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Transcription Factors CTCF and Pax6 Are Segregated to Different Cell Types During Retinal Cell Differentiation

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Abstract

We have hypothesized that the transcription factor CTCF may influence retinal cell differentiation by controlling Pax6 expression, because (1) CTCF has been shown to repress Pax6 expression in some tissues, and (2) Pax6 blocks the differentiation of retinal progenitor cells as photoreceptors and promotes their differentiation as nonphotoreceptor neurons. Our results show that, as predicted by this hypothesis, CTCF and Pax6 become segregated to different retinal cell types. The factors are initially coexpressed in the undifferentiated neuroepithelium, but already at that time they show complementary periphery-to-fundus gradients of distribution. As the retina laminates, Pax6 becomes restricted to ganglion and amacrine cells, and CTCF to the bipolar/Muller cell layer and the outer nuclear layer. Polymerase chain reaction analysis of laser capture microdissection samples and dissociated cells showed that both immature and differentiated photoreceptors are CTCF (+)/Pax6 (–). Functional studies are now under way to further analyze the role of CTCF in retinal cell differentiation.

Keywords

retinal development; retinal differentiation; retinal gradients; photoreceptors; laser capture microdissection; single cell cDNA

INTRODUCTION

CTCF (CCCTC-binding factor) is an evolutionary conserved 11 zinc-finger phosphoprotein, first identified as a transcription factor that binds to silencer elements in the chicken c-myc and lysozyme genes (Baniahmad et al., 1990; Lobanenko et al., 1990). Its diverse functions include transcriptional repression, transcriptional activation, hormone-responsive gene silencing, methylation-dependent chromatin insulation, genomic imprinting, and a central role in the enhancer blocking activity of insulator elements (reviewed by Ohlsson et al., 2001; Klenova et al., 2002; Ladomery and Dellerie, 2002; Dunn and Davie, 2003). CTCF's versatility includes the use of different combinations of zinc fingers for binding to different sequences, as well as post-translational modifications that determine switches between repressive and activating functions (Filippova et al., 1996; El-Kady and Klenova, 2005). In the chick, CTCF is a single gene with four major mRNA species (Klenova et al., 1993). Although the open

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M. V. C-S., H. H., and M. S. R. would like to express their wholehearted admiration and gratitude for Dr. Ruben Adler, who passed away after final acceptance of this article. His sudden and unexpected death filled us with the deepest sorrow for losing not only a superb mentor but a true role-model for many aspects of our lives.

reading frame predicts a protein of 82 kDa, CTCF migrates to an apparent 130-kDa molecular mass due to the electrophoretic properties of its N- and C-terminal domains (Klenova et al., 1997). In addition to the 130-kDa form, isoforms of 97, 80, 73, 70 and 55 kDa have been observed in different chicken tissues and cell lines (Klenova et al., 1993).

Our interest in CTCF was stimulated by its reported capacity to regulate the expression of Pax6, a homeobox gene whose involvement in various aspects of retinal development has been investigated in several laboratories (Schedl et al., 1996; Marquardt et al., 2001; Zhang et al., 2003; Philips et al., 2005; Curto et al., 2007), including our own (Belecky-Adams et al., 1997; Toy et al., 2002; Canto-Soler and Adler, 2006). CTCF has been shown to down-regulate Pax6 expression by interacting with a repressive element located upstream of the Pax6 P0 promoter (Li et al., 2004; Wu et al., 2006); accordingly, CTCF down-regulation enhances Pax6 expression in rabbit corneal epithelial cells, while CTCF overexpression in transgenic mice suppresses Pax6 and leads to severe microphthalmia, similar to that of *small eye* Pax6 mutants (Li et al., 2004). Pax6 is diffusely expressed in the undifferentiated retinal neuroepithelium; as cells become postmitotic and arranged in a characteristic laminar pattern; however, Pax6 remains strongly expressed in putative ganglion, amacrine, and horizontal cells, while becoming conspicuously down-regulated in photoreceptor progenitors in the outer nuclear layer (ONL), and in the cells occupying the Muller/bipolar region of the inner nuclear layer (INL; Belecky-Adams et al., 1997). Based on these observations, we hypothesized that Pax6 *downregulation* could be necessary for photoreceptor differentiation; this hypothesis was supported by the finding that Pax6 overexpression in vitro inhibited the differentiation of retinal progenitor cells as photoreceptors, while stimulating their differentiation as nonphotoreceptor neurons (Toy et al., 2002). Taken together, these observations would suggest that functional interactions between CTCF and Pax6 could be relevant to retinal cell differentiation and predict that these transcription factors should be distributed in complementary patterns within the retina and, more specifically, that CTCF should become strongly expressed in photoreceptor progenitors in which Pax6 is down-regulated.

We have investigated these predictions at different stages of development, using whole retina extracts, laser capture microdissection (LCM) samples, individual cells isolated from retinal dissociates, and tissue sections. CTCF mRNA and protein were detected in the chicken retina at all the stages studied. CTCF and Pax6 mRNA and protein showed complementary patterns of expression throughout retinogenesis; all photoreceptor subtypes were CTCF (+) and Pax6 (–). These results provide circumstantial evidence in support of the hypothesis that CTCF may influence retinal cell differentiation, and particularly photoreceptor cell differentiation, through the down-regulation of Pax6. Functional experiments are under way to test this hypothesis.

RESULTS

Analysis in Retinal Extracts

Expression of CTCF mRNA in the embryonic chick retina—CTCF mRNA expression was detected in retinal extracts by reverse transcriptase-polymerase chain reaction (RT-PCR) at all the stages studied between embryonic day (ED) 6 and 18 (Fig. 1A). Real-time quantitative PCR (QPCR) showed a fairly constant level of CTCF mRNA relative to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Fig. 1B). This persistent level of expression as the retina changes from an undifferentiated neuroepithelium to a highly heterogeneous tissue is consistent with the possible involvement of CTCF in a variety of different developmental phenomena.

