2 and ephrin A5 used for in situ hybridization, the 300 bp fragment of chick CBF2 (nucleotide residues 1418-1717; GenBank Accession Number U47276), and the 477 bp fragment of chick glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (218-794; K01458).

**DNA pull-down assay**

Chick embryonic fibroblasts were transfected with retrovirus vectors for myc-tagged CBF1 or myc-tagged CBF1AA mutant using Lipofectamine plus (Invitrogen), and cultured for a week. The nuclear extract preparation and DNA pull-down assay were performed as described previously (Mukai et al., 2002). For DNA pull-down assays, biotinylated double-stranded oligonucleotides derived from the sequence named B2 in the HNF1α promoter were synthesized by Qiagen: B2 is known as the BF-1 binding sequence (Li et al., 1995). The eluates of precipitates were subjected to SDS-PAGE, followed by immunoblot analyses with the anti-myc primary antibody 9E10 (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences). CBF1 or its mutant proteins were visualized by chemiluminescence using ECL plus (Amersham Biosciences) and the lumino-image analyzer LAS-1000plus (Fujifilm).

**Luciferase assay**

A minimal promoter construct (pGL3ti) was first made from pGL3-basic reporter vector (Promega) by inserting oligonucleotides carrying the adenovirus major-late promoter TATA box and the mouse terminal deoxynucleotidyl transferase gene initiator in the BglII site as described previously (Jonk et al., 1998). The BMP responsive reporter (pGL3ti-12GCCG) was constructed by further inserting four oligonucleotides, each containing three copies of the GCCG motif (Kusanagi et al., 2000), into the XhoI site of pGL3ti. Luciferase assays were carried out using human embryonic kidney 293 cells. Cells grown in 96-well microplates at a density of 4·10⁴ cells per well were transiently transfected with expression plasmids in a combination of the reporter (50 ng), receptor (10 ng) and Smads (10 ng each), together with or without the expression plasmid for CBF1 (20 ng) or CBF1AA (20 ng). Luciferase activity was measured with a Dual-Glo Luciferase Assay System (Promega) using Fluoroskan Ascent FL (Labsystems). Total amounts of the transfected DNAs were kept the same throughout the experiments by addition of the empty vector, and firefly luciferase activities were normalized using the Renilla luciferase activity of phRL-SV40 (Promega) co-transfected.

**Isolation of cDNA fragments encoding TGF-β family molecules**

A set of degenerative oligonucleotide primers were synthesized based on the regions conserved among TGFβ family molecules except for the growth differentiation factor (GDF) subfamily; the 5′ primer sequence was 5′-TGGVANGAYTGATHRNTNGC-3′ and the 3′
Results
Expression patterns of asymmetrically distributed molecules along the NT axis in the retina

During retinal development, two winged-helix transcription factors, CBF1 and CBF2, begin to be expressed in the nasal and temporal regions, respectively, prior to HH stage 10 (Yuasa et al., 1996; Yamagata et al., 1999). After that, two homeobox transcription factors, SOHo1 and GH6 (from HH stage 12-14), and then EphA3 (from HH stage 15) begin to be asymmetrically expressed along the NT axis in the retina (Yamagata et al., 1999; Schulte and Cepko, 2000).

Although ephrin A5 and ephrin A2 are also known to be expressed in nasal high/temporal low gradients (Cheng and Flanagan, 1994), their spatial and temporal expression patterns in the retina had not been fully investigated. Thus, we examined their expression during development of the chick retina by whole-mount and section in situ hybridization. Expression of ephrin A5 was first detected in the optic vesicle at HH stage 12, while asymmetrical distributions were not detected (Fig. 1A). At stage 18, expression of ephrin A5 was absent in the dorsal and ventral regions, but observed in the nasal and temporal retina (Fig. 1B, part a). At this stage, the expression was significantly stronger on the nasal than temporal side (Fig. 1B, part a). It was evident also by section in situ hybridization. In a section along the NT axis, ephrin A5 expression was observed in the nasal and temporal third of the retina, although the expression was stronger on the nasal than temporal side (Fig. 1B, part b). Thus, ephrin A5 was not expressed in a nasal-specific fashion at stage 18 (E3). At E6, a nasal high/temporal low gradient was clearly visible for the first time, although the expression was also observed in the periphery of the temporal retina (data not shown). At E8, a similar nasal high/temporal low expression gradient and expression in the temporal periphery was observed (Fig. 1C, parts a-c). At this stage, ephrin A5 transcripts in the central retina were mainly distributed in the ganglion cell layer (GCL), while those in the peripheral regions were observed in whole cell layers like the retina at early stages (Fig. 1C, parts a-c).

