CCCTC binding factor (CTCF), a transcriptional regulator, plays important roles in epigenetics and genetic diseases. CTCF is an evolutionarily conserved zinc finger (ZF) phosphoprotein. It binds to target sequences with high specificity. In vertebrates, the CTCF gene is encoded by a 4.1-kb mRNA with a long open reading frame (21.8 kb) and is predicted to be a 728-amino acid protein with a molecular weight of 82,000. However, the protein that appears in nuclear extracts shows a band of 130 kDa in an SDS-polyacrylamide gel. The discrepancy between the predicted and apparent sizes of the CTCF protein suggests anomalous migration of the protein in SDS-PAGE (3).

CTCF regulates important gene transcription by using its ZF to interact with CCCTC motifs in the DNA-regulatory element (1, 4, 5). CTCF suppresses c-myc expression (1) and the chicken lysozyme gene (4), but it also activates the transcription of the amyloid protein precursor gene (6). In addition, CTCF functions as an insulator, mediating the monoallelic expression of imprinted genes, insulin-like growth factor 2 (IGF2), and H19 genes (7). Expressions of IGF2 and H19 are only imprinted from the paternal and maternal alleles, respectively. CTCF plays a key role in controlling the imprinted expression of IGF2 and H19 through a DNA methylation-sensitive mechanism (7, 8). A recent study reports that CTCF is a candidate for transacting factors for X-inactivation causing the silencing of one of two female X chromosomes (9). In the eye, CTCF is highly expressed in the ciliary marginal zone, inner nuclear layer, and anterior lens epithelium (10). These findings imply that CTCF may play an important role in eye development.

Pax6 is a homeobox transcription factor in the Pax gene family containing a total of nine members in vertebrates (11). Pax6 plays a critical and evolutionarily conserved role in eye development in both vertebrates and invertebrates (12). The mutant eyeless, a homozygous mutation of Pax6, results in missing eye structures in Drosophila (13). Overexpression of Pax6 causes ectopic compound eyes in Drosophila and well-formed ectopic lenses in Xenopus laevis (14, 15). In addition, the other Pax6 mutation causes the small eye (Sey) defect in mice and ocular aniridia in humans (16–18). Pax6 is expressed essentially in all ocular structures in vertebrates, including the cornea, iris, lens, and retina (19–22). Regulation of Pax6 gene transcription is highly conserved during evolution. In most species, the Pax6 transcription is regulated via two promoters, P0 and P1 (23–25). The P0 promoter initiates the transcription in the cornea and conjunctival epithelia, the lens placode, and the retina, whereas the P1 promoter initiates the transcription mainly in the lens placode, the optic vesicle, and the central neural system (25). There is a highly conserved transcriptional control element, called the ectoderm enhancer (EE), located approximately –3.5 kbp upstream from the P0 promoter (26). The EE regulatory element is important to specific expressions of the Pax6 gene (26) and may also be important for neural retina-specific gene expressions (25). Another regulatory ele-

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ment has been suggested in the region upstream from the Pax6 gene. P0 promoter that is sufficient for direct expression in a subset of postmitotic, non-terminally differentiated photoreceptors (25). In addition, there is a regulatory element in intron 4 of the Pax6 gene to direct activities of the P0 and P1 promoters in amacrine cells, the ciliary body, and the iris (25). However, the regulation of the Pax6 transcription is still largely unknown. In pulse has been described regarding tissue-specific and time-dependent differences in the control of this process during embryonic development and in mature cells.

In the present study, we report on an important function of CTCF in regulating the Pax6 transcription in eye development. Increases in CTCF expression effectively suppressed the Pax6 transcription and protein expression in mouse embryos, resulting in underdevelopment of the eye. We have isolated a 4.2-kbp DNA fragment in the 5′-flanking region of the Pax6 gene upstream of the P0 promoter in mouse genomic DNA. The reporter fuses with the DNA fragment, and a serial deletion mutant connects to a LacZ reporter gene. Using the reporter system, P0 promoter activity was analyzed in eye-derived cells. CTCF controls Pax6 transcription through its interaction with the repressor element in the Pax6 gene to affect the ectoderm enhancer and P0 promoter activities.

