Transcriptional Regulation of Neural Retina Leucine Zipper (Nrl), a Photoreceptor Cell Fate Determinant*

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Background: The transcription factor Nrl is required for rod photoreceptor development, but mechanisms governing Nrl transcription remain largely unknown.

Results: The transcription factors CRX, OTX2, and RORB regulate Nrl by binding directly to its promoter region.

Conclusion: These three factors combinatorially control Nrl expression in the developing mouse retina.

Significance: This study elucidates a critical link in the photoreceptor cis-regulatory network.

The transcription factor neural retina leucine zipper (Nrl) is a critical determinant of rod photoreceptor cell fate and a key regulator of rod differentiation. Nrl−/− rod precursors fail to turn on rod genes and instead differentiate as cones. Furthermore, Nrl mutations in humans cause retinitis pigmentosa. Despite the developmental and clinical significance of this gene, little is known about the transcriptional regulation of Nrl. In this study, we sought to define the cis- and trans-acting factors responsible for initiation and maintenance of Nrl transcription in the mouse retina. Utilizing a quantitative mouse retinal explant electroperoration assay, we discovered a phylogenetically conserved, 30-base pair region immediately upstream of the transcription start site that is required for Nrl promoter activity. This region contains binding sites for the retinal transcription factors CRX, OTX2, and RORB, and point mutations in these sites completely abolish promoter activity in living retinas. Gel-shift experiments show that CRX, OTX2, and RORB can bind to the critical region in vitro, whereas ChIP experiments demonstrate binding of CRX and OTX2 to the critical region in vivo. Thus, our results indicate that CRX, OTX2, and RORB directly regulate Nrl transcription by binding to critical sites within the Nrl promoter. We propose a model in which Nrl expression is primarily initiated by OTX2 and RORB and later maintained at high levels by CRX and RORB.

NRL is a Maf family transcription factor that is required for mouse rod photoreceptor development (1). Nrl is expressed in the wild-type retina starting around embryonic day 12 (2), coincident with the beginning of the rod birth period (3, 4). It subsequently activates numerous rod-specific genes, including Rhodopsin (5, 6) and many components of the rod-specific phototransduction cascade (7, 8). NRL simultaneously represses cone genes in rods, either directly (9) or by activating the downstream repressor Nr2e3 (10–13). In the Nrl−/− retina, rod gene expression fails to initiate, and rod precursors show a derepression of cone genes, which results in a transfating of the cells into cones in the adult retina (1, 14). Nrl expression is the earliest known marker of rod photoreceptor identity, and expression persists at high levels in the outer nuclear layer throughout adulthood. Not surprisingly, mutations in human NRL often result in heritable retinal disease, most commonly autosomal dominant retinitis pigmentosa.

Despite the importance of Nrl in the photoreceptor transcriptional network as well as in human disease, the mechanisms by which Nrl transcription is regulated are incompletely understood. Akimoto and colleagues (2) have shown that a 2.5-kb region upstream of Nrl is sufficient to drive GFP expression in rod photoreceptors. However, specific binding sites for transcriptional regulators within this promoter region remain unknown. An earlier study of the Nrl promoter (15) utilized a heterologous cell culture assay to identify promoter elements responsive to the rod fate inducer retinoic acid (16), but these sites have not yet been tested in vivo. Nrl has also been hypothesized to be a regulatory target of other retinal transcription factors such as CRX (17, 18) and RORB (19), but it is unclear whether this regulation is direct or indirect. In this study, we sought to identify discrete cis-regulatory motifs and the transcription factors that bind them to regulate activation of the Nrl promoter.

To conduct the Nrl promoter analysis in the living mouse retina, we utilized a retinal explant electroperoration assay (20). This assay has been used previously to quantify the activity of photoreceptor-specific cis-regulatory regions (21) and represents a much more realistic assay for analyzing cis-regulation than traditional mammalian cell culture approaches. Using this system, we have found that three retinal transcription factors, OTX2, CRX, and RORB, activate Nrl transcription by binding directly to a highly conserved region of the Nrl promoter.