By contrast, the onset of ephrin A2 expression in the retina was quite late: expression of ephrin A2 was not detected until E4 (Fig. 1D) and began to be observed from E5 in the nasal retina (data not shown). From E6 onwards, a nasal high/temporal low gradient of ephrin A2 expression was observed predominantly in the GCL (Fig. 1E, parts a-c): The gradient of ephrin A2 expression in the GCL appeared to be slighter than that of ephrin A5 expression at E6 and E8 (Fig. 1E, part b and data not shown). Although ephrin A2 expression was also detected in cell layers other than the GCL, the expression appeared to be rather uniform along the NT axis, as compared with that of ephrin A5 (compare Fig. 1C, parts b,c with Fig. 1E, parts b,c). It is also known that ephrin A6 is predominantly expressed in the nasal retina (Menzel et al., 2001); however, we could not detect the signal by in situ hybridization.

Misexpression of CBF1 affects expression patterns of asymmetrically distributed molecules along the NT axis in the retina

We examined the expression of the above-mentioned molecules when a replication-competent avian retrovirus RCAS-BP vector carrying the CBF1 gene (CBF1/RCAS) was introduced into the optic vesicle of HH stage 8 embryos by in ovo electroporation. The transgene expression could be detected as early as HH stage 10, prior to the stage when the polarity along the NT axis appears to be determined, and persisted (data not shown).

At first, we examined the expression of SOHo1, GH6, EphA3 and CBF2 at HH stage 18-20 (E3) in the electroporated embryos, because these molecules clearly show graded
distributions along the NT axis in the native embryos at this stage. At E3, SOHo1 and GH6 were expressed mainly in the nasal retina and the lens ectoderm but not in the temporal retina (Fig. 2A,C), while EphA3 expression was observed in the temporal retina (Fig. 2E). Here, the EphA3 expression was more faithfully complementary to that of SOHo1 than that of GH6.

When CBF1 was misexpressed in the retina, the expression of SOHo1 (Fig. 2B; five out of eight embryos) and GH6 (Fig. 2D; four out of six embryos) was markedly induced in the temporal retina. By contrast, misexpression of CBF1 resulted in loss of EphA3 expression in the temporal retina (Fig. 2F; eight out of nine embryos). We previously reported that misexpressed CBF1 repressed the expression of the CBF2 gene (Yamagata et al., 1999). Consistent with this observation, misexpression of CBF1 completely repressed the temporal-specific expression of CBF2 at E3 (Fig. 2H; four out of five embryos).

We next examined the expression of EphA3, ephrin A5, ephrin A2 and CBF2 at E8. At this stage, these molecules distribute asymmetrically along the NT axis in the retina. Misexpression of CBF1 continuously repressed the expression of EphA3 in the GCL in the temporal retina (Fig. 3B,E; four out of five embryos). In some embryos, a complete loss of EphA3 expression was observed (two out of five embryos). Conversely, CBF1 misexpression induced ectopic expression of ephrin A5 (Fig. 3G,J; five out of five embryos) and ephrin A2 (Fig. 3L,O; five out of seven embryos) in the GCL in the temporal retina: In the control retina, the expression of these ephrins is weak in the GCL in the temporal retina (Fig. 3I,N). The expression of CBF2 in the temporal retina was completely repressed by the misexpression of CBF1 (Fig. 3Q,T; five out of six embryos). We further examined the effects of misexpressed CBF1 on expression of the EphA4 receptor, which is uniformly expressed in the developing retina (Holash and Pasquale, 1995; Düttling et al., 1999; Hornberger et al., 1999). When CBF1 was misexpressed in the developing retina, the expression of EphA4 was not affected (data not shown).

The effects of misexpressed CBF1 on the expression of EphA3, ephrin A5, ephrin A2 and CBF2 were verified by northern blot analysis. Expression of the EphA3 transcript was significantly repressed on the temporal side of the retina by misexpression of CBF1 (Fig. 3C). By contrast, CBF1 misexpression induced expression of ephrin A5 and ephrin A2 transcripts on the temporal sides (Fig. 3H,M). Interestingly, the expression of ephrin A2 and ephrin A5 was also enhanced in
the nasal retina, where these ephrins were endogenously expressed. From the in situ hybridization, however, this effect was not clearly detectable due to homogeneous enhancement. Finally, consistent with the results of in situ hybridization, the expression of CBF2 in the temporal retina was completely abolished by the misexpression of CBF1 (Fig. 3R).