MATERIALS AND METHODS
Cell Culture and Transfection—Four cell types, including human retinoblastoma (Rb), rabbit corneal epithelial (RCE), rat myeloid leukemia ML-1 cells (27–29), were employed in the present study to test promoter activity. Rb cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (Invitrogen). RCE cells were cultured in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) containing 10% fetal bovine serum and 5 ng/ml insulin. FDC-P1 cells were cultured in phenol red-free 1640 medium (Invitrogen) with 10% fetal bovine serum. ML-1 cells were cultured in 1640 medium (Invitrogen) with 10% fetal bovine serum. All cultured cells were incubated in an incubator supplied with 95% air and 5% CO2 at 37 °C. The medium was replaced every 2 days, and RCE cells were passed by digestion using 0.05% trypsin-EDTA.

CTCF-transgenic Mice and Embryo Section Preparation—Fertilized eggs from donor mice were injected with a MulliNol fragment (3.1 kb) containing the CMV promoter region and cdNA encoding full-length CTCF using a microdispenser. CTCF-injected eggs were cultured in vitro for 2 days and then transplanted into the ovarian duct of maternal recipients. They were sacrificed 7, 11, and 14 days after transplantation, and the embryos were collected from the uterus. Harvested embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) in preparation for histological examinations. Tissue sections were obtained from paraffin-fixed embryo blocks as described in Current Protocols in Molecular Biology. Embryonic tissue samples were dehydrated by serial incubations in 50, 70, 85, 95, and 100% ethanol (30 min at each concentration). After a 10-min incubation of the tissues in xylene, embryos were transferred into tubes that contained xylene/paraffin (50:50) at 60 °C and were kept at room temperature overnight. For paraffin impregnation, paraffin embryos were incubated in paraffin at 60 °C for 1 h. Finally, embryonic tissue samples were individually embedded in wax blocks that were cut into 6-μm-thick sections.

Reporter Gene Constructs and Expression—A 4.2-kbp DNA fragment upstream of the Pax6 P0 promoter was cloned by PCR using the high fidelity Taq (Platinum® high fidelity, Invitrogen), and mouse genomic DNA was used as a template. The 4.2-kbp DNA fragment was subcloned into a β-galactosidase-Basic vector (Clontech, Redwood, CA). The vector is promoterless and contains the reporter gene LacZ. All of the 5′-end deletion mutants of the 4.2-kbp DNA fragment were individually subcloned into lacZ-expressing Basic vector. The constructs were designed as P4.2, P3.5, P2.3, and P1.2, respectively. Each of the reporter constructs was individually introduced into target cells by electroporation. Briefly, 105 cells/ml were washed twice and resuspended in PBS. Cells were mixed with 5 μg of the reporter DNA and transferred into a 0.4-cm-gap cuvette (Bio-Rad). Electroporation was performed in a 200-V electric field with a time constant of 0.5 ms.

Tetracycline-inducible Expression of CTCF in RCE Cells—A 2.5-kbp DNA fragment encoding full-length human CTCF was cloned from mRNA isolated from Rb cells using reverse transcription-PCR and was confirmed by DNA sequencing. The CTCF cDNA was subcloned into pcDNA4/Tva expression vector (Invitrogen) for tetracycline-inducible expression experiments. Briefly, constructs of pcDNA4/Tva-CTCF and pcDNA4/TR (Invitrogen) were co-introduced into RCE cells by electroporation. The stably transfected cells were established by cloning the cells from a single colony and by double selection cultures in the presence of Zeocin and blasticidin. Control cells co-transfected with blank vector pcDNA4/Tva and pcDNA4/TR were also established. The stably transfected cells were cultured in Dulbecco's modified Eagle's medium/F12 medium containing 10% serum, 5 μg/ml insulin, 2 μg/ml blasticidin, and 50 μg/ml Zeocin. For induction expression experiments, 2 μg/ml tetracycline was applied into the culture medium 24 h prior to experiments.

