The Leucine Zipper of NRL Interacts with the CRX Homeodomain

A POSSIBLE MECHANISM OF TRANSCRIPTIONAL SYNERGY IN RHODOPSIN REGULATION*

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Photoreceptor-specific expression of rhodopsin is mediated by multiple cis-acting elements in the proximal promoter region. NRL (neural retina leucine zipper) and CRX (cone rod homeobox) proteins bind to the adjacent NRE and Ret-4 sites, respectively, within this region. Although NRL and CRX are each individually able to induce rhodopsin promoter activity, when expressed together they exhibit transcriptional synergy in rhodopsin promoter activation. Using the yeast two-hybrid method and glutathione S-transferase pull-down assays, we demonstrate that the leucine zipper of NRL can physically interact with CRX. Deletion analysis revealed that the CRX homeodomain (CRX-HD) plays an important role in the interaction with the NRL leucine zipper. Although binding with the CRX-HD alone was weak, a strong interaction was detected when flanking regions including the glutamine-rich and the basic regions that follow the HD were included. A reciprocal deletion analysis showed that the leucine zipper of NRL is required for interaction with CRX-HD. Two disease-causing mutations in CRX-HD (R41W and R90W) that exhibit reduced DNA binding and transcriptional synergy also decrease its interaction with NRL. These studies suggest novel possibilities for protein-protein interaction between two conserved DNA-binding motifs and imply that cross-talk among distinct regulatory pathways contributes to the establishment and maintenance of photoreceptor function.

Gene activation is a stringently controlled process, involving combinatorial and cooperative action of multiple regulatory proteins with promoter and enhancer DNA elements (1–6). Recent studies, including reconstitution experiments, have suggested that target specificity and transcriptional synergy are achieved by specific and precise interactions among various activator proteins during the assembly of higher order nucleo-protein complexes, including the “enhanceosome” (7–11). The elucidation of these protein-protein and protein-DNA interactions is critical to our understanding of the mechanisms of cell type- and tissue-specific gene expression.

Generation of multiple neuronal cell types during retinal development is an evolutionarily conserved biological process, which offers a convenient model system to investigate tissue-specific gene regulation. More than 30 transcription factors representing several classes of DNA-binding proteins are expressed in developing and mature mammalian retina; nevertheless, the precise function of a majority of these proteins remains to be elucidated (12–14). Rhodopsin, the G-protein-coupled light receptor, is expressed specifically in the rod photoreceptors of retina and is a pivotal protein for visual function. Its expression is correlated to rod differentiation and maintained at high levels afterward, throughout life (15). Altered expression of rhodopsin and mutations that affect its function in mature rods result in retinal degeneration (15, 16). Regulation of rhodopsin expression is primarily at the level of transcription and is mediated by two distinct regions: a proximal sequence from −176 to +70 bp, which determines photoreceptor specificity (called the rhodopsin proximal promoter region (RPPR)),1 and another more upstream region required for high level expression (called the rhodopsin enhancer region) (17–22). A number of DNA sequence elements that bind to nuclear proteins have been identified within RPPR; these include Ret-1/PCE-1 (23, 24), BAT-1 (25), eopsin-1 (26), Ret-4 (27), and NRE (28) (Fig. 1). NRL, a basic leucine zipper (bZIP) protein of the MaF subfamily (29), was the first transcription factor shown to bind to NRE in the RPPR region and transactivate the rhodopsin promoter in cultured cells (28, 30). Soon thereafter, CRX, a photoreceptor-specific paired-like homeodomain protein, was identified as the Ret-4 and BAT-1 binding protein by yeast one-hybrid screening and shown to activate the promoters of rhodopsin and other retinal genes (31). NRL and CRX demonstrated transcriptional synergy in rhodopsin promoter activation when transfected together in cultured cells (31). Recently, the Ret-1/PCE-1 element was shown to bind two other homeodomain proteins, Erx (32) and Rx (33).

During embryonic development in mice, Nrl transcripts are detected in all postmitotic neurons and lens; however, its expression becomes restricted primarily to the retinal photoreceptors in the adult (34). CRX is expressed specifically in photoreceptors and pinealocytes and plays a significant role during photoreceptor differentiation (31, 35, 36). Consistent with their