EXPERIMENTAL PROCEDURES

Mouse Husbandry—Adult CD1, C57BL/6, Crx−/− (22) and Otx2−/− (23) mice were maintained on a 12-h light/dark schedule.
directed mutagenesis was also used to create one- or two-nucleotide (mutated nucleotides underlined), 5’ to 3’

| Construct no. | Sequence of site-directed mutagenesis
|---------------|------------------------------------------|
| 1             | TTAGACTCTCGGTTAGTTGGCAGACAACACCATTTTTAAACG |
| 2             | GAGTTTTTTTACCAAGACATCAAGCCGCGAAACACCTCGAAGAG |
| 3             | TACATTAGAGGCTTACATTTTGCTGACACACAGGAAGCTTCAAACC |
| 4             | CTTATTTAAGCAGCTTCTTAT-12CTAT-10CAGAGTCTTGGCTA |
| 5             | CAATCTCCACAGGTTGCTTACAAACGTACACGACGACCTTGAATA |
| 6             | CATCCAGGACCTTAATAGCCAGGACACACCTCCTGCCCTTCTT |
| 7             | GCTAATCTCTTACACTATGACAAGGAGGACGAGGATGAGTA |
| 8             | ACTCTCCCGCCCTCCTAGTGTGTTCCTGCTATAGGCTGTG |
| 9             | CTCTCCACAGGACCTTAATAGCCAGGACACACCTCCTGCCCTTCTT |
| 10            | TACATTAGAGGCTTACATTTTGCTGACACACAGGAAGCTTCAAACC |
| 11            | TACATTAGAGGCTTACATTTTGCTGACACACAGGAAGCTTCAAACC |
| 12            | TGACACTCTTACAGGAGCTTACATTTTGCTGACACACAGGAAGCTTCAAACC |
| 13            | AGAAAGTCTGGTGCCTGACACACAGGAAGCTTACATTTTGCTGACACACAGGAAGCTTCAAACC |
| 14            | AGGCGGATCGGACAGTGGACAAGCAGGAAGCTTACATTTTGCTGACACACAGGAAGCTTCAAACC |
| 15            | GCTGAAAATGTAGGTCACACCCAGCCTGAGTTTCCGGAGCGAGAGAGAAG |
| 16            | TAGGTCACACACCCCAGCCTGAGTTTCCGGAGCGAGAGAGAAG |

TABLE 1

Sequences of sense primers used in site-directed mutagenesis reactions

Nrl Regulation by CRX, OTX2, and RORβ

at 22 °C with free access to food and water. The Otx2−/− mutant (accession no. CDB0013K, Riken BioResource Center) was obtained as described (23). The health of the animals was regularly monitored. All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee (approval no. 20110089).

DNA Constructs—The pNrl(3.2 kb)-DsRed and pCAG-GFP vectors have been described previously (20, 24). pNrl(3.2 kb)-GFP was created by replacing the DsRed coding sequence in pNrl(3.2 kb)-DsRed with GFP, using the EcoRI and NotI restriction sites.

To create the Nrl promoter truncation constructs in Fig. 1, PCR was used to amplify the following regions of the mouse Nrl promoter relative to the transcription start site (RefSeq NM_00136074): pNrl(2.9 kb), −2063 to +865; pNrl(2.5 kb), −1695 to +865; pNrl(1.1 kb), −202 to +865; pNrl(0.8 kb), +35 to +865; and pNrl(0.3 kb), +549 to +865. The promoter fragments were subcloned upstream of DsRed in the no-basal vector (17) using the XbaI and Smal restriction sites.

Scanning mutagenesis constructs were created via site-directed mutagenesis of pNrl(1.1 kb)-DsRed. The QuikChange II XL Site-Directed Mutagenesis kit (Agilent, Santa Clara, CA) was used with the primers listed in Table 1. (Both sense and antisense primers were added to the mutagenesis PCR reaction although only sense primers are listed.) In general, G→T and C→A substitutions were made unless mutagenesis generated a CRX binding site (constructs 8 and 12), in which case the de novo site was disrupted by an alternative substitution. Site-directed mutagenesis was also used to create one- or two-nucleotide mutations in the CRX and RORβ binding sites within the pNrl(1.1 kb)-DsRed vector.

