Effect of CTCF-Binding Motif on Regulation of PAX6 Transcription

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PURPOSE. Previous studies indicate that the CCCTC binding transcription factor (CTCF) regulates homeobox PAX6 gene transcription in corneal epithelial cells. In the present study, the effect was investigated of CTCF activity on PAX6 transcription through interaction with five essential motifs located in an 80-bp region upstream from the PAX6 P0 promoter.

METHODS. An electrophoretic mobility shift assay (EMSA) was used to determine the interaction between CTCF and DNA binding motifs. DNA mutagenesis was applied in identification of DNA motif functions. Immunohistochemistry and Western blot analyses were performed to detect the stress-induced effect on CTCF activity.

RESULTS. The five identified CTCF-binding motifs were mutated one by one or in different combinations. Interactions of CTCF with these mutated motifs were determined by EMSA and DNA-binding competitions. All five CCCTC motifs were functional for the CTCF binding and DNA-binding activity of CTCF was proportionally decreased after increases in mutations of motif numbers. In addition, ultraviolet (UV) irradiation and epidermal growth factor (EGF) induced suppression and activation of CTCF expression, respectively. Effects of UV and EGF induction were due to alterations in CTCF expression and activity resulting in changes in CTCF DNA binding activity to the PAX6 promotor region detected by EMSA.

CONCLUSIONS. These findings indicate that CTCF regulates PAX6 expression in response to stress-induced conditions and that the molecular base of CTCF controlling PAX6 expression is through five functional and specific motifs in the region upstream from the PAX6 P0 promoter in corneal epithelial cells. (Invest Ophthalmol Vis Sci. 2006;47:2422–2429) DOI: 10.1167/iovs.05-0536

The PAX6 gene is one of the important genes in the homeobox gene family. Deficient expression of the PAX6 gene causes death in mice shortly after birth. Although PAX6 is downregulated or fades away in most tissues and cell types after differentiation, it remains detectable in several mature cell types of eye including corneal epithelia, lens, and retina. Expression of the PAX6 gene in ocular tissues is essential for the structural development of the eye and ocular cell differentiation in vertebrates. In the developed cornea, PAX6 is a positive transcription factor essential for controlling transcription of the cornea-specific differentiation marker keratin 12, suggesting that PAX6 may play a major role in early differentiation and maintaining differentiation pattern in these cells. In our previous studies, we found that inhibition of PAX6 expression is a prerequisite for epidermal growth factor (EGF) to act as a control of cell growth and fate. Apparently, downregulation of PAX6 expression is necessary in EGF-induced corneal epithelial proliferation, because knockdown of mRNA expression with PAX6-specific small interfering (si)RNAs in corneal epithelial cells significantly promotes EGF-induced proliferation.

The regulatory mechanisms of PAX6 gene expression are just beginning to be revealed. There are at least two enhancers in upstream sequences of the PAX6 P0 promoter that are apparently responsible for regulating gene expression. One has been identified as a 341-bp enhancer located in the 5′ region of the PAX6 gene. This enhancer is highly conserved in the PAX6 gene of mouse, humans, and puffer fish (Fugu). It is a dominant factor in the preplacodal phase of PAX6 expression and has been designated as the PAX6 ectoderm enhancer (EE), whereas the existence of the other (enhancer 2) is implied. Recently, studies in our laboratory reveal that a repressor element is located at ~1.2 kb upstream from the PAX6 P0 promoter in the mouse. The repressor element contains five repeat binding sites in an 80-bp region for CTCF, a transcription factor binding to CCCTC DNA sequence.