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1 The abbreviations used are: RPPR, rhodopsin proximal promoter region; bZIP, basic leucine zipper; GST, glutathione S-transferase; bCRX, bovine CRX; HD, homeodomain.
role in rhodopsin regulation, detection of NRL and CRX transcripts precedes rhodopsin expression during rod development in mammals. In addition, mutations in the human CRX and NRL genes have been identified in retinopathies, and these mutations result in altered transcriptional synergy in rhodopsin promoter activation assays (37–41). Based on these findings, we hypothesized that the transcriptional synergy between NRL and CRX in rhodopsin regulation results from cooperativity in binding to adjacent NRE and Ret-4 (or BAT-1) sites and/or from direct physical interaction, leading to the formation of a stable enhanceosome and/or initiation complex. In this paper, we demonstrate direct interaction between the leucine zipper of NRL and the homeodomain of CRX using the yeast two-hybrid interaction trap and \textit{in vitro} glutathione S-transferase (GST) pull-down assays.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Constructs for Yeast Two-hybrid Screening—}The two-hybrid screening in yeast was carried out according to the published procedure (42), with minor modifications. The bait construct was generated by cloning the \textit{SacII}-PvuII fragment of the human NRL cDNA (29) at the PvuII site of the pHybLex/Zeo vector (Invitrogen, Carlsbad, CA). The resulting construct, called pLex-NRL-ZIP, encoded the NRL leucine zipper (NRL-ZIP; amino acids 171–231) fused in frame with the LexA protein and did not display autologous activation of the reporter gene \textit{lacz} or \textit{HIS3} upon transformation in the yeast L40 strain (MATa his3 \textit{A102} leu2-3,112 ade2 lys2::4lexAop-HIS3) URA3::(LexAop-lacZ) GAL4. The following constructs were obtained as part of the “Hybrid Hunter System” (from Invitrogen) and used for positive and negative interaction control experiments: pHybLex/Zeo-Fos2, pHyLex/Zeo-Laminin, and pYESTrp-Jun, which expressed the c-Fos leucine-zipper domain fused to LexA, laminin fused to LexA, and the c-Jun leucine zipper fused to the B42-activation domain (B42-AD), respectively. A bovine retina cDNA library (40) in \textit{pACTII} (prey vector with Gal4 activation domain) (a generous gift of Dr. C. H. Sung) was used to isolate interacting clones.

\textbf{Yeast Two-hybrid Screening and Interaction Testing—}Yeast strain L40 was sequentially transformed with pLex-NRL-ZIP and then with 10 \mu g of DNA from the retina library or purified clones in the prey vector, essentially as described (44). Double transformants were selected for presence of the bait and the prey vectors, and possible interactors were selected by growth on the appropriate yeast minimal medium (250 \mu M zeocin, minus Leu His) and by filter lift assay of \textit{\beta}-galactosidase activity. Mixed bait and prey construct DNAs were recovered from double-positive yeast transformants after Zymolase-20T/SDS treatment (ICN Biomedicals, Aurora, OH). The \textit{pACTII} retina library clones were separated from the bait vector DNA by electrophoresis into \textit{Escherichia coli} XL1-Blue bacteria and growth on LB-ampicillin plates. Plasmid DNA, prepared by the alkaline lysis method, was used for sequencing and to test for false positives by retransforming \textit{S. cerevisiae} with the wild-type bCRX and primers corresponding to the appropriate end sequences with added \textit{BamHI} (5’ or EcoRI (3’) site (see Fig. 5A). The resulting polymerase chain reaction fragments were digested with \textit{BamHI} and EcoRI, gel-purified, and subcloned into \textit{BamHI/EcoRI}-digested plasmid DNA 3.1/HisC vector. All deletions are fused in frame with the His\textsubscript{6} tag at the N terminus and contain a stop codon at the C terminus. The sequence of each deletion construct was confirmed using a Perkin-Elmer ABI Prism DNA sequencing kit and ABI Prism 310 Genetic Analyzer.

\textbf{Purification of GST Fusion Proteins—}The GST, GST-NRL, and GST\textit{bCRX} proteins were produced in \textit{E. coli} strain BL21, essentially as described (45). Briefly, the transformed bacteria were grown at 37°C for 4 h (A\textsubscript{600} ≈ 0.8) and induced with 0.5 mM isopropyl-\textit{b}-\textit{D}-galactopyranoside for 3 h at 27°C. Cells were disrupted by sonication in the lysis buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM EDTA, and 1× Complete protease inhibitor mixture (Amersham Pharmacia Biotech)). \textit{E. coli}-expressed proteins were purified using glutathione-Sepharose beads, as suggested by the manufacturer (Amersham Pharmacia Biotech). Protein concentrations were estimated using bicinchoninic acid reagent (Sigma). The GST-CRX-HD fusion protein was expressed and purified from \textit{E. coli} BL21, as described previously (31).