To create the CR-DsRed construct, the following oligonucleotides containing the Nrl critical region were kinased and annealed (restriction site-compatible ends underlined): 5’-ctaggttacagttggtgccacctgctctctc-3’ and 5’-cggtggagctgactctctctc-3’, and the resultant product was subcloned upstream of DsRed in the Rho-basal vector (17) with the XbaI and KpnI restriction sites. To create (Rho-prox)-DsRed, a 205-nucleotide region of the mouse Rho promoter was obtained by PCR from mouse genomic DNA using the following primers: 5’-gtacttttcagctgctgctgctgcctc-3’ and 5’-gtacgccagacacagcgagctc-3’. This product was digested with XbaI and KpnI and subcloned upstream of DsRed in the no-basal vector. (CR)-(Rho-prox)-DsRed and mutation-containing variants (see Fig. 2C) were created by subcloning CR2-containing oligonucleotides into the (Rho-prox)-DsRed vector with the restriction sites Sall and XbaI.

To create the Nrl promoter truncation constructs in Fig. 3, PCR was used to amplify the following regions of the mouse Nrl promoter relative to the transcription start site: pNrl(0.99 kb), −122 to +865; pNrl(0.97 kb), −102 to +865; pNrl(0.95 kb), −82 to +865; and pNrl(0.92 kb), −57 to +865. The promoter fragments were subcloned upstream of DsRed in the no-basal vector (17) using the XbaI and Smal restriction sites.

Two non-fluorescent DNA constructs were electroporated in this study. The Crx RNAi construct has been described previously by Matsuda and Cepko (20). pNrl(1.1 kb)-Cre was created by subcloning the Cre-coding sequence from pCAG-Cre (24) in place of DsRed in the pNrl(1.1 kb)-DsRed vector, using the restriction sites EcoRI and NotI.

Electroporation and Culture of Explanted Retinas—Electroporation and explant culture of mouse retinas were performed as described previously (17). Briefly, eyes were removed from decapitated P0 mouse pups, and the retinas were dissected, leaving the lens in place. The retinas were then electroporated in a DNA solution consisting of the experimental DsRed reporter construct and a control GFP reporter construct, each at a final concentration of 0.5 μg/μl. Following the explant culture period, retinas were fixed, whole-mounted for quantitative imaging, and embedded for cryo-sectioning as described previously (21). Wild-type CD1 mice were used unless otherwise stated.

Quantitation of Promoter Activity in Whole-mount Retinas—DsRed fluorescence levels were measured and normalized to control GFP values as described previously (21). Briefly, eight to nine whole-mounted electroporated retinas for each reporter construct were imaged at 40× magnification under epifluorescent illumination. Images were captured in both the red and green channels using a monochromatic camera (ORCA-ER; Hamamatsu, Japan). Circles of interest were defined and mean pixel values were recorded for five regions overlaying the electroporated retina and three control regions outside the retina (for background subtraction) in both the red and green channels using ImageJ software (National Institutes of Health, Bethesda, MD). For normalization, the background-subtracted mean pixel value of the experimental red channel was divided by the background-subtracted mean pixel value of the control green channel. S.D. was calculated based on all normalized fluorescence measurements (five measurements per retina, eight to nine retinas per construct).

Reverse Transcription Quantitative PCR for Nrl Transcript—Retinal tissue was isolated from wild-type (C57BL/6) and Crx−/− mice (on a C57BL/6 background). Three biological replicates (three to four retinas each) were collected for each time point (for background subtraction) in both the red and green channels using ImageJ software (National Institutes of Health, Bethesda, MD). For normalization, the background-subtracted mean pixel value of the experimental red channel was divided by the background-subtracted mean pixel value of the control green channel. S.D. was calculated based on all normalized fluorescence measurements (five measurements per retina, eight to nine retinas per construct).

The abbreviations used are: CR, critical region; qPCR, quantitative real-time PCR; P12, postnatal day 12.