**Repressing construct of CBF1 similarly regulates expression of SOHo1, GH6, EphA3, CBF2, and ephrin A5 as the wild-type, but not expression of ephrin A2**

We next analyzed the mode of action of CBF1. As BF-1 is suggested to act as a transcripational repressor (Li et al., 1995; Bourguignon et al., 1998), we examined whether this function is essential to regulate the expression of SOHo1, GH6, EphA3, CBF2, ephrin A5 and ephrin A2 in the developing retina. We designed a RCAS vector (CBF1-eve/RCAS) expressing a chimeric protein that consists of an even-skipped repression domain (Han and Manley, 1993) and a winged-helix DNA binding domain (WH domain) of CBF1 as shown in Fig. 4A. It has been demonstrated that nuclear localization of CBF1 is determined by a sequence within the WH domain (Chang et al., 1996). Consistent with this report, the chimeric protein was found to be concentrated in the nucleus when expressed in the chick embryonic fibroblasts (Fig. 4B), and in the chick retinal cells (data not shown).

In ovo electroportation of CBF1-eve/RCAS at HH stage 8 in the optic vesicle resulted in an expansion of SOHo1 (Fig. 4C, part a; 12 out of 20 embryos) and GH6 expression (Fig. 4C, part b; 15 out of 24 embryos) into the temporal side of the retina at E3. On the other hand, expression of EphA3 (Fig. 4C, part c; 14 out of 17 embryos) and CBF2 (Fig. 4C, part d; three out of three embryos) in the temporal retina was repressed by electroporation of CBF1-eve/RCAS. At E8, continuous repression of EphA3 expression (Fig. 4D, part a; eight out of eight embryos) and induction of ephrin A5 expression (Fig. 4D,
part d; six out of eight embryos) were observed in the temporal retina as expected. However, surprisingly, the expression of ephrin A2 was not affected by the introduction of RCAS/CBF1-eve (Fig. 4D, part f; eight out of eight embryos). These results suggest that CBF1 functions as a transcriptional repressor for the regulation of expression of SOHo1, GH6, EphA3, CBF2 and ephrin A5. However, expression of ephrin A2 appeared to be regulated by CBF1 through a different mechanism.

Mutant CBF1 deficient in DNA binding ability exerts similar effects on expression of SOHo1, GH6, EphA3, CBF2 and ephrin A2 as the wild-type CBF1, but not on expression of ephrin A5

We next tested whether DNA binding of CBF1 is essential for the activity to regulate the expression of asymmetrically distributed molecules. Two amino acid residues in the WH domain of murine BF-1, Asn219 and His223, are suggested to be important for binding to DNA, and it has been shown that the mutant generated by changing these two residues to alanine (AA mutant) is devoid of DNA binding ability (Dou et al., 2000). We prepared CBF1AA/RCAS to express the mutant CBF1 protein in which Asn189 and His193 were changed to alanine: These two are equivalent to Asn219 and His223 in murine BF-1, respectively (Fig. 5A). We examined the ability of the wild-type CBF1 and CBF1AA mutant proteins to bind to the BF-1 binding sequence by DNA pull-down assay beforehand. The wild-type protein was co-precipitated with the double-stranded DNA fragment containing the BF-1 binding sequence (Fig. 5B, part a). However, CBF1AA was not co-precipitated (Fig. 5B, part a), although sufficient amounts of mutant proteins were expressed (Fig. 5B, part b). Thus, the mutation at Asn189 and His193 abolishes the ability of CBF1 to bind the BF-1 binding sequence.

Then, we misexpressed the CBF1AA mutant in the developing retina by in ovo electroporation at HH stage 8. When the mutant protein was misexpressed, positive regions for SOHo1 (Fig. 5C, part a; 21 out of 34 embryos) and GH6 (Fig. 5C, part b; six out of 14 embryos) expanded into the temporal sides in the E3 retina. On the other hand, expression of EphA3 (Fig. 5C, part c; five out of six embryos) and CBF2 (Fig. 5C, part d; four out of four embryos) in the temporal