Analysis of β-Galactosidase Activity—Cells were rinsed twice with ice-cold PBS and suspended in an ice-cold lysis buffer containing 100 mM NaCl, 1 mM dithiothreitol, pH 7.8. Cells were subjected to three cycles of freeze-thaw and precleared by centrifugation at 15,000 × g for 5 min. Supernatants were kept at −80 °C until the analysis of β-galactosidase activity. β-Galactosidase activity was tested by a luminescent β-galactosidase system utilizing a chemiluminescent substrate named Galacton-Star (Clontech). Chemiluminescent signals were detected by exposure of x-ray film or by using a Sirius lumimenter (Berthold Detection Systems, Pforzheim, Germany). To normalize LacZ activity, an internal control vector pBRL-TK (0.2 μg/transfection) from Invitrogen was introduced into RCE or Rb cells together with the Pax6 P0 reporter and mutants. Luciferase activity (for internal controls) was measured using an assay kit supplied by Invitrogen. Reporter activity was normalized by taking a ratio of measured β-galactosidase activity over luciferase activity obtained from the internal control vector (β-galactosidase activity/luciferase activity).

Northern Analysis of CTCF—The total RNA from cells was extracted using a guanidium thiocyanate procedure (30, 31). Cells (1 × 106) were collected for each sample. After rinsing with ice-cold PBS, the cells were immediately lysed with 1 ml of guanidium solution (5 m guanidine hydrochloride, 50 mM Tris-HCl, pH 8, 0.5% N-lauroylsarcosine, 100 mM β-mercaptoethanol). The lysate was extracted three times with 50:50 phenol/chloroform. Finally, the RNAs were washed in ethanol and spun down by centrifugation at 12,000 rpm for 15 min. RNA (20 μg) from each sample was loaded in 1% agarose gel denatured with 2:2:2 formamide/borate/EDTA and then transferred onto a nylon membrane for later hybridization procedures with α-32P-labeled probes and visualized by the exposure of x-ray films at −80 °C overnight.

Western Analysis and Immunohistochemistry—For Western analysis, 5 × 105 cells were harvested in a 0.5-ml lyses buffer containing 137 mM NaCl, 1.5 mM MgCl2, 2 mM EDTA, 10 mM Na-pyrophosphate, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na-orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml leupeptin, 20 μg/ml Tris, pH 7.5. Cell lysates were preincubated by centrifugation at 13,000 × g for 20 min. Samples were denatured by adding an equal volume of 2× Laemmli buffer and then boiled for 5 min. Samples were fractionated by electrophoresis in 8% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and then incubated with primary antibodies (1:4,000) and secondary anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (1:1,500) (Santa Cruz Biotechnology, Santa Cruz, CA). For immunostaining experiments, tissue sections were made to be membrane-permeable for 30 min in PBS and 0.1% Triton X-100 (PBS-T), blocked for 1 h with 10% normal horse serum in PBS-T at room temperature, and then incubated overnight at 4 °C with the primary antibody, anti-Pax6 (Covance, Princeton, NJ), at 1:100–1:200 dilution in PBS-T. After rinsing three times with PBS, fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG antibody (1:100, Jackson Immunoresearch Lab) was applied for 2 h at room temperature. Sections were washed and mounted in Vectashield (Vector Labs). Analysis was then made with an Olympus fluorescence microscope (Olympus Fluoview with a x40 objective lens).