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point. Total RNA was extracted using TRIzol (Invitrogen) and quantified using a Nanodrop device (Thermo Fisher Scientific). cDNA was synthesized using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen) with 1 μg total RNA and oligo(dT)$_{20}$ primers. Reverse transcription quantitative PCR (RT-qPCR) was performed using SYBR-Green (Applied Biosystems, Carlsbad, CA) and the following primers (25): Nrl transcript, 5′-aacctctgagactctggca-3′ and 5′-taaagactgctgacctggaca-3′; GAPDH transcript, 5′-ctccctcaagccgaaatc-3′ and 5′-cgcttgaaggagatgtttgat-3′. Three technical replicate PCRs were performed for each biological replicate. For each biological replicate, Nrl transcript levels were normalized to control GAPDH levels ($\Delta C_t$). $\Delta C_t$ values from the three biological replicates were then averaged, and fold change relative to the wild-type P7 Nrl expression level was calculated, along with the S.D. across the biological replicates.

**EMSA**—The DNA binding domains of Otx2 and Rorb were amplified from mouse retinal cDNA using the following PCR primers: Otx2, 5′-tcggatatccgggaagggagga-3′ and 5′-tcagctggcgccggcttcctgg-3′; Rorb, 5′-tcggatatcctctgtggagacggc-3′ and 5′-tcagctggcgccggcttcctgg-3′. The products were digested with EcoRI and NotI and subcloned into the pGEX-5X-1 vector (Amersham Biosciences/GE Healthcare) to generate an N-terminal GST fusion. A third GST replicate PCRs were performed for each of the two biological replicates. The fold enrichment of each target was calculated as 2 to the power of the cycle threshold ($C_t$) difference between IgG- and CRX-ChIP or IgG- and OTX2-ChIP.

**RNA in Situ Hybridization**—In situ hybridization on retinal tissue sections was performed as described previously (26). Briefly, either whole eyes or retinas were isolated from C57BL/6 mice at various time points. The tissue was fixed in 4% paraformaldehyde at 4°C overnight, equilibrated in 30% sucrose/PBS, and embedded in OCT (Tissue Tek). The embedded tissue was cryosectioned, mounted on glass slides, and hybridized with RNA riboprobes synthesized from PCR products derived from templates described in previous studies (8, 17).

**RESULTS**

A 30-Nucleotide Region Is Critical for Nrl Promoter Activity—To identify the cis- and trans-acting factors required for regulation of Nrl, we undertook a detailed analysis of its proximal promoter region. A previous study demonstrated that a 3.2-kb fragment of the mouse Nrl promoter (from −2345 to +865 bp relative to the Nrl transcription start site) is sufficient to drive strong rod photoreceptor-specific expression in electroporated retinas (24). This fragment contains three blocks of high phylogenetic conservation (denoted P1, P2, and P3 in Fig. 1A), which correspond to regions bound by CRX in vivo as identified in a recent genome-wide ChIP-seq study (18). We created a series of Nrl promoter truncations to narrow down the minimal promoter region required for Nrl expression (Fig. 1A). These promoter truncations were fused to the fluorescent reporter DsRed and electroporated into P0 retinal explants along with the loading control pNrl(3.2 kb)-GFP. The 3.2- and 2.9-kb constructs (both encompassing P1, P2, and P3) expressed strongly in explant cultures after 8 days (Fig. 1B). Interestingly, the 2.5- and 1.1-kb constructs that encompassed only P2 and P3 drove equally strong expression (Fig. 1B). Elimination of P2 in the 0.8- and 0.3-kb constructs, however, caused complete loss of pNrl-DsRed expression, suggesting that P2 harbors cis-regulatory motifs critical for Nrl expression.

To identify these motifs within the P2 block, we performed scanning mutagenesis over a 160-bp region (Fig. 1C). Ten adja-
The Critical Region Contains Functional CRX- and RORβ-binding Sites—Examination of the 30-nucleotide critical region revealed near-consensus binding sites for two key photoreceptor transcription factors, CRX and RORβ, spaced seven nucleotides apart (Fig. 2A). The 

mut1” and “RORβ mut2”) (21, 27). All three constructs resulted in a complete loss of expression in retinal explants (Fig. 2A). Next, we tested whether the critical region has intrinsic promoter and/or enhancer activity (Fig. 2B). To test promoter activity, we cloned the critical region (CR) upstream of a minimal basal promoter from bovine rhodopsin which, by itself, does not drive any expression in photoreceptors (17). This construct failed to drive DsRed in explants (Fig. 2B, top panels). To determine whether the critical region could enhance the activity of an adjacent active promoter, we cloned it upstream of a promoter fragment from mouse rhodopsin (“Rho-prox”), which can drive moderate levels of DsRed expression by itself (Fig. 2B, middle panels). Fusion of the critical region to Rho-prox markedly boosted DsRed expression (Fig. 2B, bottom panels). Thus, the critical region lacks autonomous promoter activity but can act as a potent enhancer.