CTCF is a zinc finger (ZF) phosphoprotein that binds to target sequences with its DNA-binding domain containing 11 ZFs. In mammalian and avian species, CTCF protein is evolutionarily conserved in a wide range of adult tissues and cell lines. CTCF regulates important gene transcriptions by its ZF interaction with CCCTC motif in the DNA regulatory element. The effect of CTCF on the regulation of gene expression is based on its DNA binding sequences and modification of the DNA binding site. It has been known that methylation modification of the DNA binding site can affect CTCF function linking to epigenetics and genetic diseases. CTCF mediates monoallelic expression of imprinted genes, such as insulin-like growth factor 2 (IGF2) and H19 genes. Expressions of IGF2 and H19 are imprinting only from maternal allele and from the maternal allele, respectively. CTCF plays a key role in controlling imprinting expression of IGF2 and H19 through a DNA methylation-sensitive mechanism. The effects of CTCF on regulation of gene expression are through different mechanisms. CTCF downregulates the expression of...
c-myc and chicken lysozyme genes.\(^{12,15}\) Overexpression of the CTCF gene in corneal epithelial cells induces a decrease in PAX6 activity and deletion of a region containing potential CTCF binding sequences attenuated the decrease, indicating that CTCF serves as a repressive protein in PAX6 gene expression.\(^{11}\) CTCF can also upregulate transcription of the amyloid protein precursor gene.\(^{18}\) The diverse effects of CTCF on gene regulation indicate that CTCF is a unique “multivalent” transcriptional factor. In addition, CTCF is a phosphoprotein with different phosphorylated forms. The phosphorylation of CTCF is associated with cell proliferation/differentiation.\(^{19,20}\)

In the eye, CTCF is highly expressed in the ciliary marginal zone, inner nuclear layer, and anterior lens epithelium.\(^{21}\) These findings imply that CTCF may play an important role in eye development. In our previous studies, we reported an important function of CTCF in regulating PAX6 transcription in eye development. In the present study, we identified the nuclear structure of the CTCF binding site. We further report that CTCF controls PAX6 transcription through its interaction with an 80-bp DNA sequence containing five necessary and functional motifs upstream from the P0 promoter in the PAX6 gene. CTCF binding activity to these motifs was regulated by UV irradiation and growth factor stimulation, suggesting that it is one of the important proteins in the pathway of stress-induced cellular responses.

**MATERIALS AND METHODS**

**Cell Culture, Transfection, and UV Irradiation**

There were three cell types used in this study, including rabbit corneal epithelial (RCE), human corneal epithelial (HCE), and bovine endothelial cell (BCE) cells. Corneal epithelial and endothelial cells were cultured in 50/50 DMEM/F-12 medium (PAA Laboratories, Linz, Austria), 10% fetal bovine serum (FBS), 5\(\mu\)g/mL insulin, 10,000 U/mL penicillin, and 10,000 \(\mu\)g/mL streptomycin. Cells were incubated in...
95% air and 5% CO₂ at 37°C. The medium was replaced every 2 days, and cells were passed weekly by using 0.05% trypsin-EDTA digestion. UV irradiation was applied to cells by placing cultured cells under an UV-C light source (wave length, 254 nm) at an intensity of 45 JH262 J/cm².

For transfection experiments, 10⁷/mL cells were washed twice and resuspended in phosphate-buffered saline (PBS). Cells were mixed with 5 JH262 Jg DNA constructs and transferred into a 0.4-cm gap cuvette (Bio-Rad Laboratories, Hercules, CA). Electroporation was performed in a 300-V electric field with a time constant of 0.5 ms.

Detection of PAX6 and CTCF Expression

Total RNA were isolated and purified from the eye, pancreas, brain, skin, heart, or kidney of 2-month old mice. RT-PCR was performed to amplify PAX6 and CTCF cDNA fragments. Primers used for amplification of the cDNA fragments were PAX6, 639U24 (sense) 5’-ACT AAG GAT GTT GAA CCG GCA GAC 3’ and 1088L21 (antisense) 5’-GCT GTG GGA TTG GCT GGT GGT AGA 3’, and CTCF, U455 (sense) 5’-GCA TGA TAT GTC ACA CCT AAC 3’ and L803 (antisense) 5’-AGC ATG TGT AAT TGC AAA GCT CAC 3’. PCR products were loaded onto 1.5% agarose gels and displayed by electrophoresis.