Expression of CTCF protein at different stages of retinogenesis—In agreement with a previous report (Klenova et al., 1993) analysis of chicken heart tissue by Western blot showed two CTCF isoforms of 130 and 55 kDa (Fig. 1C). As shown in Figure 1D, the retina

contained not only the 130- and 55-kDa isoforms, but also three additional isoforms of approximately 70, 97, and 120 kDa. The 70- and 97-kDa isoforms have been previously described in some chicken cell lines and tissues (Klenova et al., 1993) but, to the best of our knowledge, the 120-kDa isoform has not been previously reported.

As also illustrated in Figure 1D, comparative analysis of various developmental stages showed different temporal patterns of isoform expression in the retina. The 130 kDa isoform was down-regulated after ED12 (Fig. 1D); in contrast, the 70-kDa isoform, which has been suggested to interact with the 130-kDa isoform (Klenova et al., 1997), appeared progressively up-regulated between ED12 and ED18 (Fig. 1D). The 97- and 120-kDa bands were weak at ED6, increased between ED8 and ED12, and returned thereafter to low levels similar to those at ED6 (Fig. 1D). The 55-kDa band was the weakest one throughout development and became practically undetectable after ED12 (Fig. 1D).

In Situ Hybridization Analysis of CTCF and Pax6 mRNA Distribution in the Chick Embryo Retina

Initial in situ hybridization showed a distribution of CTCF mRNA that appeared complementary to the previously reported distribution of Pax6 mRNA at several stages of chick retinal development (Belecky-Adams et al., 1997); both transcription factors, therefore, were analyzed in parallel in this study. At early (ED 3–4) stages, the undifferentiated retinal neuroepithelium showed mRNA for these two factors distributed in complementary gradients: periphery-low to fundus-high for CTCF, and periphery-high to fundus-low for Pax6 (Fig. 2A,B). By ED6, CTCF and Pax6 signals appeared fairly widespread throughout the undifferentiated neuroepithelium (Fig. 2C,D). However, in the vitreal region of the retina, presumably occupied by recently “born” ganglion cells, CTCF signals appeared weaker and Pax6 labeling was stronger than in the rest of the neuroepithelium (Fig. 2C,D). As retina lamination became evident by ED8, CTCF mRNA signals were more intense in the ONL and the outer part of the INL than in the ganglion cell layer (GCL) and the inner part of the INL (Fig. 2E). Pax6 distribution again showed a complementary pattern, with signals being stronger in the GCL and the inner part of the INL than in the outer part of the INL and in the ONL (Fig. 2F). These patterns appeared more sharply delineated by ED18, when the lamination of the retina is already well established; CTCF mRNA appeared restricted to the ONL and outer region of the INL, with very low or undetectable levels in the GCL and inner region of the INL (Fig. 2G); Pax6 expression was predominantly confined to the GCL and the inner region of the INL, but lighter Pax6 signal was also seen in horizontal cells, particularly at higher magnification (Fig. 2H; and data not shown).

Distribution of CTCF and Pax6 mRNA in LCM Samples and Isolated Photoreceptor Cells

LCM-isolated ONL and inner retina samples—Putative photoreceptor cells are already postmitotic by ED8, when they occupy the ONL, which is clearly demarcated from the rest of the retina by an incipient outer plexiform layer (OPL). LCM was, therefore, used to isolate the ONL layer, as well as an inner retina (IR) fraction that extended from the OPL to the vitreal surface of the retina. After cDNA synthesis and global PCR amplification, possible cross-contamination was investigated by PCR analysis of mRNAs known to be restricted to the ONL (e.g., IRBP) or the IR (CRALBP, CAC, Vsx2/Chx10 and Prox1). ONL and IR samples showing no signs of cross-contamination ($n = 4$) were processed for PCR detection of CTCF and Pax6 mRNA. As expected, both CTCF and Pax6 were detected in the highly heterogeneous IR samples; on the other hand, ONL samples, containing only photoreceptor progenitors, were positive for CTCF but devoid of Pax6 (Fig. 3A).

Analysis of CTCF and Pax6 mRNA expression in isolated photoreceptors—The data described in previous sections did not elucidate whether CTCF mRNA is expressed in all

or only in subsets of photoreceptors. This question was investigated by PCR analysis of globally amplified cDNA from individual rods and cones isolated from dissociated ED20 chicken retinas. Rods, double cones, and red and green single cones were identified by morphological criteria, including an elongated shape, the presence of a rod-like or cone-like outer segment and, in the case of single cones, a brightly colored oil droplet whose pigmentation is characteristic of particular cone subtypes (Bowmaker et al., 1997; Lopez et al., 2005; Huang et al., 2007; Wahlin et al., 2007). Blue and violet cones were not included in this analysis because of their scarcity. The identity of the cells was corroborated by PCR amplification of an mRNA expressed in both rods and cones (Crx), and of photoreceptor type-specific visual pigment mRNAs (rhodopsin for rods, iodopsin for double cones and red cones, and green opsin for green cones; Fig. 3B). CTCF expression was detected in all photoreceptor sub types analyzed (Fig. 3B). Signals were seen in 4/6 rods, 4/6 red cones, 5/6 green cones, and 5/6 double cones. It is unclear at this stage whether individual variation reflects real differences between the cells or represents limitations of the technique, as previously reported by others (Chiang and Melton, 2003; Tietjen et al., 2003; Wahlin et al., 2004). Pax6 PCR products were not observed in any of the photoreceptors studied (not shown).