Fig. 5. Misexpression of CBF1AA mutant proteins alters expression of SOHo1, GH6, EphA3, CBF2 and ephrin A2, but not ephrin A5. (A) Schematic representation of myc-tagged CBF1AA mutant. The CBF1AA mutant is deficient for DNA-binding activity because of substitutions of asparagine 189 and histidine 193 with alanines. (B) DNA pull-down assays using the nuclear extracts prepared from chick embryonic fibroblasts transfected with myc-tagged CBF1 or CBF1AA (a). Western blot analysis using anti-myc primary antibody indicated the amounts of nuclear extracts used in the DNA pull-down assays (b). (C) Whole-mount in situ hybridization of E3 (stage 18-20) chick embryos transfected with CBF1AA/RCAS using antisense probes for SOHo1 (a), GH6 (b), EphA3 (c) or CBF2 (d). The normal expression of SOHo1, GH6, EphA3 or CBF2 in the control eyes, is shown in insets. Arrowheads indicate the border of the endogenous expression. (D) Horizontal section in situ hybridization of E8 retina transfected with CBF1AA/RCAS using antisense probes for EphA3 (a,b), ephrin A3 (c,d) or ephrin A2 (e,f). The temporal regions of untransfected retinae (control) are shown in the left panels (a,c,e), and those of the transfected ones in the same embryos are shown in the right panels (b,d,f). Scale bar: 100 μm.
region (a). When BMP2/RCAS was electroporated in the retina, expression of ephrin A2 was completely repressed (b). (E) Horizontal section in situ hybridization of E8 temporal retina transfected with CBF1/RCAS (b) or CBF1AA/RCAS (c) with a probe for BMP2. Electroporation of CBF1/RCAS (b) or CBF1AA/RCAS (c) resulted in repression of BMP2 expression in the GCL in the temporal retina of E8 embryos. The temporal region of untransfected retinae (control) are shown in a.c.

CBF1 inhibits the BMP signaling in the retina

We previously demonstrated that a BMP antagonist, Vimentin, promotes expression of ephrin A2 but not ephrin A5 in the developing retina (Sakuta et al., 2001). In addition, Noggin, a structurally unrelated BMP antagonist, has the same activity as Vimentin in the promotion of ephrin A2 expression (Sakuta et al., 2001). These results indicate that BMP signaling regulates the expression of ephrin A2, which prompted us to examine the possibility that CBF1 modulates BMP signaling. BMPs transduce their signals into the cell through a family of mediator proteins known as Smads. Upon phosphorylation by the BMP receptors, Smad1 associates with Smad4 and

Fig. 6. CBF1 inhibits BMP signaling. (A) A luciferase reporter assay using a BMP-responsive reporter construct in the presence of CBF1 or CBF1AA mutant. HEK 293 cells transfected without (−) or with (+) pcDNA/ALK3-CA, Smad1, and Smad4 were measured for luciferase activities in the presence of pcDNA (vector), CBF1/pDNA (CBF1) or CBF1AA/pDNA (CBF1AA) by co-transfection. The values are represented by fold induction compared with the basal activity of pcDNA (vector). Data are shown as the means±s.d. of triplicate experiments. (B) Expression of BMP2 and Vimentin in the E8 chick retina. Section in situ hybridization was performed with antisense probes for BMP2 (a,b) and Vimentin (c,d). BMP2 was expressed in a double-gradient pattern that is complementary to Vimentin. (a,c) Horizontal sections. Nasal (anterior) is upwards, temporal (posterior) is downwards. (b,d) Coronal sections. Dorsal is upwards, ventral is downwards. Scale bars: 600 μm. (e) Schematic representation of the expression patterns of BMP2 and Vimentin in the retina. (C) Horizontal section in situ hybridization of E8 temporal retina transfected with Vimentin/RCAS (b) or BMP2/RCAS (d). In the temporal region of a control retina, BMP2 was expressed in the GCL and INL (a). When Vimentin/RCAS was electroporated, expression of BMP2 was completely repressed (b). In the nasal region of a control retina, Vimentin was expressed in the INL (c). When BMP2/RCAS was electroporated, expression of Vimentin was completely repressed (d). Scale bar: 100 μm. (D) Horizontal section in situ hybridization of E8 temporal retina transfected with BMP2/RCAS (b) with a probe for ephrin A2. Ephrin A2 was mainly expressed in the GCL in the nasal region (a). When BMP2/RCAS was electroporated in the retina, expression of ephrin A2 was completely abolished. At E8, repression of the EphA3 expression was continuously observed in the temporal retina (Fig. 5D, part b; four out of four embryos). In addition, induction of ephrin A2 expression (Fig. 5D, part f; four out of five embryos) was observed in the temporal retina. However, expression of ephrin A5 was not affected by the introduction of CBF1AA/RCAS (Fig. 5D, part d; six out of six embryos). These results suggest that the DNA binding activity of CBF1 is necessary for the regulation of the expression of ephrin A5. However, expression of SOHo1, GH6, EphA3, CBF2 and ephrin A2 can be regulated by CBF1 through a DNA binding-independent mechanism.
translocates into the nucleus where the complex recruits DNA-binding proteins to activate specific gene transcription.

Indeed, the reporter activity was stimulated when a constitutively active BMP receptor, Smad1 and Smad4 expression plasmids were co-transfected into HEK293 cells with a reporter construct that consists of a BMP-responsive promoter and the luciferase gene (Fig. 6A). Using this reporter assay system, we examined whether CBF1 interferes with BMP signaling. We found that CBF1 completely inhibited the activation of the reporter gene by the Smad complex (Fig. 6A). In addition, the CBF1AA mutant also blocked the activation of the reporter gene (Fig. 6A). These results indicate that CBF1 inhibits BMP signaling by a DNA binding-independent mechanism.