Lastly, we evaluated the relative contributions of the CRX and RORβ sites to the critical region enhancer activity (Fig. 2, C and D). Three new constructs were created on the (critical region)-(Rho-prox)-DsRed backbone, which eliminated the CRX site alone, the RORβ site alone, or both. These constructs were electroporated into retinal explants (Fig. 2C), and fluorescence levels were quantified (Fig. 2D). Mutation of the RORβ site elicited a greater reduction in enhancer activity than the CRX site mutation, whereas mutation of both sites caused an

enriched isoform ROR with somewhat different DNA-binding preferences. Whereas CRX binds the 30-nucleotide AGGTCA core element of the putative ROR binding site with near-consensus binding affinity (21), ROR binds the same sequence with reduced affinity (27). The critical region contains both the CRX and RORβ binding sites (Fig. 1C). To test the functionality of these two sites within the pNrl(1.1 kb)-DsRed reporter, we created point mutations predicted to abolish CRX binding (“CRX mut”) or RORβ binding (“RORβ mut1” and “RORβ mut2”) (21, 27). All three constructs resulted in a complete loss of expression in retinal explants (Fig. 2A). Next, we tested whether the critical region has intrinsic promoter and/or enhancer activity (Fig. 2B). To test promoter activity, we cloned the critical region (CR) upstream of a minimal basal promoter from bovine rhodopsin which, by itself, does not drive any expression in photoreceptors (17). This construct failed to drive DsRed in explants (Fig. 2B, top panels). To determine whether the critical region could enhance the activity of an adjacent active promoter, we cloned it upstream of a promoter fragment from mouse rhodopsin (“Rho-prox”), which can drive moderate levels of DsRed expression by itself (Fig. 2B, middle panels). Fusion of the critical region to Rho-prox markedly boosted DsRed expression (Fig. 2B, bottom panels). Thus, the critical region lacks autonomous promoter activity but can act as a potent enhancer.

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almost complete loss of critical region enhancer activity (Fig. 2D).

A Conserved Sequence Upstream of Critical Region Contains Additional cis-Motifs—With one exception, scanning mutagenesis of individual 10-bp blocks upstream of the critical region (constructs 1–11) resulted in modest decrements in promoter activity, in some cases down to /811011/50% of wild-type levels (Fig. 1C). This observation, and the fact that this region is highly conserved phylogenetically, suggested that it might contain multiple transcription factor binding sites that coordinately regulate promoter activity. To test this idea, we engineered five additional truncations of the Nrl promoter across this region and electroporated them into retinal explants as DsRed fusion constructs (Fig. 3, A and B). As quantified in Fig. 3C, graded truncation of the Nrl promoter correlated with graded loss of promoter activity (Fig. 3C). Thus, the 110-bp sequence immediately upstream of the Nrl critical region is indispensable for Nrl promoter activity, despite the fact that no specific 10-bp block within the region is strictly required for expression.

Coordinate Regulation of Nrl by CRX and OTX2—Explant electroporation experiments determined that a putative CRX-binding site is essential for Nrl promoter activity. In addition, a prior study showed a marked decrement in Nrl transcript levels in P28 Crx<sup>−/−</sup> retinas by in situ hybridization (17). To deter-
mine the temporal dependence of Nrl expression on CRX, we measured Nrl transcript levels across developmental time in wild-type and Crx−/− retinas (Fig. 4A). RT-qPCR showed that Nrl transcript levels are similar in wild-type and Crx−/− retinas until P7, after which the levels fall off sharply in Crx−/− compared with wild-type (Fig. 4A). This result suggests that CRX is not required for the initiation of Nrl expression in vivo but that it is necessary for maintenance of Nrl transcription at later time points.