Comparative Analysis of CTCF and Pax6 Protein Expression

It has been recently reported that the expression of several genes involved in retinal cell differentiation is regulated posttranscriptionally (Decembrini et al., 2006). It appeared therefore important to determine whether the distribution of CTCF and Pax6 proteins matched that of their cognate mRNAs at all the developmental stages included in this study. Immunocytochemical analysis at ED3–4 showed that CTCF was highly expressed in the fundal region of the retina with lower expression levels at the periphery, while Pax6 conversely showed stronger signals at the periphery than in the fundus (Fig. 4A–C). These gradients were much less conspicuous by ED6. In the fundal region, in particular, CTCF and Pax6 signals overlapped with a fairly diffuse distribution pattern; however, putative ganglion cells showed stronger signals for Pax6 than for CTCF, while the opposite was true in the ONL (Fig. 4D,D'). The periphery-to-fundus gradients were no longer observed at ED8, when overlapping expression in cells located in the middle region of the INL (Fig. 4E') was accompanied by the emergence of a pattern of *complementary laminar* distribution of CTCF and Pax6. Thus, CTCF signals were strong and Pax6 signals were almost undetectable in the ONL and the outer region of the INL, whereas signals for CTCF were very weak and those for Pax6 were strong in ganglion cells and the inner region of the INL (Fig. 4E,E'). These laminar differences became even clearer as the retina matured at ED12 (not shown) and ED18 (Fig. 4F–H). Given the heterogeneity of the INL, the cellular distribution of CTCF within this retinal region was further investigated by colabeling with antibodies against Lim1, Lim3, or AP2 α , transcription factors known to be expressed in horizontal, bipolar and amacrine cells, respectively. This analysis corroborated that CTCF is expressed in horizontal and bipolar cells (Fig. 4I–N) but not in amacrine cells (Fig. 4I–Q). It is worth noting that horizontal cells were the only cell type in which CTCF and Pax6 could be concomitantly detected at this stage with the techniques used in this study (Fig. 4F–H, asterisks; and F'–H').

DISCUSSION

The results of this study can be summarized as follows: (1) CTCF mRNA and protein were identified in whole retina extracts at all the stages studied; (2) Western blots showed several CTCF retinal isoforms, including one that has not been reported so far in other tissues; bands corresponding to all isoforms showed characteristic temporal variations in intensity; (3) CTCF and Pax6 mRNA showed complementary patterns of distribution at all the stages studied: (i) At ED3–4, CTCF and Pax6 showed complementary periphery-to-fundus gradients of distribution; (ii) At ED6, the vitreal surface of the retina already showed weaker signals for

CTCF and stronger signals for Pax6 than the rest of the neuroepithelium; (iii) At ED8, CTCF mRNA was abundant and Pax6 mRNA scarce or undetectable in the ONL and the outer region of the INL, while Pax6 signals were strong and CTCF signals low or undetectable in ganglion cells and the inner region of the INL; (iv) This complementary laminar distribution became even better defined by ED18; (4) PCR analysis showed that photoreceptor precursors as well as differentiated rods, double cones and red and green single cones were positive for CTCF but devoid of Pax6 mRNA; (5) CTCF and Pax6 protein also showed complementary patterns of distribution at all the stages studied; (6) Within the heterogeneous INL, colocalization of CTCF immunoreactivity with that for Lim1 or Lim3 but not with Ap2 α demonstrated that CTCF is present in horizontal and bipolar cells, but is absent from differentiated amacrine cells.

Zinc-finger proteins are the largest family of transcriptional regulators in the human proteome (Ladomery and Dellaire, 2002; Ganss and Jheon, 2004; Merzdorf, 2007). Their possible involvement in photoreceptor differentiation has been recognized in *Drosophila* for some time (Moses et al., 1989; Moses and Rubin, 1991; Della et al., 1993; Begemann et al., 1997; Lai et al., 1997; Dickson, 1998; Lai and Li, 1999). Their analysis in vertebrate retinas includes reports of the down-regulation of IRBP by Mok2 (Arranz et al., 2001), the embryonic distribution and function in lens development of the muscleblind gene (Kanadia et al., 2003a,b), the interaction of Nrl with the zinc-finger protein Fiz1 (Mitton et al., 2003), the repression of the rhodopsin and IRBP promoters by Kruppel-like factor 15 (Otteson et al., 2004), the expression of RZF in human photoreceptors (Sharma et al., 2004), and the synergistic interactions between Sp4 and Crx (Lerner et al., 2005). We detected CTCF among several zinc-finger transcription factors found in developing photoreceptors by suppression subtractive hybridization (Huang and Adler, unpublished observations), and its presence has now been verified with several complementary techniques.

Although QPCR analysis showed a fairly constant relative level of CTCF mRNA in the retina throughout development, a much more dynamic behavior was observed with other methods. Examples are the bands corresponding to five different CTCF isoforms detected by Western blot analysis, which showed isoform-specific variations in intensity during development. Their biological significance in the retina is still unclear, as is the role of CTCF isoforms in other tissues (Klenova et al., 1993; Dunn and Davie, 2003). It has been suggested that the smaller forms of CTCF could act as modulators of the full length protein (Klenova et al., 1997; Dunn and Davie, 2003), but further analysis will be necessary to determine the validity of this and other possible functions.

A very dynamic pattern of expression, involving CTCF and Pax6, was also observed by in situ hybridization and immunocytochemistry. Although opposite fundus-to-periphery CTCF and Pax6 gradients were observed at ED3–4, both factors were detectable together in the undifferentiated retinal neuroepithelium until approximately ED6. Their coexpression appeared transient, however, and was no longer detectable after ED8; as the layers of the retina became established at this time, CTCF and Pax6 became segregated into a complementary laminar pattern. Even at ED6, moreover, signals for Pax6 were stronger in putative ganglion cells located at the vitreal surface of the retina, whereas CTCF signals appeared more intense in the region of putative photoreceptors (future ONL). Taken together, these changes are consistent with a model proposing that the fate of developing retinal cells (including the type of transcription factors that they express) is influenced by position-dependent microenvironmental factors that the cells encounter as they migrate toward their definitive laminar positions within the retina (Adler, 2000). Horizontal cells represented a noteworthy exception, because they did show signals for both CTCF and Pax6. There is an interesting correlation between this “hybrid” phenotype of horizontal cells and their peculiar migratory behavior (Edqvist and Hallbook, 2004), because after being “born” they move first toward the inner retina (an environment in which amacrine and ganglion cells become rich in Pax6 and

poor in CTCF), and only subsequently return to the boundary between the INL and the OPL (where cells are rich in CTCF and poor in Pax6). It must be noted that, at this time, it is uncertain whether these complementary patterns of expression are peculiar to the chick, or are also found in other species (Burke et al., 2002a).