\textbf{In Vitro Translation and GST Pull-down Assay—}bCRX constructs in pcDNA3.1/HisC vector (0.3 \mu g of double-stranded DNA) were translated in a 25-\mu l reaction in the presence of [\textit{\beta}35]methionine (>1000 \mu Ci/mmol; Amersham Pharmacia Biotech) using the T7-TNT Quick Coupled Transcription/Translation System\textsuperscript{TM} (Promega). For \textit{in vitro} interaction experiments, 7 \mu l of the \textit{\beta}35-labeled protein was incubated with glutathione-Sepharose-bound GST, GST-NRL, or GST\textit{bCRX} protein (>100 \mu g) in the binding buffer (20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.2% Nonidet P-40). After 18 h of incubation at 4°C on a nutorator, the beads were washed five times in buffer containing 20 mM Tris-Cl, pH 9.0, 150 mM NaCl and, 0.2% Triton X-100. After the final wash, glutathione-Sepharose-bound proteins were resuspended in 60 \mu l of 2× SDS sample buffer, boiled for 5 min, and separated by SDS-polyacrylamide gel electrophoresis. To estimate the amount of labeled protein used in binding, 20% of the \textit{in vitro} translated products were also examined on a parallel gel. The radiolabeled proteins were visualized by fluorography after treatment of the gel with Amplify\textsuperscript{TM} (Amersham Pharmacia Biotech), as described (46).

For \textit{in vitro} translation studies, the NRL cDNA (29) was subcloned in pcDNA3.1 (Invitrogen). The plasmid construct was linearized by different restriction enzymes that digest at a unique site, and \textit{\beta}35-labeled full-length or truncated NRL proteins were produced using the T7-TNT Quick Coupled Transcription/Translation System\textsuperscript{TM} (Promega).

\textbf{RESULTS}

\textbf{NRL-CRX Interaction Detected by Yeast Two-hybrid Assay—}As an initial approach to evaluate the possibility of direct interaction between NRL and CRX, the yeast two-hybrid method was employed. We generated a bait vector that produced a fusion protein with LexA and the leucine zipper domain of NRL (NRL-ZIP). This bait construct did not autoactivate the \textit{HIS3} and \textit{lacz} reporter genes upon transformation in L40 yeast. The NRL-ZIP bait was used for screening a bovine retinal cDNA library in the \textit{pACTII} vector (43). Twenty-eight yeast double transformants that displayed fast growth on minus His medium were selected for further analysis. Filter lift tests of these His\textsuperscript{6} clones identified 26 clones that expressed \textit{\beta}-galactosidase activity as well. Sequence analysis of the double positive clones revealed that 21 of them had bCRX sequence fused in frame to the Gal4 activation domain. These clones could be divided into five subsets; four subsets included the 5’ noncoding sequence of bCRX, whereas one began at codon 14 of bCRX (Fig. 2). All of the bCRX clones obtained from the screen contained the homeodomain, some with or without the additional sequence from the 5’-untranslated region of the \textit{bCRX} gene residing in L40 yeast. The presence of both the bait vector pLex-NRL-ZIP and bCRX-prey clones was found to be essential for growth on minus His medium (Fig. 3).

Specificity of interaction between NRL-ZIP and bCRX was confirmed by transformation of \textit{pACTII}-bCRX(N14) with pLex-NRL-ZIP or pHybLex/Zeo-Laminin and streaking the double
transformants onto minus His plates containing 50 mM amino-triazole. As shown in Fig. 3B, only yeast double transformants with pLex-NRL-ZIP grew on minus His plates, while the presence of the laminin bait construct did not result in the activation of the HIS3 reporter gene. Under identical assay conditions, double transformants of L40 yeast with the c-Fos (pHybLex/Zeo-Fos2) bait and c-Jun (pYESTrp-Jun) prey constructs showed excellent growth on minus His plates, whereas the double transformants with the laminin bait (pHybLex/Zeo-Laminin) and c-Jun prey did not (Fig. 3C).

CRX Interaction in Vitro with GST-NRL by Pull-down Assay—To confirm the direct interaction of NRL and CRX, we employed a pull-down assay with GST fusion proteins. Both GST-NRL (expressing GST fused with the full-length NRL protein) and GSTANRL (GST fused with the C-terminal 117 amino acids of NRL; primarily the bZIP domain), but not GST alone, were able to interact with the *in vitro* translated full-length CRX protein (Fig. 4). This was consistent with the yeast two-hybrid screening, where NRL-ZIP bait was used. The addition of micrococcal nuclease to the GST pull-down reactions (in order to remove the DNA template) did not significantly alter the interaction of CRX with NRL (data not shown). To further define the region of bCRX protein that interacts with NRL, various deletion constructs (shown in Fig. 5A) were used for *in vitro* translation. In the pull-down assay, full-length (construct bCRX) and truncated proteins containing the HD and the glutamine-rich plus basic region (constructs N34, C160, and C208) demonstrated strong binding to GST