Cloning DNA Fragments and Reporter Gene Constructs

P4.2, a 4.2-kb DNA fragment located upstream from the P0 promoter of the PAX6 gene, was cloned by PCR using mouse genomic DNA as a template. Both sense and antisense primers used in the PCR were designed based on the PAX6 gene sequence in GenBank with accession numbers of AF098639 and AF008212, respectively. Deletion mutations were performed by PCR with primers, cut up2856 (5’-CAG CCT AGT CCA CCC TGG TCT CC-3’) and cut low2731 (5’-AGA GTC TTG GGG AAA AGG CCC TGG GG-3’), using P4.2 as the template. High-fidelity Taq polymerase (platinum H-F; Invitrogen-Gibco, Carlsbad, CA) was used in PCR experiments to clone the various DNA fragments. A 2.5-kb cDNA fragment encoding the full-length human CTCF was cloned from mRNA isolated from Rb cells, by using RT-PCR, and was confirmed by DNA sequencing. The CTCF cDNA fragment was subcloned into pcDNA4/To/A (Invitrogen-Gibco) for tet-inducible expression experiments. P4.2 and its various mutants were subcloned into a β-gal-basic vector (BD-Clontech, Redwood, CA). The vector is promoterless and contains a reporter gene, LacZ. All the deletion mutants were individually subcloned in the pβ-gal-basic vector. These constructs were named P4.2, PxbaI P4.2-80, and P4.2-F. The reporter genes were introduced into target cells by electroporation. Cells transfected with β-gal control vectors, a SV40 promoter and β-gal-basic vector only, served as positive and negative controls, respectively.

Analysis of β-Gal Activity

Cells were rinsed twice with ice-cold PBS and suspended in ice-cold lysis buffer containing (mM): 100 KH₂PO₄, 1 dithiothreitol [pH 7.8]. Cells were lysed by three cycles of freeze-thaw and precleared by centrifugation at 13,000g for 5 minutes. Supernatants were kept in –80°C until analysis of β-galactosidase activity. β-galactosidase activity was tested by a luminescent β-gal system using a chemiluminescent substrate (Galacton-star; BD-Clontech). Chemiluminescent signals were detected by exposure to x-ray film or by luminometer (Sirius; Berthold Detection System, Pforzheim, Germany). To normalize LacZ
activity, an internal control vector pBRL-TK (0.2 μg per transfection; Invitrogen-Gibco) was introduced into RCE or Rb cells, together with the PAX6 P0 reporter and various mutants. Luciferase activity (for internal controls) was measured with an assay kit (Invitrogen Gibco). Finally, LacZ reporter activity was normalized by taking the ratio of measured β-galactosidase activity over luciferase activity obtained from the internal control vector (β-galactosidase activity/luciferase activity).

**Western Blot Analysis of CTCF and PAX6**

Western blot assay was performed as described previously.22 In brief, 5 × 10^5 cells were harvested in 0.5 mL lysate buffer containing (mM): 137 NaCl, 1.5 MgCl_2, 2 EDTA, 10 sodium pyrophosphate, 25 β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 Na-orthovanadate, 1 phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin, and 20 Tris (pH 7.5). Cell lysates were rinsed by PBS and precleared by centrifugation at 13,000 g for 20 minutes. Samples were denatured by adding an equal volume of 2× Laemmli buffer and boiling for 5 minutes. Twenty micrograms total protein was loaded into each well and then fractionated by electrophoresis in 8% SDS-PAGE gel. Proteins were transferred to PDF membranes and then incubated with goat anti-CTCF or anti-PAX6 antibodies (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (HRP, 1:1000; Santa Cruz Biotechnology). Gel band intensities of Western blot analysis were scanned by densitometry, and data were collected from three independent experiments.