As mentioned in the Introduction section, our interest on possible interactions between CTCF and Pax6 derived from the hypothesis that Pax6 down-regulation is necessary for progenitor cells to develop as photoreceptors (Belecky-Adams et al., 1997; Toy et al., 2002), and from reports that CTCF can regulate Pax6 expression in corneal and retinoblastoma cells by interacting with a repressor element located upstream of the Pax6 P0 promoter (Li et al., 2004, 2006). In the context of this hypothesis, it is relevant that it was possible to demonstrate the **complete absence** of Pax6 in CTCF (+) photoreceptors and their precursor cells, by PCR analysis of isolated single cells and LCM samples. These data are consistent with the possibility that CTCF up-regulation may influence the development of retinal precursors as photoreceptors through Pax6 down-regulation. It is not inconceivable, however, that CTCF could also influence photoreceptor differentiation through other mechanisms. For example, CTCF binding sites are often found in close proximity to thyroid hormone response elements (TREs), and synergistic interactions between CTCF and thyroid hormone receptor (THR) are necessary for the repression of several genes (Lutz et al., 2003; reviewed by Burke et al., 2002b). Interestingly, THRs are expressed in retinal progenitor cells and differentiating photoreceptors, and play a role in the control of photoreceptor genesis and differentiation (Ng et al., 2001; Yanagi et al., 2002; Roberts et al., 2006; Mader and Cameron, 2006; reviewed by Harpavat and Cepko, 2003). Additional analysis of the Pax6 gene sequence with Mapper (Marinescu et al., 2005a,b; <http://mapper.chip.org>) revealed several TREs in the vicinity of the active CTCF binding sites, making it possible to speculate that CTCF may repress Pax6 expression in developing photoreceptors by synergistic interaction with THRs.

EXPERIMENTAL PROCEDURES

Experimental Animals

White Leghorn chick embryos from B&E Eggs (York Spring, PA), staged as in Hamburger–Hamilton (Hamburger and Hamilton, 1951), were used.

RT-PCR and Real-Time QPCR

Total RNA was isolated from chick embryo retinas at ED6, 8, 10, 12, 15, and 18 with Trizol (Invitrogen, Carlsbad, CA), reverse-transcribed into cDNA with Superscript III (Invitrogen) and primed with oligo(dT) at 50°C for 60 min. CTCF primers (see Table 1) were designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) according to the published chicken CTCF sequence (NM_205332). Real-time QPCR was performed in 96-well plates with the Bio-Rad IQ5 system, as described in (Huang et al., 2005) with some modifications. Each 20 µl of reaction contained 10 µl of 2× SYBR Green Supermix, 100–250 nM of primer mix, and 20 ng of a cDNA template. QPCR conditions included an initial denaturing step for 3 min at 95°C, followed by 35–38 cycles (95°C for 15 sec, 55–58°C for 20 sec, and 72°C for 25 sec). Primers were optimized by melting curve profiles and agarose gel analysis. For quantification, a standard curve was generated from a series of cDNA dilutions. Relative expression of each gene was calculated using the second derivative maximum values from the linear regression of threshold cycle (C_T) number versus log concentration of the amplified gene. The housekeeping gene GAPDH was used for normalization.

Single Cell PCR

Enucleated eye cups from ED20 chicken embryos were incubated in Ca²⁺ and Mg²⁺ free HBSS (CMF) for 20 min at 37°C; after removing the retinal pigment epithelium, the retina

was dissociated after a 5-min incubation in CMF containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 5 U/ml papain, preactivated with the reducing agent L-cysteine (2.7 mM). Individual rods, double cones, and green and red single cones, identified by morphological criteria described in the Results section, were captured with a capillary pipette, and microgram amounts of cDNA were synthesized and exponentially amplified from each cell (Dulac, 1998; Wahlin et al., 2004). Cell identity was verified by PCR amplification of cell type-specific visual pigment genes as described in the Results section, with primers listed in Table 1.

LCM

The anterior chamber, lens, and vitreous body were removed from enucleated ED8 chick embryo eyes, and the resulting eyecups were infiltrated at 4°C in 6.75, 12.5, and 25% sucrose in 0.1 M phosphate buffer for 10, 20, and 30 min, respectively, equilibrated in 2:1 25% sucrose: OCT for 1 hr, and frozen by immersion in a mixture of dry ice and methylbutane. Seven micrometer cryosections were mounted onto slides with charged PEN-foil membranes (Leica Microsystems, Bannockburn, IL), fixed in 70% cold ethanol for 30 sec, briefly rinsed in DEPC-treated water, stained with hematoxylin for 10 seconds, and dehydrated in 70% and 100% ethanol for 30 and 60 sec, respectively. Outer nuclear layer and inner retina samples, delimited as described in the Results section, were separately collected using an LMD6000 LCM microscope (Leica Microsystems). From each sample, cDNA was synthesized and exponentially amplified according to Dulac (1998) and Wahlin et al. (2004) with some modifications.