**BMP2 begins to be expressed in the dorso-temporal retina**

At the late developmental stages of the retina (E6 onward), we have speculated that unknown TGF-β family molecule other than BMP4 begins to be expressed double-gradiently with an expression pattern complementary to that of Ventratin. To identify this unknown TGFβ family molecule, we performed RT-PCR with degenerated primers based on the conserved amino acid sequences of the TGFβ family molecules. We identified cDNA fragments encoding *BMP2, BMP4, BMP7, growth differentiation factor 5 (GDF5), GDF6 and GDF7* from E8 chick retinal RNA. By analyzing expression patterns of these molecules by in situ hybridization, we found that *BMP2* is expressed in a pattern complementary to *Ventratin* along both the NT and DV axes (Fig. 6B, parts a,b). Expression of the other molecules appeared to be uniform along the NT axis or was not detected by in situ hybridization due to low levels in the chick retina (data not shown).

Weak expression of *BMP2* was first observed in the dorso-temporal retina at E5 (data not shown). From E6 onwards, a temporal high/nasal low expression gradient as well as a dorsal high/ventral low gradient of *BMP2* expression became evident (Fig. 6B, parts a, b). The *BMP2* expression was positive in the GCL and inner nuclear layer in the central retina, and in the whole layers in the periphery (Fig. 6B, parts a, b). This is somewhat different from that of *Ventratin*: Expression of *Ventratin* is not observed in the GCL. The *BMP2* expression was restricted to the dorso-temporal one third of the retina, while the *Ventratin* expression expanded into the dorso-temporal retina (Fig. 6B, parts a-e).

**BMP2 controls *ephrin A2* expression along the NT axis**

The complementary expression patterns of *Ventratin* and *BMP2* suggest that these molecules repress the expression of their counterpart. To test this possibility, we examined effects of misexpression of these molecules. When *Ventratin* was misexpressed in the retina by in ovo electroporation, *BMP2* expression in the temporal regions was completely repressed (Fig. 6C, part b; seven out of seven embryos). Similarly, misexpression of *BMP2* inhibited expression of *Ventratin* in the nasal retina (Fig. 6C, part d; six out of six embryos). We previously demonstrated that misexpression of *Ventratin* induces *ephrin A2* expression in the temporal retina (Sakuta et al., 2001). Therefore, we next tested effects of *BMP2* misexpression on *ephrin A2* expression. Misexpression of *BMP2* repressed expression of *ephrin A2* in the nasal retina (Fig. 6D, part b; seven out of nine embryos), suggesting that the counteraction between Ventratin and BMP2 controls the graded distributions of *ephrin A2* along the NT axis.

Finally, to address whether CBF1 is involved in the regulation of BMP signaling in the developing retina, we examined the expression of *Ventratin* and *BMP2* when *CBF1* was misexpressed in the retina. Expression of *BMP2* in the GCL was repressed by misexpression of *CBF1*, while expression in the inner nuclear layer was not reduced (Fig. 6E, part b; five out of nine). On the other hand, expression of *Ventratin* appeared not to be altered by misexpression of *CBF1* (data not shown; six out of six embryos). These phenotypes were recapitulated when the CBF1AA mutant was misexpressed (Fig. 6E, part c; four out of nine embryos). These results suggest that CBF1 represses the expression of BMP2 in the ganglion cells by inhibiting BMP signaling. In conclusion, these results are consistent with our view that CBF1 regulates the *ephrin A2* expression through inhibition of BMP signaling (Sakuta et al., 2001).

**Discussion**

*In ovo* electroporation of replication-competent retrovirus vector allows immediate and sustained expression of transgenes

We previously reported that misexpression of *CBF1* in the retina did not alter the distribution of *EphA3* (Yuasa et al., 1996; Yamagata et al., 1999). Another group reported that the expression of *SOhO1* and *GH6* was not affected by misexpression of *CBF1* (Schulte and Cepko, 2000) using our retroviral construct CBF1/RCAS (B), a replication-competent retroviral vector carrying the *CBF1* gene (Yuasa et al., 1996). These experimental results were obtained through the infection of retroviral particles or *in ovo* electroporation of the pMiw expression plasmid vector to misexpress *CBF1* in the developing retina. By virus infection, it usually takes more than 12 hours for the expression of transgenes to start, whereas by *in ovo* electroporation of the plasmid vector, the expression of transgenes begins immediately (within 3 hours) but it stops in a few days. In the present study, the expression level of the CBF1 transgene was improved by modification of the last RCAS construct (Yuasa et al., 1996): We replaced the long 5'-untranslated region of *CBF1* in the previous construct with the 5'-untranslated region of the *src* gene that confers efficient expression on heterologous coding sequences (Morgan and Fekete, 1996; Suzuki et al., 2000) (see Materials and methods). In addition, we employed *in ovo* electroporation of CBF1/RCAS DNA instead of virus infection.