**Electrophoretic Mobility Shift Assay**

Cells (5 × 10^7) were washed twice with cold PBS and suspended in ×5 volumes of ice-cold cell homogenization buffer containing (mM): 10 HEPES-KOH, 1.5 MgCl_2, 10 KCl, 0.5 dithiothreitol, and 0.5 phenylmethylsulfonyl fluoride (pH 7.9). After centrifugation at 250 g for 10 minutes, cells were resuspended in ×5 volumes of ice-cold homogenization buffer with 0.05% nonidet-40 and homogenized with 20 strokes (Dounce homogenizer; Bellco Glass, Vineland, Nj). Intact nuclei released from cells were collected by centrifugation at 250 g for 10 minutes at 4°C, and then resuspended in 1 mL of nuclear suspension buffer containing (mM): 400 KCl, 1 dithiothreitol, 0.1 phenylmethylsulfonyl fluoride, 0.1% aprotinin, and 40 HEPES-KOH (pH 7.9). After adding NaCl to a final concentration of 300 mM, the suspension was gently mixed and incubated on ice for 30 minutes. Nuclear proteins were extracted by centrifugation at 100,000 g for 20 minutes and transferred into a fresh tube for storage at −80°C. ^32P-labeled DNA probes were used in gel shift assays. For each gel shift assay, 10 μg nuclear extracts (10 μL) were incubated with 30 fm ^32P-labeled DNA probe (1 μL), 1 μg poly(dI-dC) (1 μL), 20% single-density gradient (5 μL; Ficoll 400), and 3 μL water. Competition reactions were conducted by adding 1 pM nonlabeled DNA probe. The reactions were performed by incubation for 30 minutes at 4°C. DNA-protein complexes were displayed by electrophoresis on 5% nondenatured polyacrylamide gels. These gels were dried and exposed to x-ray film for autoradiography.

**RESULTS**

To study the motif-specific effect of CTCF on PAX6 transcription, we first made a survey in different tissue and cell types that express both CTCF and PAX6. Expressions of CTCF and the PAX6 mRNA in various tissues of mice were determined by RT-PCR. Expression of the CTCF mRNA was highly detectable in all tested tissues, indicating that CTCF is expressed in all tissue types (Fig. 1A). However, the PAX6 mRNA was detectable only in the brain, eyes, and pancreas (Fig. 1A). Furthermore, we examined expression of PAX6 in eye-derived BCE and RCE cells by RT-PCR and found that the PAX6 mRNA was detectable only in RCE cells but not BCE cells (Fig. 1B). These results are consistent with previous reports that PAX6 is expressed in these tissues in mature animals.

Corneal epithelial cell growth control is determined by stimulation of growth factors and cytokines. We used UV
irradiation to stimulate cells and to determine the ability of CTCF to control PAX6 transcription, because UV irradiation-induced effects resemble cytokine-induced cellular responses. UV irradiation induced a downregulation of CTCF expression and an upregulation of PAX6 expression within 8 hours (Fig. 2A). UV-induced decreases in CTCF expression and increases in PAX6 expression were detected after UV exposure (Figs. 2B, 2C). CTCF binding activity on an 80-bp region upstream from the PAX6 P0 promoter detected by electrophoretic mobility shift assay (EMSA) was altered in UV-treated cells (Fig. 2D). Statistical analysis indicated that CTCF-binding activity was significantly decreased by UV irradiation ($P < 0.01$, $n = 3$, Fig. 2E).

UV-induced cellular changes share similar signaling pathways with other stress-induced mechanisms such as cytokine stimulation. Our previous studies found that CTCF is involved in EGF-induced downregulation of PAX6 expression, which is necessary for the corneal epithelial cell proliferation stimulated by this growth factor. We tested and found that EGF increased the expression levels of CTCF over time (Fig. 2A). EGF treatment also increased the binding activity of CTCF on the 80-bp region, as assayed by EMSA, and the increased CTCF binding activity was statistically significant compared with that of the control group (Figs. 3B, 3C). Our results in RCE cells indicate that upregulation or downregulation of CTCF expression by UV irradiation or EGF treatment, respectively, effectively altered CTCF binding activity to its DNA motifs to control PAX6 transcription.

To study further the binding of CTCF to the DNA motifs upstream from the PAX6 P0 promoter, a P4.2 PAX6 transcription reporter and various mutants were constructed in a β-gal reporter system (Fig. 4A). These constructs were cotransfected with or without cDNA encoding the full length CTCF. Cotransfection of CTCF in RCE cells significantly inhibited P4.2 PAX6 reporter activity and activity of P4.2-F2 in which a CCCTC sequence in the second perspective motif mutated to GGATC ($P < 0.01$, $n = 3$). However, overexpression of EGF had no effect on P4.2Pax6 (containing a 500-bp deletion) and p4.2-80 (carrying an 80-bp deletion) reporter activity, in that all five perspective CCCTC binding motifs for CTCF were eliminated by these deletion mutants (Fig. 4B). These findings suggest that the capability of CTCF to suppress PAX6 transcription is dependent on its ability to bind to its binding motifs in the 80-bp region upstream from the PAX6 P0 promoter.