In a second experiment, we co-electroporated pNrl(3.2 kb)-DsRed and control pCAG-GFP into P0 wild-type and Crx−/−/− retinal explants. After 10 days in culture, pNrl(3.2 kb)-DsRed expression was moderately reduced in Crx−/− explants relative to wild-type (Fig. 4B), confirming that CRX plays only a minor role in activating the Nrl promoter in the early postnatal period.

These two results are seemingly at odds with the observation that mutation of a single putative CRX-binding site within the critical region completely abolishes Nrl promoter activity in electroporated retinas in the early postnatal period (Fig. 2A). To account for this discrepancy, we hypothesized that the retinal transcription factor OTX2 might be able to compensate for CRX at early time points. OTX2 and CRX are both K50 home-domain transcription factors required for photoreceptor development (28–30), and they have nearly identical DNA-binding preferences (21, 31). In addition, Otx2 is required for initiation of Crx expression (30). Otx2 and Crx are both expressed at high levels in differentiating photoreceptors through P3.5 (Fig. 4C). By P10.5, however, Otx2 levels have decreased in photoreceptors, whereas Crx levels remain high (Fig. 4C). At the adult stage, expression of Otx2 persists in photoreceptors at only very low levels, whereas Crx is maintained at high levels (17). Thus, it is reasonable to surmise that both OTX2 and CRX may bind to the Nrl promoter critical region in the early postnatal period to activate Nrl transcription. As photoreceptor OTX2 levels decline later, CRX may become the predominant activator of Nrl.

To test the hypothesis that both CRX and OTX2 activate the Nrl promoter in the early postnatal period, we performed a series of explant electroporations utilizing retinas from the Otx2f/f mouse, which is homozygous for a conditional (“floxed”) allele of Otx2 (Fig. 4D). Electroporation of pNrl(3.2 kb)-DsRed and control pCAG-GFP into Otx2f/f explants resulted in strong DsRed expression after 10 days (Fig. 4D, upper first panel). To
eliminate CRX alone, we co-electroporated a CRX RNAi construct (20) along with the fluorescent reporters, which resulted in a decrease in pNrl-DsRed expression (Fig. 4D, upper second panel). Interestingly, the decrease in expression was greater than what was observed when the pNrl-DsRed construct was electroporated into Crx−/− retinas (Fig. 4B). This difference might be explained by the fact that in the Crx−/− retina, there is an ~2-fold increase in Otx2 expression (17). Thus, in the acute RNAi-mediated knockdown of Crx, there may be insufficient time for compensatory Otx2 up-regulation.

To acutely knock-out Otx2 alone, we co-electroporated a plasmid encoding Cre recombinase along with the fluorescent reporters and again observed decreased pNrl-DsRed expression (Fig. 4D, upper third panel). Because initiation of Crx expression depends on Otx2 (30), one might expect that knock-out of Otx2 would result in loss of both Otx2 and Crx. However, some residual expression of the DsRed reporter was still apparent in photoreceptors when Otx2 was knocked out (Fig. 4D, upper third panel). We therefore hypothesized that there may be some perdurance of Crx transcripts in Otx2 knock-out. Thus, to decrease Crx and Otx2 transcript levels simultaneously, we co-electroporated both CRX RNAi and Cre recombinase. This dual knockdown approach reduced pNrl-DsRed expression to nearly undetectable levels (Fig. 4D, upper fourth panel), supporting our hypothesis that CRX and OTX2 are both necessary for activation of the Nrl promoter during rod photoreceptor development. In addition, these findings are consistent with the idea that both CRX and OTX2 mediate Nrl activation by binding to the “CRX” site present in the critical region.