This strategy allowed the immediate and sustained expression of the transgene, because the mRNA is directly transcribed from the incorporated RCAS vector DNA shortly after the gene transfer, which is followed by the production of retroviral particles and widespread infection in the retina. When CBF1/RCAS was introduced by *in ovo* electroporation into the optic vesicle of HH stage 8 embryos, the transgene expression was detected at 6 hours after the electroporation, as early as HH stage 10, just before the stage at which the polarity along the NT axis appears to be determined by the endogenous CBF1 expression (Dütting and Meyer, 1995; Dütting and Thanos, 1995; Thanos et al., 1996), indicating that this in ovo
electroporation method fulfills the requirements for the functional study of CBF1.

**CBF1 controls all of the asymmetrically distributed molecules along the NT axis in the developing retina**

We show that misexpression of CBF1 in the temporal retina results in induction of SOHo1, GH6, ephrin A2 and ephrin A5, and down-regulation of EphA3 and CBF2 expression (Fig. 7). These results indicate that all of the asymmetric molecules so far identified to be involved in the formation of the topographic map along the AP axis (Yuasa et al., 1996; Drescher et al., 1995; Nakamoto et al., 1996; Schulte and Cepko, 2000; Dütting et al., 1999; Hornberger et al., 1999) are downstream genes of CBF1. Endogenous expression of CBF1 begins around HH stage 8-9 in the retina, and is confined to the nasal retina prior to HH stage 11 (Yuasa et al., 1996; Yamagata et al., 1999), preceding all other genes that are asymmetrically expressed along the NT axis during development. CBF2 is first asymmetrically expressed in the temporal retina at HH stage 11 (Yamagata et al., 1999). Expression of SOHo1 and GH6 is restricted in the nasal retina from HH stage 12-14 (Yamagata et al., 1999; Schulte and Cepko, 2000). EphA3 expression is confined to the temporal retina at HH stage 15 (Yamagata et al., 1999; Schulte and Cepko, 2000). In addition, as shown in this report, graded distributions of ephrin A5 and ephrin A2 are observed after HH stage 18 (E3) and E5, respectively. The order of onset of these genes thus supports the view that CBF1 determines the NT specificity, thereby controlling the expression of a series of asymmetrically distributed molecules in the developing retina. It is likely that misexpression of CBF1 endues the temporal retinal ganglion cells with the character of nasal ganglion cells by inducing the expression of SOHo1, GH6, ephrin A5 and ephrin A2, and inhibiting the expression of CBF2 and EphA3.

In this study, we also misexpressed CBF2 in parallel experiments. Unexpectedly, misexpression of CBF2 had no effect on the expression of asymmetrically distributed molecules, including CBF1 (data not shown). As misexpression of CBF2 also results in retinotectal projection errors along the AP axis (Yuasa et al., 1996), CBF2 is also likely to be involved in the formation of the retinotectal map by regulating the expression of unknown topographic molecule(s). In our experiments, misexpressed CBF1 completely repressed the expression of CBF2 (see also Yamagata et al., 1999), suggesting that CBF1 can control the expression of these unknown topographic molecules through the regulation of CBF2 expression. One potential candidate is a receptor for a membrane-associated glycoprotein, RGM, which was recently reported to be distributed in a gradient with increasing concentrations from the anterior-to-posterior pole of the embryonic tectum and collapse the temporal growth cones (Stahl et al., 1990; Müller et al., 1996; Monnier et al., 2002).

**CBF1 regulates the expression of asymmetrically distributed molecules through multiple mechanisms**

BF-1 has been shown to act primarily as a transcriptional repressor (Li et al., 1995; Bourguignon et al., 1998). In addition to the functions which are dependent on their ability to bind to DNA, several transcription factors have been reported to function even when their DNA binding-ability is deficient (Beato et al., 1995; Um et al., 1995; Plaza et al., 1997; Schuur et al., 2001; Zhao et al., 2001). This DNA binding-independent function is also observed in murine BF-1 (Dou et al., 2000; Rodriguez et al., 2001). To clarify the modes of action of CBF1, we examined effects of misexpression of CBF1-eve and CBF1AA mutants. As a result, we found that the downstream asymmetric molecules can be classified into three distinct categories by the mode of action of CBF1 (Fig. 7A): (1) those whose expression is affected only by the CBF1-eve mutant (written in red), (2) those whose expression is affected only by the CBF1AA mutant (in green), and (3) those whose expression...
is affected by both the CBF1-eve and CBF1AA mutants (in blue).