Western Blot Analysis

Chicken retina and heart tissues were homogenized in cold lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100) containing “Complete Mini™,” protease inhibitor (Roche, Indianapolis, IN), incubated for 30–45 min on ice, and centrifuged at 12,000 rpm for 15 min and the supernatant containing the crude protein lysate saved for analysis. Protein concentration was measured with the DC Protein Assay Kit (Bio-Rad, Hercules, CA) according to manufacturer’s instructions. Equal amounts of protein were electrophoresed on Nu-PAGE Novex 4–12% bis-tris gels (Invitrogen), and transferred into a nitrocellulose membrane using an iBlot Dry Blotting System according to the manufacturer’s instructions (Invitrogen). Membranes were incubated in primary antibody (CTCF monoclonal antibody kindly provided by Dr. Loukinov, National Institutes of Health, 1:2000) overnight at room temperature, followed by incubation with an alkaline phosphatase-conjugated secondary antibody (Sigma; 1:3,000) and Vector’s alkaline phosphatase detection kit (Vector labs, Burlingame, CA). Gels run in parallel were processed with a primary antibody against chicken tubulin (NICHD-funded Developmental Studies Hybridoma Bank maintained by The University of Iowa, Iowa City, IA) as a loading control. As an additional loading control, gels were stained with Coomassie blue after the transfer step.

Immunohistochemistry

Chick embryos were euthanized by decapitation before ED10, and by halothane overdose thereafter. Whole heads were fixed at ED3–4, and enucleated eyes (devoid of cornea, lens, and vitreous body) were used thereafter. At ED12, 15, and 18, moreover, the dorso-fundal region of the optic cup was isolated with surgical blades for fixation. Tissues were fixed in cold acetic ethanol (75% ethanol - 25% acetic acid) for 1 hr on ice; briefly rehydrated in 95%, 75%, and 50% ethanol; washed in 1× phosphate buffered saline (PBS) and subsequently immersed in 5, 10, and 15% sucrose in phosphate buffer for several hours each, followed by overnight incubation in 20% sucrose and 1-hr incubation in a 2:1 ratio of 20% sucrose and OCT Tissue-Tek (Ted Pella; Redding, CA), and by snap freezing on dry ice/methylbutane. Immunohistochemistry was carried out in 10-μm cryostat sections. For double labeling with

CTCF (monoclonal antibody kindly provided by Dr. Loukinov, National Institute of Health) and Pax6 (rabbit polyclonal antibody kindly provided by Dr. Reed, Johns Hopkins University), sections were blocked and permeabilized in 10% goat serum/0.25% Triton X-100 for 1 hr at room temperature and incubated overnight in a primary antibody mixture (CTCF 1:4,000/Pax6 1:1,000) in 2% goat serum containing 0.05% Triton X-100 at 4°C. Antibody binding was detected with Alexa 594 goat anti-mouse and Alexa 488 goat anti-rabbit (Molecular Probes Inc., Eugene, OR; 1:1,000). Double immunostaining involving CTCF and a second monoclonal antibody (Lim3, Lim1 or AP2 α ; NICHD-funded Developmental Studies Hybridoma Bank maintained by The University of Iowa, Iowa City, IA) was performed using tyramide amplification according to the manufacturer's instructions with some modifications (TSA-PLUS Fluorescein System; Perkin Elmer, Boston, MA Perkin Elmer). Briefly, sections were incubated in 1% H₂O₂ for 30 min at room temperature to quench endogenous peroxidase activity, blocked and permeabilized in 10% goat serum/0.25% Triton X-100 for 1 hr at room temperature, and incubated overnight at 4°C in primary antibody diluted in 1× PBS (Lim1, 1:2,500 and Lim3, 1:200) or in 2% goat serum containing 0.05% Triton X-100 (AP2 α , 1:350). Control reactions showed that, at these concentrations, the antibodies were detectable after tyramide amplification but undetectable with fluorescent secondary antibodies. Antibody binding was detected with sequential incubations with biotinylated goat anti-mouse (1:1,000), horseradish peroxidase–streptavidin (1:1,000) and fluorescein-tyramide (1: 500). Sections were then profusely washed in 1× PBS, followed by overnight incubation with CTCF antibody (1:4,000) and visualization with Alexa 594 goat anti-mouse secondary antibody.

In Situ Hybridization

In situ hybridization was carried out as in Canto-Soler and Adler (2006) Pax6 sense and antisense probes were generated from cDNA generously provided by Dr. Olof Sundin (Johns Hopkins University, Baltimore, MD). CTCF probes were generated by PCR amplification of a 345-bp segment from embryonic chick retina cDNA using the primers listed in Table 1. Probes were labeled with digoxigenin RNA labeling mix, and hybridization was detected with an alkaline phosphatase-labeled anti-digoxigenin antibody and the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) alkaline phosphatase enzymatic reaction (Roche; Indianapolis, IN).

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ABBREVIATIONS

AC	amacrine cells
BC	bipolar cells
ED	embryonic day
GC	ganglion cells

GCL	ganglion cell layer
HC	horizontal cells
INL	inner nuclear layer
IR	inner retina
LCM	laser capture microdissection
ONL	outer nuclear layer
OPL	outer plexiform layer
PGCL	putative ganglion cell layer
PONL	putative outer nuclear layer