Electrophoretic Mobility Shift Assay (EMSA)—Cells (5 × 106) were washed twice with ice-cold PBS and suspended in ice-cold cell homogenization buffer containing 10 mM HEPES-KOH, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4. These cell nuclei were subsequently resuspended in 1 ml of nuclear suspension buffer containing 400 mM KCl, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 40 mM HEPES-KOH, pH 7.9. Nuclear proteins were extracted by centrifugation at 100,000 × g for 20 min and transferred into a new tube for storage at −80 °C. A 32P-labeled DNA probe (80 bp) that contained five CTCF binding sites was labeled with [γ-32P]-ATP and synthesized by agarose gel electrophoresis. In addition, a mutant probe was made by replacing five CCCTC repeats (putative CTCF binding sites) by a 32P-labeled probe conjugated by agarose gel electrophoresis. In addition, a mutant probe was made by replacing five CCCTC repeats (putative CTCF binding sites) by a 32P-labeled probe conjugated by agarose gel electrophoresis. In addition, a mutant probe was made by replacing five CCCTC repeats (putative CTCF binding sites) by a 32P-labeled probe conjugated by agarose gel electrophoresis.
32P-labeled DNA probe (1 μl), 1 μg of poly(dI-dC) (1 μl), 20% Ficoll 400 (5 μl), and 5 μl of H2O. Competitive reactions were conducted by adding 1 μM non-labeled DNA probe into each sample. The reactions were carried out through incubation for 30 min at 4 °C. DNA-protein complexes were displayed by electrophoresis on a 5% non-denaturing polyacrylamide gel. The gel was dried with a vacuum gel drier and exposed to x-ray film for autoradiography. For the supershift assay, DNA-protein complexes were further incubated for an additional 30 min at 4 °C with the anti-CTCF antibody and other control antibodies (Santa Cruz Biotechnology).

RESULTS

We studied the functional correlation between CTCF and Pax6 expression by introducing a CTCF transgene in the early developmental stages of the mouse embryo. Control mice were only injected with the CMV promoter. Embryos were collected at stages E11 and E14. We found that there were significant defects in eye development in CTCF-overexpressed mouse embryos. Injected mice had underdeveloped eyes (Fig. 1A). In transgenic mice with small eye defects, the lens size was also markedly reduced and deformed. Eye sections of CTCF-transgenic mice were compared with the controls. The eyecup of CTCF-transgenic mice did not fully develop (Fig. 1B). In addition, there were reduced populations of cells in the retina, lens, and cornea in transgenic mice. Mouse embryos analyzed in our experiments were collected at stages E11 and E14. There were a total of 81 CTCF-transgenic mouse embryos found with the small eye phenotype. These results suggest that there is a correlation between CTCF function and eye development.

Levels of exogenous CTCF DNA in mouse embryonic tissues were determined by PCR using specific primers according to the promoter region sequence of the CMV-CTCF construct. Genomic DNA templates were individually isolated from 26 transgenic mice and 26 control mice. We found a single band of exogenous CMV-CTCF DNA with an expected molecular size of 0.4 kbp detected in 24 of 26 transgenic mice but not in the control mice (Fig. 2A). Expressions of the CTCF protein in transgenic and control mice were detected with immunostaining using a specific anti-CTCF antibody (Fig. 2B). immunofluorescence activity of the CTCF protein was markedly increased in the eye obtained from the E14 transgenic mouse embryo compared with the control eye. Because Pax6 is crucial for eye development, further
analysis was done to probe for endogenous Pax6 expressions in the eye of the E14 mouse embryo by immunostaining using the anti-Pax6 antibody. There was evidence for immunoreactive staining of Pax6 in the lens and corneal epithelial cells in control mice. In contrast, endogenous Pax6 expression was markedly diminished in the eye of CTCF-transgenic mice (Fig. 2C). Overexpression of CTCF in mouse embryos resulted in decreases in endogenous Pax6 expression, suggesting that the Pax6 gene expression may be a downstream event from CTCF. These results demonstrated that an increase in CTCF expression in transgenic mice suppresses the Pax6 gene expression, resulting in defective eye development.