Ephrin A5 belongs to the first category. Consistent with the reports that CBF1 acts as a transcriptional repressor, misexpression of CBF1-eve shows similar activity for the expression of ephrin A5 as the wild-type CBF1. Because misexpression of CBF1AA mutant protein has no effect on the expression of ephrin A5, the expression of this molecule is regulated only by the function of CBF1 as a transcriptional repressor (Fig. 7A). How does CBF1, a transcriptional repressor, induce the expression of ephrin A5? The simplest explanation is that CBF1 functions as a transcriptional repressor of some other putative intermediate repressor(s) which inhibits the expression of ephrin A5 (indicated by 'X' in Fig. 7B). The onset of ephrin A5 expression appears to be triggered first by a transcription activator(s) at stage 12 to yield the homogeneous expression in the retina (Fig. 1A). Subsequently, the factor X may begin to be expressed in the temporal retina where CBF1 is absent, and represses expression of ephrin A5 in the temporal retina. In misexpressed embryos, ectopic CBF1 probably abrogates the repression by factor X from the temporal retina, and then expression of ephrin A5 is re-induced by the transcriptional activator(s) which is ubiquitously expressed in the retina.

Ephrin A2 belongs to the second category. Surprisingly, expression of ephrin A2 is not affected by misexpression of the CBF1-eve fusion protein. By contrast, CBF1AA exerted the same effect on the expression of ephrin A2 as the wild-type protein. Therefore, expression of ephrin A2 is regulated only by a DNA binding-independent mechanism, which clearly contrasts with ephrin A5 whose expression is regulated only by a DNA binding-dependent mechanism (Fig. 7A). In the retina, both ephrin A2 and ephrin A5 are expressed in a nasal high/temporal low gradient. Although these ephrins commonly function as ligands for EphA receptors, their expression patterns are not identical. The gradient of ephrin A2 expression in the GCL is gentle and wide along the NT axis, when compared with that of ephrin A5 expression. In addition, the onsets of their expression are also distinct: ephrin A5 begins to be expressed at HH stage 12 (E2), but ephrin A2 does not appear until E5. These differences may reflect the distinct regulatory mechanisms for their expression. We previously demonstrated that expression of ephrin A2 is induced by misexpression of Vimentin, a BMP4 antagonist (Sakuta et al., 2001). We show here that BMP2 begins to be expressed in a temporal high/nasal low gradient along the NT axis from E5. Furthermore, Vimentin counteracts BMP2 as well as BMP4, and BMP2 represses ephrin A2 expression. Therefore, it is likely that the DNA binding-independent control mechanism used by CBF1 on ephrin A2 is relevant to BMP2 signaling (Fig. 7B). This view is consistent with the expression patterns of BMP2 and ephrin A2 in the retina: BMP2 expression is restricted to the temporal third of the retina, while the expression of ephrin A2 expands into the temporal side, making a gentle gradient along the NT axis. In the present study, we demonstrated by in vitro reporter assay that CBF1 indeed interferes with BMP signaling through a DNA binding-independent mechanism. In addition, misexpression of CBF1 represses the expression of BMP2 in the GCL. This notion is supported by a recent study describing the interaction between BF-1 and Smad1 (Rodriguez et al., 2001): Smad1 is an intracellular signaling intermediate for the receptors for BMP2 and BMP4 (Piek et al., 1999), and it is reported that BF-1 interferes with BMP signaling via interaction with Smad molecules (Rodriguez et al., 2001). The loss of the BF-1 gene in mice leads to ectopic expression of BMP4 in the dorsal telencephalic neuroepithelium (Dou et al., 1999). All these results indicate that CBF1 interferes with the BMP signaling.