**FIGURE 5.** Effect of mutation of each CTCF-binding DNA sequence on CTCF binding. (A) DNA-binding activity of CTCF detected by using 80-bp and mutated probes. $^{32}$P-labeled 80-bp probes with single mutated binding sequence at different sites (sites 1–5) were used to detect CTCF binding activity. (B) Competing CTCF DNA binding with wild-type (wt) and mutant probes in EMSA. (C) DNA binding activity of CTCF detected by using combinations of mutated probes. $^{32}$P-labeled 80-bp probes with different combinations of site 1 and other mutated sites were used to detect CTCF binding activity. (D) Competing CTCF DNA binding with wild-type (wt) and double-mutant probes in EMSA. (E) DNA binding activity of CTCF detected by using combinations of mutated probes. $^{32}$P-labeled 80-bp probes with different combinations of site 2 and other mutated sites were used to detect CTCF binding activity. (F) Competing CTCF DNA binding with wild-type and double-mutant probes in EMSA. (G) DNA binding activity of CTCF detected by using multiple combinations of mutated probes. $^{32}$P-labeled 80 bp probes with multiple combinations of mutated sites were used to detect CTCF-binding activity. (H) CTCF DNA binding competition with wild-type and multiple mutation probes in EMSA. Cold 80-bp and mutant probes were used in competitive binding experiments with CTCF DNA, and the interaction between nuclear proteins and $^{32}$P-labeled probes was determined as the inhibitory rate (arbitrary density with cold probes/arbitrary density without competent cold probes).
To determine the dependence of CTCF DNA binding on these five CCCTC binding motifs in the 80-bp region upstream from the PAX6 P0 promoter, each of these motifs mutated from CCCTC to GGATC and was tested in EMSA binding and competing experiments. First, mutation of single CTCF-binding motifs in each mutant showed slightly reduced CTCF-binding activity, and respective cold mutant probes were able to compete with CTCF binding activity in competition experiments (Figs. 5A, 5B). Second, combination of the first motif mutation with an additional mutation in different motifs in the 80-bp sequence further reduced CTCF binding in nuclear extracts and there was also competition with CTCF binding by respective combinations of cold mutant sequences (Figs. 5C, 5D). Third, combinations of the second motif mutation with motifs 3, 4, or 5 in the 80-bp sequence effectively suppressed CTCF-binding activity, and CTCF binding activity with mutant probes was competitively suppressed by respective combinations of cold mutant probes (Figs. 5E, 5F). Finally, multiple mutations of CTCF-binding motifs in different combinations in the 80-bp sequence markedly decreased CTCF binding activity in nuclear extracts and CTCF binding activity with mutant probes was competitively inhibited by the respective multimutated cold probes (Figs. 5G, 5H). These results suggest that five potential CTCF-binding motifs in the 80-bp region are all involved in CTCF DNA binding.

In the 80-bp region upstream from the PAX6 P0 promoter, there are five potential CCCTC binding motifs for the interaction of CTCF. Further analysis of the mutation and CTCF binding results indicated that CTCF binding activity decreased proportionally after increases in mutation motif numbers (Fig. 6A). Statistical significance was determined by performing ANOVA and multiple comparison analysis ($n = 3$, $P < 0.01$). Specificities of nuclear CTCF DNA binding activity were assured by competing DNA binding with cold probes that were...
mutated in different numbers of motifs in EMSA (Fig. 6B). There was a significantly reduced effect on CTCF binding when the number of mutation sites were increased \((n = 3, P < 0.01)\). Our results indicate that there is a clear correlation between the number of motifs and binding activity of CTCF in EMSA in the 80-bp region upstream from the \(P0\) promoter.