The effect of CTCF on Pax6 expression was also studied in eye-derived RCE cells. CTCF cDNA was subcloned into a TREx system (the construct is named as pcDNA4/To-CTCF DP), and the sequence was confirmed with DNA sequencing using a T7 primer. Gene and protein expressions of CTCF in RCE cells were detected by Northern and Western analyses using the cloned cDNA probe and a specific antibody, respectively. A stable RCE cell line co-transfected with pcDNA4/To-CTCF and pcDNA6/TR was established by double selections with Zeocin (50 μg/ml) and blasticidin (2 μg/ml). After the induction of tetracycline, CTCF mRNA levels were markedly increased, whereas the Pax6 protein expression fell upon induction of RCE cells with tetracycline (Fig. 3B; n = 3, p < 0.01). In addition, CTCF protein levels were detected with
To further prove that CTCF regulates Pax6 expression, we knocked down endogenous CTCF gene production using a siRNA technique. A Silencer™ siRNA construction siPORT...
FIG. 6. Interaction of CTCF with a repressor element upstream from the Pax6 P0 promoter. A, DNA sequence deduced from −1.7 to −1.2 kbp upstream from the Pax6 P0 promoter. A typical sequence with five repeats of CCCTC for the interaction of CTCF was identified (highlighted region) in an 80-bp region in the sequenced fragment. B, formation of the specific EMSA complex with a probe representing CTCF binding sites. C, interaction of a CTCF-specific antibody with the EMSA complex causing a band shift. JNK, c-Jun NH2-terminal kinase. D, specific
lipid system supplied by Ambion was used. Two 5'-end and 3'-end primers were designed with a total of 29 nucleotides (21 nucleotides plus 8 nucleotides complementary to the T7 promoter) according to the sequence of CTCF mRNA. SIRNA was synthesized using a T7 RNA polymerase. Synthesized siRNA was transfected into eye-derived RCE cells by lipofection. CTCF siRNA effectively knocked down CTCF mRNA in transfected cells over the next 3 days as revealed by Northern analysis (Fig. 4A). CTCF siRNA also markedly suppressed the CTCF protein expression resulting in an increase in Pax6 protein expression (Fig. 5B). Our results firmly establish that CTCF down-regulates Pax6 expression in Pax6-expressing cell types, which is consistent with the results obtained from in vivo studies.

To study the molecular interaction, a 4.2-kbp fragment (P4.2) upstream from the Pax6 P0 promoter was cloned from mouse genomic DNA and subcloned into a promoterless β-galactosidase-Basic vector (Clontech) to generate a Pax6 reporter construct (Fig. 5A). Pax6 P0 promoter activity was examined by transient expression of these constructs into several cell lines, including eye-derived RCE and Rb cells and hematopoietic FDC-P1 and ML-1 cells. For control experiments, cells were transfected with SV40-β-galactosidase vector. β-Galactosidase activities in RCE and Rb cells were elevated after transfection of the P4.2 ββ-galactosidase construct, indicating that the P4.2 fragment upstream from the Pax6 P0 promoter is able to initiate the reporter gene expression (Fig. 5A). P4.2 ββ-galactosidase-transfected FDC-P1 and ML-1 cells did not display β-galactosidase activity, although strong β-galactosidase activity initiated by the SV40 promoter was observed in these cells. The results reveal that the P4.2 construct preserves the Pax6 P0 promoter activity and is capable of selectively expressing the reporter gene in eye-derived cells. Mutants of the reporter construct with 5'-end deletions were also generated and subcloned into the promoterless ββ-galactosidase-Basic vector (P3.5, P2.3, and P1.2) to screen for the regulator element in the 3.5-kbp region upstream from the Pax6 promoter. Deletion of the P0 promoter contains an EE for Pax6 gene expression. In the normal eye, Pax6 plays a dominant role in determining the size of the eyeball and mediating cell differentiation (32–35). Both appropriate eyeball size and cell differentiation are essential for the normal visual function. Our studies also provide evidence for a new role of CTCF in the control of eye development. Basal CTCF levels during early mouse embryonic stages can affect eye development through changing Pax6 expression patterns because CTCF directly controls Pax6 transcription. Future studies are needed to identify additional molecular interactions and cellular pathways underlying the CTCF function in the genetics of eye development.