**FIGURE 4. CRX and OTX2 coordinately regulate Nrl expression in the developing mouse retina.** A, RT-qPCR analysis of Nrl transcript levels in C57BL/6 wild-type mice (blue line) and Crx null mice (orange line). Error bars represent S.D. across three biological replicates. B, wild-type and Crx null retinas were co-electroporated with pNrl(3.2 kb)-DsRed and the pCAG-GFP loading control and cultured for 10 days. DsRed expression was modestly reduced in Crx−/− retinas compared with wild-type. C, in situ hybridizations for the Otx2, Crx, and Nrl transcripts in wild-type retinas at various developmental time points. The embryonic day 14.5, P3.5, and P10.5 Crx images have been previously published (17) and are reproduced here for the sake of comparison. Note that Crx and Otx2 possess nearly identical expression patterns in the retina from embryonic day 12.5 through P3.5, with the exception that Otx2 is also expressed in the retinal pigmented epithelium. However, after P3.5, Otx2 levels diminish in the outer nuclear layer, whereas high Crx levels are sustained. The orange arrow at P10.5 denotes persistent, high level Otx2 expression in bipolar cells of the inner retina. D, retinas from homozygous Otx2 floxed mice (Otx2f/f) were co-electroporated with pNrl(3.2 kb)-DsRed and the pCAG-GFP and cultured for 10 days. Retinas that were additionally electroporated with CRX RNAi (upper second panel) or a Cre recombinase-encoding plasmid (upper third panel) showed a reduction in pNrl(3.2 kb)-DsRed expression. Retinas that received both CRX RNAi and Cre recombinase (upper fourth panel) showed nearly undetectable levels of pNrl(3.2 kb)-DsRed. Note that the fluorescence enclosed by the white dashed lines indicates lens autofluorescence. Residual DsRed expression in photoreceptors is indicated by white arrows.
OTX2, CRX, and RORβ bind directly to Nrl promoter. Next, we wished to determine whether OTX2, CRX, and RORβ bind the bioinformatically identified sites within the critical region of the Nrl promoter. First, we performed an EMSA with biotinylated probes containing the sequence of the critical region (Fig. 5). Recombinant proteins consisting of the DNA-binding domains of OTX2, CRX, and RORβ were purified, mixed with the probes, and run on a non-denaturing gel. OTX2 and CRX protein were both able to shift the biotinylated probe containing the wild-type critical region sequence (Fig. 5, top two panels, third row), and the shift was eliminated by non-biotinylated competitor probe (fourth row). Mutation of the CRX site alone completely abolished the shift (fifth row); mutation of the RORβ site alone did not affect the shift (sixth row); and mutation of both the CRX and RORβ sites again abolished the shift (seventh row). Likewise, RORβ was able to shift the wild-type probe (bottom panel, third row) and the probe containing a CRX mutation (fifth row) but was unable to shift the probes containing mutations of the RORβ site (sixth row) or RORβ and Nrl Regulation by CRX, OTX2, and RORβ.
CRX sites (seventh row). This result indicates that OTX2, CRX, and RORβ bind strongly and specifically to their cognate sites in the Nrl critical region in vitro.

To test whether OTX2, CRX, and RORβ bind to the Nrl critical region in vivo, we performed a chromatin immunoprecipitation experiment utilizing mouse retinal tissue isolated at three postnatal time points. Antibodies to OTX2 and CRX produced enrichment of the Nrl critical region at all three time points, with peak OTX2 binding at P3 and peak CRX binding at P7 (Fig. 6). As a control, the promoter of the microglial gene Tyrobp, which is not predicted to bind either CRX or OTX2, was not enriched. Two different polyclonal antibodies against RORβ did not show enrichment of the critical region (data not shown). However, these antibodies also failed to show enrichment of the blue cone opsin promoter region, which has been shown previously to be a direct target of RORβ (32). Thus, the absence of enrichment at the Nrl promoter in this case is likely attributable to the inability of these antibodies to immunoprecipitate their target.

DISCUSSION

In this study, we have shown that OTX2, CRX, and RORβ directly regulate Nrl expression in developing mouse rod photoreceptors. Binding sites for these factors reside in a highly conserved region immediately upstream of the Nrl transcription start site, and directed point mutations in these sites are sufficient to completely abolish pNrl-DsRed expression in mouse retinal explants. EMSA experiments confirm direct binding of OTX2, CRX, and RORβ at this region of the Nrl promoter. Direct binding of OTX2 and CRX was further validated in ChIP experiments with mouse retinal lysates. While we were preparing our manuscript, a report appeared, which corroborates our findings with respect to the regulation of the Nrl promoter by RORβ (33).