**DISCUSSION**

Regulations of corneal epithelial cell proliferation/differentiation in response to growth factors, cytokines, and stress stimulation determine corneal epithelial renewal and wound healing. UV irradiation-induced cellular responses share common signaling mechanisms in cytokine- and stress-induced pathways that relate to programmed cell death.25–26 Our previous studies suggested a novel role of CTCF in the development of eyes—direct control of \(PAX6\) expression11—but the regulatory mechanism remains unclear. In the present study, we found that UV and EGF stimulation can regulate \(PAX6\) transcription by decreasing and increasing CTCF expression, resulting in reduction and enhancement of nuclear binding ability of CTCF, respectively. We reported previously that EGF stimulates ERK1/2 (MAP kinases) signaling pathways to increase CTCF transcription and enhancement of nuclear binding ability of CTCF, resulting in decreasing and increasing CTCF expression, respectively. However, it is not known how UV irradiation suppresses CTCF expression levels. In contrast, different responses of CTCF expression to different stimuli indicate that stress-induced regulatory effects of CTCF expression and its DNA binding ability are through complicated signaling cascades in corneal epithelial cells. Because CTCF is highly conserved in most tissue and cell types, the specificities of CTCF control of \(PAX6\) expression in response to UV and EGF stimulation are determined by tissue-specific expression of the \(PAX6\) gene (Fig. 1). These findings are consistent with previous studies in that \(PAX6\) is found to be downregulated in most cells after differentiation, it remains detectable in several mature cell types of eye including corneal epithelia, lens, and retina.27

Previous studies indicate that CTCF controls gene transcription by interactions with repeating CCCTC DNA motifs in a short DNA sequence region. We found that CTCF regulates \(PAX6\) transcription through interactions with five CTCF-binding CCCTC motifs in an 80-bp region upstream of the \(PAX6\) promoter. The inhibitory effect of CTCF on \(PAX6\) transcription can be abolished by deletions of either a 500-bp fragment containing an 80-bp region or only the 80-bp region upstream from the \(P0\) promoter (Fig. 4). In experiments of \(PAX6\) transcription readouts, site-directed mutations of putative CTCF-binding motifs further confirmed that the effect of CTCF on \(PAX6\) transcription is through interactions with these motifs in the 80-bp region. Our results indicate that the regulatory effect of CTCF on gene transcription may be involved in one of the following possible mechanisms: (1) blockage of the access of enhancer region for promoters in \(H19\) and \(IGF-2\) transcription—namely, boundary functions16,28,29; (2) blockage of elongation stages in transcription; and (3) prevention of the target gene from being transcribed by removing acetyl groups from histone proteins in the chromatin, or by inhibiting the transcription-initiation complex.30

To address the question of whether all the five motifs in the 80-bp region are involved in CTCF interaction and functional regulation of \(PAX6\) transcription, we designed a series of experiments to replace CCCTC motifs with mutants, and the resultant nuclear CTCF binding activity was examined in EMSA. First, we mutated individual CCCTC motifs to GGATC in the 80-bp sequence and examined the effect of the single-motif mutation in nuclear CTCF protein binding activity. In general, the single-motif mutation weakened binding activity to nuclear CTCF. Second, combinations of motif mutations significantly impaired binding ability to nuclear CTCF. Finally, multiple motif mutations in different combinations in the 80-bp region significantly affected nuclear CTCF binding activity (Fig. 6). Increases in the number of mutated sites proportionally reduced nuclear CTCF binding activity in EMSA. These results suggest that an 80-bp region containing five CTCF-binding motifs upstream from the \(PAX6\) \(P0\) promoter is essential for CTCF interaction and regulation of \(PAX6\) transcription. The functional role of these motifs is to mediate interactions with nuclear CTCF. Any one of the motifs is involved in nuclear CTCF binding activities. Apparently, more motif involvements are necessary for nuclear CTCF to bind onto its DNA regulatory sites and to regulate \(PAX6\) transcription.

**References**


4. Walther C, Gruss P. \(Pax-6\), a murine paired box gene, is expressed in the developing CNS. Development. 1991;113:1435–1449.