References

- Adler R. A model of retinal cell differentiation in the chick embryo. *Prog Retin Eye Res* 2000;19:529–557. [PubMed: 10925242]
- Arranz V, Dreuillet C, Crisanti P, Tillit J, Kress M, Ernoult-Lange M. The zinc finger transcription factor, MOK2, negatively modulates expression of the interphotoreceptor retinoid-binding protein gene, IRBP. *J Biol Chem* 2001;276:11963–11969. [PubMed: 11278819]
- Baniahmad A, Steiner C, Kohne AC, Renkawitz R. Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell* 1990;61:505–514. [PubMed: 2159385]
- Begemann G, Paricio N, Artero R, Kiss I, Perez-Alonso M, Mlodzik M. muscleblind, a gene required for photoreceptor differentiation in *Drosophila*, encodes novel nuclear Cys3His-type zinc-finger-containing proteins. *Development* 1997;124:4321–4331. [PubMed: 9334280]
- Belecky-Adams T, Tomarev S, Li HS, Ploder L, McInnes RR, Sundin O, Adler R. Pax-6, Prox 1, and Chx10 homeobox gene expression correlates with phenotypic fate of retinal precursor cells. *Invest Ophthalmol Vis Sci* 1997;38:1293–1303. [PubMed: 9191592]
- Bowmaker JK, Heath LA, Wilkie SE, Hunt DM. Visual pigments and oil droplets from six classes of photoreceptor in the retinas of birds. *Vision Res* 1997;37:2183–2194. [PubMed: 9578901]
- Burke LJ, Hollemann T, Pieler T, Renkawitz R. Molecular cloning and expression of the chromatin insulator protein CTCF in *Xenopus laevis*. *Mech Dev* 2002a;113:95–98. [PubMed: 11900981]
- Burke LJ, Zhang R, Lutz M, Renkawitz R. The thyroid hormone receptor and the insulator protein CTCF: two different factors with overlapping functions. *J Steroid Biochem Mol Biol* 2002b;83:49–57. [PubMed: 12650701]
- Canto-Soler MV, Adler R. Optic cup and lens development requires Pax6 expression in the early optic vesicle during a narrow time window. *Dev Biol* 2006;294:119–132. [PubMed: 16564518]
- Chiang MK, Melton DA. Single-cell transcript analysis of pancreas development. *Dev Cell* 2003;4:383–393. [PubMed: 12636919]

- Curto GG, Lara JM, Parrilla M, Aijon J, Velasco A. Modifications of the retina neuronal populations of the heterozygous mutant small eye mouse, the Sey(Dey). *Brain Res* 2007;1127:163–176. [PubMed: 17113047]
- Decembrini S, Andreazzoli M, Vignali R, Barsacchi G, Cremisi F. Timing the generation of distinct retinal cells by homeobox proteins. *PLoS Biol* 2006;4:e272. [PubMed: 16903786]
- Della NG, Senior PV, Bowtell DD. Isolation and characterisation of murine homologues of the *Drosophila* seven in absentia gene (*sina*). *Development* 1993;117:1333–1343. [PubMed: 8404535]
- Dickson BJ. Photoreceptor development: breaking down the barriers. *Curr Biol* 1998;8:R90–R92. [PubMed: 9443906]
- Dulac C. Cloning of genes from single neurons. *Curr Top Dev Biol* 1998;36:245–258. [PubMed: 9342532]
- Dunn KL, Davie JR. The many roles of the transcriptional regulator CTCF. *Biochem Cell Biol* 2003;81:161–167. [PubMed: 12897849]
- Edqvist PH, Hallbook F. Newborn horizontal cells migrate bidirectionally across the neuroepithelium during retinal development. *Development* 2004;131:1343–1351. [PubMed: 14973293]
- El-Kady A, Klenova E. Regulation of the transcription factor, CTCF, by phosphorylation with protein kinase CK2. *FEBS Lett* 2005;579:1424–1434. [PubMed: 15733852]
- Filippova GN, Fagerlie S, Klenova EM, Myers C, Dehner Y, Goodwin G, Neiman PE, Collins SJ, Lobanenko VV. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Mol Cell Biol* 1996;16:2802–2813. [PubMed: 8649389]
- Ganss B, Jheon A. Zinc finger transcription factors in skeletal development. *Crit Rev Oral Biol Med* 2004;15:282–297. [PubMed: 15470266]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol* 1951;88:49–92.
- Harpavat S, Cepko CL. Thyroid hormone and retinal development: an emerging field. *Thyroid* 2003;13:1013–1019. [PubMed: 14651785]
- Huang H, Frank MB, Dozmorov I, Cao W, Cadwell C, Knowlton N, Centola M, Anderson RE. Identification of mouse retinal genes differentially regulated by dim and bright cyclic light rearing. *Exp Eye Res* 2005;80:727–739. [PubMed: 15862179]
- Huang H, Wahlin K, McNally M, Irving N, Adler R. Developmental regulation of muscleblind-like (MBNL) gene expression in the chicken embryo retina. *Dev Dyn*. 2007in press
- Kanadia RN, Johnstone KA, Mankodi A, Lungu C, Thornton CA, Esson D, Timmers AM, Hauswirth WW, Swanson MS. A muscleblind knockout model for myotonic dystrophy. *Science* 2003a;302:1978–1980. [PubMed: 14671308]
- Kanadia RN, Urbinati CR, Crusselle VJ, Luo D, Lee YJ, Harrison JK, Oh SP, Swanson MS. Developmental expression of mouse muscleblind genes *Mbnl1*, *Mbnl2* and *Mbnl3*. *Gene Expr Patterns* 2003b;3:459–462. [PubMed: 12915312]
- Klenova EM, Nicolas RH, Paterson HF, Carne AF, Heath CM, Goodwin GH, Neiman PE, Lobanenko VV. CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. *Mol Cell Biol* 1993;13:7612–7624. [PubMed: 8246978]
- Klenova EM, Nicolas RH, U S, Carne AF, Lee RE, Lobanenko VV, Goodwin GH. Molecular weight abnormalities of the CTCF transcription factor: CTCF migrates aberrantly in SDS-PAGE and the size of the expressed protein is affected by the UTRs and sequences within the coding region of the CTCF gene. *Nucleic Acids Res* 1997;25:466–474. [PubMed: 9016583]
- Klenova EM, Morse HC III, Ohlsson R, Lobanenko VV. The novel BO-RIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. *Semin Cancer Biol* 2002;12:399–414. [PubMed: 12191639]
- Ladomery M, Dellaire G. Multifunctional zinc finger proteins in development and disease. *Ann Hum Genet* 2002;66:331–342. [PubMed: 12485467]
- Lai ZC, Fetchko M, Li Y. Repression of *Drosophila* photoreceptor cell fate through cooperative action of two transcriptional repressors Yan and Tramtrack. *Genetics* 1997;147:1131–1137. [PubMed: 9383057]