SOHo1, GH6, EphA3 and CBF2 belong to the third category. The expression of these molecules is affected by misexpression of both the CBF1-eve fusion protein and CBF1AA mutant protein, indicating that the expression of these molecules is regulated by CBF1 through not only a DNA binding-dependent but also a DNA binding-independent mechanism (Fig. 7A). Regulation of these four molecules, especially the induction of SOHo1 and GH6 in the temporal retina, by the misexpressed CBF1-eve fusion protein or CBF1AA mutant protein appeared to be milder than that by the wild-type CBF1 (compare Fig. 2B and 2D with Fig. 4C, parts a,b and Fig. 5C, parts a,b). Thus, cooperation of DNA binding-dependent and DNA binding-independent mechanisms of CBF1 should be necessary for the full regulation of SOHo1 and GH6 by CBF1. As SOHo1 and GH6 repress the expression of EphA3 in the retina (Schulte and Cepko, 2000), repression of EphA3 expression by CBF1 likely resulted from induction of SOHo1 and GH6 by CBF1. It might also be possible that CBF1 directly downregulates the expression of EphA3 not through SOHo1 or GH6. This is, however, unlikely because EphA3 is homogenously expressed in the optic vesicle at HH stage 12, when CBF1 is topographically expressed (Yamagata et al., 1999). In addition, CBF1 and EphA3 are co-expressed in the same region of the chick forebrain (H.T., T.S., H.S. and M.N., unpublished). Thus, CBF1 probably represses EphA3 expression through induction of SOHo1 and GH6 expression. To explain the induction of SOHo1 and GH6 expression by CBF1 through a DNA binding-dependent mechanism, the existence of a putative intermediate repressor(s), which inhibits expression of SOHo1 and GH6, must be postulated (indicated by 'Y' in Fig. 7B). As SOHo1 and GH6 are induced by a transcriptional activator(s) which is ubiquitously expressed in the retina.

Expression of SOHo1, GH6 and CBF2 is also regulated by CBF1 through a DNA binding-independent mechanism (Fig. 7A). As expression of SOHo1, GH6 and CBF2 is not affected by misexpression of Vimentin or BMP2 (Sakuta et al., 2001) (H.T., T.S., H.S. and M.N., unpublished), these molecules are not regulated by BMP signaling. Several transcription factors are known to regulate the expression of their downstream target genes by interacting with other transcription factors (Beato et al., 1995; Um et al., 1995; Plaza et al., 1997; Schuur et al., 2001; Zhao et al., 2001). Thus, it is probable that CBF1 regulates expression of SOHo1, GH6 and CBF2 via interaction with other transcription factors which directly regulate the expression of SOHo1, GH6 and CBF2.

What is the meaning of using two distinct mechanisms, DNA binding-dependent and DNA binding-independent (Fig. 7A), to regulate the expression of topographic molecules? It may be for security to maintain regulation of the EphA/ephrin A system: For example, if the DNA binding-dependent mechanism is lost by a mutation in the WH domain, CBF1 can still regulate the asymmetrical distribution of EphA3 and
BMP2 and BMP4 are implicated in many different processes of vertebrate development (for a review, see Balemans and Hul, 2002). The extent of BMP action is controlled in part by the influence of antagonists such as Noggin, Chordin and Follistatin. Counteractions between BMPs and their antagonists, which appear to play important roles in BMP signaling, are suggested to play important roles in vertebrate development.

At the early developmental stages of the eye (stage 11 to E5), the counteraction between Ventroptin and BMP4 governs the regional specification in the retina along the DV axis (Sakuta et al., 2001). In the embryos misexpressing Ventroptin in the retina, projections of almost all the dorsal retinal axons shifted dorsally on the tectum (Sakuta et al., 2001). At the later stages (E6 onward), in proportion to the disappearance of BMP4 expression from the dorsal retina, Ventroptin begins to be expressed in both a nasal high/temporal low and a ventral high/dorsal low gradient in the retina. In the present study, we found that BMP2 begins to be expressed from E5 in a pattern complementary to that of Ventroptin along both the NT and DV axes. In addition, BMP2 and Ventroptin repressed the expression of each other in the developing retina. Therefore, at the later stages when retinal axons actively begin to invade the tectum, the counteraction between Ventroptin and BMP2 appears to play an important role in the topographic projection along the AP axis in addition to the DV axis. This view is consistent with our previous results that almost all temporal axons labeled with Dil extended to the posterior end of the tectum, when Ventroptin was misexpressed, owing to the induction of ephrin A2 expression in the temporal retina (Sakuta et al., 2001).

In the present study, we showed that misexpression of CBF1 moderately inhibits expression of BMP2, while misexpression of Ventroptin does it completely. These results suggest that BMP signaling in the nasal retina is mainly repressed by Ventroptin. Thus, CBF1 may fine-tune BMP signaling. From this point of view, CBF1 presumably triggers the shift in the expression pattern of Ventroptin to the double gradient. Around E5, BMP4 expression in the dorsal retina begins to disappear. Concomitantly, the expression of Ventroptin would be enhanced more in the nasal retina because of the inhibition of BMP signaling by CBF1, which leads to the nasal high/temporal low expression gradient of Ventroptin. Then, BMP2 begins to be expressed with a double-gradient, in a fashion counter to Ventroptin, and this counter gradient would be fixed afterwards. Our study thus demonstrated that two distinct BMP signals sequentially play pivotal roles in the topographic projection along the two axes.

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