We have found in the present study that CTCF is involved in regulation of developmental homeobox Pax6 gene expression and interacts with a repressor element located in the region of −1.2 kbp upstream from the Pax6 P0 promoter. Deletion of the repressor element reveals a similar result as previous reports describing that in the region upstream from −3.5 to −3.3 kb the P0 promoter contains an EE for Pax6 P0 promoter activity (25). Now we have found for the first time that EE activity is inhibited by the further downstream repressor element in response to CTCF activation. A working model is proposed that Pax6 P0 promoter activity is up-regulated by the EE in the 3.5-kbp region upstream from the P0 promoter and is down-regulated by a CTCF-controlled repressor in the −1.2-kbp region upstream from the P0 promoter (Fig. 7). To identify possible binding sites that interact with CTCF, the region containing the repressor element was sequenced and confirmed with EMSA and supershift assay.

The CTCF protein is an evolutionarily conserved DNA-binding protein with a DNA-binding domain containing 11 ZFs (1, 27583). To confirm the potential CTCF interaction element, EMSA and the supershift assay were performed using a synthesized 80-bp 32P-labeled DNA probe that contained the identified sequence of the 80-bp fragment. Nuclear extracts prepared from RCE cells were used in EMSA. Results revealed a single protein-DNA complex that was outcompeted by an excess of unlabeled probe but not by unlabeled DNA fragments of NFκB and pBluescript vector (Fig. 6B). The mobility of the protein-DNA complex was super-shifted by incubation of nuclear extracts with a CTCF antibody (Fig. 6C). A control experiment was performed by using a series of antibodies against c-Jun, c-Jun NH2-terminal kinase, and cyclin B instead of the CTCF antibody. All of these antibodies did not affect the protein-DNA complex mobility. Further EMSA experiments were performed by using a mutated 80-bp probe in which all five repeats of CCCTC sequences were replaced by five CGATC sequences. There were no bands in lanes containing the mutant probe. In addition, the mutated probe could not compete with the wild type probe for binding CTCF proteins in EMSA because it lost binding capability with nuclear proteins isolated from RCE cells (Fig. 6D). These results indicate that CTCF is capable of binding to the DNA probe specifically derived from the sequence of the 80-bp repressor region but not to the mutant probe.

DISCUSSION

Our results suggest a genetic model in which CTCF participates in key cellular mechanisms underlying early eye development by selectively regulating Pax6 gene expression. In the normal eye, Pax6 plays a dominant role in determining the size of the eyeball and mediating cell differentiation (32–35). Both appropriate eyeball size and cell differentiation are essential for the normal visual function. Our studies also provide evidence for a new role of CTCF in the control of eye development. Basal CTCF levels during early mouse embryonic stages can affect eye development through changing Pax6 expression patterns because CTCF directly controls Pax6 transcription. Future studies are needed to identify additional molecular interactions and cellular pathways underlying the CTCF function in the genetics of eye development.
 binding sites. When CTCF binds on specific sites in the region, a region (80 bp) between the EE and the P0 promoter containing CTCF from the P0 promoter to promote promoter activity is suppressed resulting in inhibition of Pax6 expression (B).

(25, 26). The result provides additional evidence to support the mechanism that CTCF binding sites located between the enhancer and the P0 promoter are just insulator binding sites to regulate Pax6 gene expression. Pax6 is evolutionarily conserved in its genetics and function (43, 44). On the other hand, transcriptional regulation of Pax6 is also highly conserved in evolution evidenced by the finding that the conserved EE located at −3.5 kbp upstream from the Pax6 gene P0 promoter in bird, mouse, and human (25, 26). Interestingly, similar to Pax6, CTCF also is highly conserved in evolution with 93% homology between avian and mammalian CTCF (1). The conservation of both Pax6 and CTCF in evolution further suggests that CTCF plays an important role in regulation of Pax6 transcription in the development. In summary, the present study has identified a new functional role of CTCF in regulating Pax6 expression to affect eye development. CTCF can interact with the regulatory element and inhibit activity of the Pax6 P0 promoter.

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Fig. 7. A schematic working model for CTCF regulating Pax6 transcription. There is an ectoderm enhancer at −3.5 kbp upstream from the P0 promoter to promote Pax6 gene transcription (A). There is a region (80 bp) between the EE and the P0 promoter containing CTCF binding sites. When CTCF binds on specific sites in the region, Pax6 P0 promoter activity is suppressed resulting in inhibition of Pax6 expression (B).
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