- Lai ZC, Li Y. Tramtrack69 is positively and autonomously required for *Drosophila* photoreceptor development. *Genetics* 1999;152:299–305. [PubMed: 10224262]
- Lerner LE, Peng GH, Gribanova YE, Chen S, Farber DB. Sp4 is expressed in retinal neurons, activates transcription of photoreceptor-specific genes, and synergizes with Crx. *J Biol Chem* 2005;280:20642–20650. [PubMed: 15781457]
- Li T, Lu Z, Lu L. Regulation of eye development by transcription control of CCCTC binding factor (CTCF). *J Biol Chem* 2004;279:27575–27583. [PubMed: 15096508]
- Li T, Lu Z, Lu L. Pax6 regulation in retinal cells by CCCTC binding factor. *Invest Ophthalmol Vis Sci* 2006;47:5218–5226. [PubMed: 17122106]
- Lobanenko VV, Nicolas RH, Adler VV, Paterson H, Klenova EM, Polotskaja AV, Goodwin GH. A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5'-flanking sequence of the chicken c-myc gene. *Oncogene* 1990;5:1743–1753. [PubMed: 2284094]
- Lopez R, Lopez-Gallardo M, Busturia I, Anezary L, Prada C. Spatial and temporal patterns of growth and differentiation of cone oil droplets in the chick retina. *J Neurosci Res* 2005;79:401–411. [PubMed: 15605374]
- Lutz M, Burke LJ, LeFevre P, Myers FA, Thorne AW, Crane-Robinson C, Bonifer C, Filippova GN, Lobanenko V, Renkawitz R. Thyroid hormone-regulated enhancer blocking: cooperation of CTCF and thyroid hormone receptor. *EMBO J* 2003;22:1579–1587. [PubMed: 12660164]
- Mader MM, Cameron DA. Effects of induced systemic hypothyroidism upon the retina: regulation of thyroid hormone receptor alpha and photoreceptor production. *Mol Vis* 2006;12:915–930. [PubMed: 16917485]
- Marinescu VD, Kohane IS, Riva A. The MAPPER database: a multi-genome catalog of putative transcription factor binding sites. *Nucleic Acids Res* 2005a;33:D91–D97. [PubMed: 15608292]
- Marinescu VD, Kohane IS, Riva A. MAPPER: a search engine for the computational identification of putative transcription factor binding sites in multiple genomes. *BMC Bioinformatics* 2005b;6:79. [PubMed: 15799782]
- Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P. Pax6 is required for the multipotent state of retinal progenitor cells. *Cell* 2001;105:43–55. [PubMed: 11301001]
- Merzdorf CS. Emerging roles for zic genes in early development. *Dev Dyn* 2007;236:922–940. [PubMed: 17330889]
- Mitton KP, Swain PK, Khanna H, Dowd M, Apel IJ, Swaroop A. Interaction of retinal bZIP transcription factor NRL with Flt3-interacting zinc-finger protein Fiz1: possible role of Fiz1 as a transcriptional repressor. *Hum Mol Genet* 2003;12:365–373. [PubMed: 12566383]
- Moses K, Ellis MC, Rubin GM. The glass gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. *Nature* 1989;340:531–536. [PubMed: 2770860]
- Moses K, Rubin GM. Glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing *Drosophila* eye. *Genes Dev* 1991;5:583–593. [PubMed: 2010085]
- Ng L, Hurley JB, Dierks B, Srinivas M, Salto C, Vennstrom B, Reh TA, Forrest D. A thyroid hormone receptor that is required for the development of green cone photoreceptors. *Nat Genet* 2001;27:94–98. [PubMed: 11138006]
- Ohlsson R, Renkawitz R, Lobanenko V. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *Trends Genet* 2001;17:520–527. [PubMed: 11525835]
- Otteson DC, Liu Y, Lai H, Wang C, Gray S, Jain MK, Zack DJ. Kruppel-like factor 15, a zinc-finger transcriptional regulator, represses the rhodopsin and interphotoreceptor retinoid-binding protein promoters. *Invest Ophthalmol Vis Sci* 2004;45:2522–2530. [PubMed: 15277472]
- Philips GT, Stair CN, Young Lee H, Wroblewski E, Berberoglu MA, Brown NL, Mastick GS. Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type. *Dev Biol* 2005;279:308–321. [PubMed: 15733660]
- Roberts MR, Srinivas M, Forrest D, Morreale de Escobar G, Reh TA. Making the gradient: thyroid hormone regulates cone opsin expression in the developing mouse retina. *Proc Natl Acad Sci U S A* 2006;103:6218–6223. [PubMed: 16606843]

- Schedl A, Ross A, Lee M, Engelkamp D, Rashbass P, van Heyningen V, Hastie ND. Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities. *Cell* 1996;86:71–82. [PubMed: 8689689]
- Sharma S, Dimasi D, Higginson K, Della NG. RZF, a zinc-finger protein in the photoreceptors of human retina. *Gene* 2004;342:219–229. [PubMed: 15527981]
- Tietjen I, Rihel JM, Cao Y, Koentges G, Zakhary L, Dulac C. Single-cell transcriptional analysis of neuronal progenitors. *Neuron* 2003;38:161–175. [PubMed: 12718852]
- Toy J, Norton JS, Jibodh SR, Adler R. Effects of homeobox genes on the differentiation of photoreceptor and non-photoreceptor neurons. *Invest Ophthalmol Vis Sci* 2002;43:3522–3529. [PubMed: 12407164]
- Wahlin KJ, Lim L, Grice EA, Campochiaro PA, Zack DJ, Adler R. A method for analysis of gene expression in isolated mouse photoreceptor and Muller cells. *Mol Vis* 2004;10:366–375. [PubMed: 15205663]
- Wahlin K, Moreira E, Huang H, Yu N, Adler R. Molecular dynamics of photoreceptor synapse formation in the developing chick retina. *J Comp Neurol* 2008;506:822–837. [PubMed: 18076030]
- Wu D, Li T, Lu Z, Dai W, Xu M, Lu L. Effect of CTCF-binding motif on regulation of PAX6 transcription. *Invest Ophthalmol Vis Sci* 2006;47:2422–2429. [PubMed: 16723452]
- Yanagi Y, Takezawa S, Kato S. Distinct functions of photoreceptor cell-specific nuclear receptor, thyroid hormone receptor beta2 and CRX in one photoreceptor development. *Invest Ophthalmol Vis Sci* 2002;43:3489–3494. [PubMed: 12407160]
- Zhang X, Heaney S, Maas RL. CreloxP fate-mapping of Pax6 enhancer active retinal and pancreatic progenitors. *Genesis* 2003;35:22–30. [PubMed: 12481295]