An interesting conclusion from our experiments was the functional redundancy of CRX and OTX2 in the activation of Nrl transcription during the late embryonic and early postnatal periods. Both OTX2 and CRX are required for photoreceptor development (22, 28, 30) but because OTX2 is required for initiation of Crx transcription (30), it is difficult to distinguish the roles of these two factors in the regulation of specific genes. A study utilizing the Ottx2^+/crx^−/−,Crx^−/− double knock-out suggested that OTX2 itself is an active regulator of certain photoreceptor genes in the early postnatal period, such as Rho and Pde6b (34). In the present study, we demonstrated by ChIP that OTX2 and CRX are both bound at the Nrl promoter at P3, P7, and P21. Moreover, OTX2 activation of Nrl can partially compensate for the loss of Crx in the first postnatal week, whereas loss of both OTX2 and CRX results in a dramatic loss of Nrl promoter activation. Our findings suggest the following model for the initiation and maintenance of Nrl transcription (Fig. 7). During the initiation phase, OTX2 and RORβ are the main activators of the Nrl promoter. In the later maintenance phase, however, CRX supplants OTX2 as the main activator of Nrl transcription.

Although our analysis has identified two critical cis-regulatory motifs in the Nrl promoter, there are undoubtedly a number of other important regulatory sites that remain unknown.

For example, the 110-base pair region upstream of the critical region likely contains binding sites for multiple transcription factors that may act synergistically to activate Nrl expression. We found that disruption of individual sites via scanning mutagenesis is not sufficient to abolish Nrl promoter activity, whereas elimination of the entire 110-bp region causes total loss of activity. A couple of low affinity CRX sites are present in this region and may play a role in activation (data not shown). It is possible that binding sites for other factors are also present.

Retinoic acid has long been known to foster rod photoreceptor differentiation (16, 35); thus, retinoic acid receptors represent attractive candidate regulators of Nrl expression. Several years ago, Khanna and colleagues (15) identified three elements within the Nrl promoter region that appear to be sensitive to retinoic acid in a heterologous tissue culture assay system. We sought to test the importance of these putative retinoic acid response elements in living mouse retinas by mutating each retinoic acid response element individually in the context of the pNrl(1.1 kb)-DsRed reporter. Mutation of the individual retinoic acid response elements resulted in only modest changes in DsRed expression relative to the non-mutated control (data not shown). Therefore, although these sites might contribute to the modulation of Nrl expression in vivo, they are not individually required for activation of the Nrl promoter.

A recent CRX ChIP-seq study found that many photoreceptor genes are surrounded by a spatially distributed network of CRX-bound regions that are hypothesized to act in a combinatorial fashion to regulate expression of their associated gene (18). The Nrl gene also appears to follow this pattern as a total of four highly conserved noncoding regions, which correspond to CRX ChIP-seq peaks, were identified around the locus: P1, P2, P3, and an element within the third intron (Fig. 1A). The initial promoter truncation analysis demonstrates that the P1 element is not critical for Nrl promoter activation, but P1 may be involved in fine-tuning of Nrl expression levels in rods. In addition, the P1 element may also contribute to maintenance of normal levels of Nrl expression at later developmental time-points and in the adult.
The roles of the P3 region and the intronic element also remain to be explored. Preliminary bioinformatic analyses identified a potential CRX site in the P3 region, but point mutation of this site in the context of the full-length pNrl(3.2 kb)-DsRed resulted in only minimal changes in expression (data not shown). It is possible that this region and the intronic element play a role in modulating Nrl expression outside the time window assayed in the present study.

The close proximity of the OTX2/CRX and RORβ binding sites in the Nrl promoter critical region is noteworthy. We previously showed that clusters and pairs of transcription factor binding sites, often within 50 bp of one another, are present in many photoreceptor cis-regulatory regions (17, 18). In fact, as few as three closely clustered CRX sites in the context of a synthetic cis-regulatory element are sufficient to drive photoreceptor-specific expression in electroporated retinas (18). Many endogenous photoreceptor cis-regulatory regions, however, appear to be comprised of clusters of both CRX sites and other sites for factors such as NRL, NR2E3, and bHLH factors such as NEUROD1 (9, 18, 36). Thus, the paired OTX2/CRX-cis sites in the Dow assayed in the present study.

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