Fig. 1. CTCF mRNA and protein are expressed in the chicken retina throughout development. **A,B:** CTCF mRNA was investigated at different developmental stages by reverse transcriptase-polymerase chain reaction (RT-PCR, A) and real-time quantitative PCR (B). **C,D:** Western blot analysis. **C:** CTCF protein isoforms present in embryonic day (ED) 18 heart. **D:** CTCF isoforms detected in the chicken retina at different stages of development.

Fig. 2.

CTCTF and Pax6 mRNA show complementary patterns of distribution throughout retinogenesis. **A,B:** Transverse sections of embryonic day (ED) 4 chicken embryo eyes. CTCTF (A) and Pax6 (B) showed complementary fundus-to-periphery patterns of distribution. **C–H:** Transverse sections of embryonic chicken retina showing distribution of mRNA for CTCTF (C,E,G) and Pax6 (D,F,H) at ED6 (C–D), ED8 (E,F) and ED18 (G,H). Retinal sections are shown with the pigment epithelium at the top and the vitreal surface of the retina at the bottom of the pictures. Note that CTCTF signals were stronger in the outer nuclear layer (ONL) and the outer region of the inner nuclear layer (INL), whereas Pax6 signals were stronger in the ganglion cell layer

(GCL) and the inner region of the INL. The asterisk indicates horizontal cells. f, fundus; p, periphery.

Fig. 3.

Photoreceptor precursors and differentiated photoreceptors express CTCF but are devoid of Pax6. **A:** Hematoxylin and eosin (H&E) panels show transverse sections of embryonic day (ED) 8 chicken retina from which outer nuclear layer (ONL) and inner retina (IR) samples were isolated by laser capture microdissection (LCM). Polymerase chain reaction (PCR) detected both CTCF and Pax6 mRNA in IR samples, while only CTCF was detected in ONL samples. **B:** Differential interference contrast microscopy images of single photoreceptors isolated from dissociated ED20 chick retinas. Individual photoreceptor subtypes were identified as described in the Results section. Note that CTCF mRNA was detected in all photoreceptor subtypes analyzed.

Fig. 4.

CTCF and Pax6 protein show complementary patterns of expression throughout retinogenesis. **A–E:** Transverse sections of chicken embryo optic cups at embryonic day (ED) 4 (A–C), ED6 (D, D') and ED8 (E, E') processed for double-labeling immunocytochemistry for CTCF (red) and Pax6 (green). **F–Q:** Transverse sections of ED18 chicken retina processed by double-labeling immunocytochemistry for CTCF (red) in combination with Pax6 (F–H and F'–H') Lim1 (I–K), Lim3 (L–N), AP2α (O–Q) (green). Retinal sections are shown with the pigment epithelium at the top and the vitreal surface of the retina at the bottom of the pictures. Note CTCF expression in horizontal (asterisks) and bipolar cells and its exclusion from amacrine cells. AC, amacrine cells; BC, bipolar cells; GC, ganglion cells; GCL, ganglion cell layer; HC, horizontal cells; INL, inner nuclear layer; ONL, outer nuclear layer; PGCL, putative ganglion cell layer; PONL, putative outer nuclear layer.

TABLE 1**List of Primers Used in This Study**

Gene	Accession no.	Application	Forward primer	Reverse primer
CAC	Z14957.1	LCM	GGCAACATCTTGTCTCCATA	GCTCTCCAAACAGCCAATA
Vsx2/ Chx10	AF178671	LCM	TTCTGAGCCTGAAGGGAATG	CACAGCGTGGGCTATAATCT
CRALBP	BX935536	LCM	TAACCCCAACCACCTCCATA	CTGGCTGTGCAGACAGTGT
CRX	AF 285171	Single cell PCR	ATGCCACAAGCACACAGGCA	GCATCGGCAAACACAGGAAGCA
CTCF	NM 205332	RT-PCR Single cell PCRn In situ probe synthesis LCM	AAGGGCAAACGTGGCCGAAA	AGGCTGAGCTTCCTCCTCTT
CTCF	NM 205332	QPCR	AGAGCCTCAGTCTTGCTGAT	CCTTCCCAGCTTTCAAACCC
GAPDH		Single cell PCR	AGGGTAGTGAAGGCTGCTGCTGA	ATGCAGAACTGAGCGGTGGT
Green opsin	NM 205490	Single cell PCR	ATCCCTGCAGAGCTGCACAT	AGCAGTCTTGTGCTGACACGA
Iodopsin	NM 205440	Single cell PCR	TCCCGCATGGTGGTGGTGATGAT	AACCGGAAGCTGCGGTGTGGA
IRBP		LCM	CGATGCCGGACATCAAGTTC	ACCCACCACTGGTCTGTTCT
Pax6	D87837	Single cell PCRLCM	CACCACTTCCACAGGTCTCAT	TCGTGCCGAACCGATATAAT
Prox1	U46563	LCM	ATAACCTGCGCCAGTAATCG	AGACCAAGACGTGGCAATTT
Rhodopsin	D00702.1	Single cell PCR	ACACGCTGAAGCCGGAGATCAA	TTCTTGCCGCAGCAGAGGGTTGT

^a LCM, laser capture microdissection; RT-PCR, reverse transcriptase-polymerase chain reaction; QPCR, real-time quantitative PCR.