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**FIG. 2.** DNA sequences of the Gal4AD-CRX prey clones obtained from yeast two-hybrid interaction screen of a bovine retinal library using the LexA-NRL leucine zipper domain. The arrows indicate the first nucleotide of the CRX clones (A–D and N14), identified in yeast two-hybrid screening using the NRL-leucine zipper bait. The number in parenthesis, beside the clone name, shows the redundancy, i.e. the number of times a particular clone was isolated. CRX-A and -B shared the same 5'-end but were different in nucleotides indicated by an asterisk above the DNA sequence. The CRX polypeptide is shown in boldface type below the nucleotide sequence. The CRX clones A–D contained an additional upstream sequence (derived from the 5'-untranslated region) that was in frame with the Gal4 activation domain. The CRX polypeptide in the clone N14 begins at residue 14.

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**FIG. 3.** NRL-leucine zipper domain interacts with CRX in the yeast two-hybrid assay. A, the pACTII prey clones obtained from the screening of a bovine retinal library were transformed into yeast L40 with and without the bait vector pLex-NRL-ZIP and replica-streaked onto minimal medium (minus Leu His, plus 10 mM aminotriazole) or minimal medium (minus Leu His, plus 50 mM aminotriazole). Three examples are shown here: Gal4-AD/CRX fusions without the 5'-noncoding sequence (clone CRX-N14), with the 5'-noncoding sequence (clone CRX-A), and a novel clone (KM1698–1). Only L40 double transformants with the bait vector grew on minus His plates. B, interaction of CRX is specific for the NRL-leucine zipper domain. Bait yeast strains L40/pHybLex/Zeo-Laminin and L40/pLex-NRL-ZIP were transformed with the CRX-N14 DNA, and double transformants were streaked onto minimal medium (minus Leu, minus His, plus 250 mM zeocin and 50 mM aminotriazole). The yeast transformants with the laminin bait and CRX prey did not grow, whereas NRL-ZIP and CRX double transformants activated the HIS3 reporter gene and grew well. C, LexA-c-Fos (pHybLex/Zeo-Fos2) interacts with B42-AD-c-Jun (pYESTrp-Jun) in L40 yeast under identical assay conditions, whereas double transformants of LexA-laminin (pHybLex/Zeo-Laminin) with B42-AD-c-Jun did not show any interaction. Double transformants with c-Jun-c-Fos grew well on minimal medium (minus Trp, minus His, plus 250 mM zeocin and 50 mM aminotriazole), while those with laminin-c-Fos did not.
although at a reduced level (Fig. 5B). SDS-PAGE analysis of the in vitro translated bCRX proteins revealed a comparable intensity of the labeled protein in binding reactions (Fig. 5C). Taken together, the data strongly suggest that amino acids 34–88 (i.e. the homeodomain) constitute a region of CRX that is important for interaction with NRL-bZIP. Nonetheless, the binding efficiency was enhanced dramatically when the glutamine-rich plus basic region that follows the HD was included. Together with the yeast two-hybrid experiments, these results suggest that CRX-HD provides the primary interface for physical interaction with the leucine zipper motif of NRL.

DISCUSSION

Using two independent methods, we have provided evidence for direct physical interaction of NRL and CRX, two transcription factors implicated in rhodopsin regulation. The yeast two-hybrid studies show that the interaction is stable and functional within a cellular environment, whereas the GST pull-down experiments demonstrate direct association in vitro. These studies also indicate that the leucine zipper domain of

**Fig. 4.** CRX interacts with NRL, in vitro. 35S-Labeled CRX protein was prepared by in vitro translation. Interaction of CRX with glutathione-Sepharose bound GST-NRL or GST (control) was detected by SDS-PAGE, followed by autoradiography. 35S-CRX bound strongly to GST-NRL but not GST.

**Fig. 5.** CRX-Homeodomain interacts with NRL. A, schematic representation of the CRX constructs used for in vitro translation and GST pull-down assay. The top bar shows the CRX protein and various domains identified within. The lines below represent various deletion constructs. B, autoradiographs of GST pull-down. C, autoradiographs of 20% of the input protein used for the assay after separation on SDS-PAGE gel. Results with only GSTΔNRL pull-downs are shown here. The 35S-labeled CRX protein was prepared in vitro and incubated with GSTΔNRL. All interacting CRX polypeptides contained all or part of the homeodomain region. Stronger interaction is detected when the Gln stretch and the basic region are also included with HD.

**Fig. 6.** NRL leucine zipper is required for interaction with the CRX homeodomain. GST-CRX-HD (homeodomain) fusion protein was used for pull-down assays. Autoradiographs show GST pull-downs and 20% of the input protein used for the assay after separation on SDS-PAGE gel. The 35S-labeled full-length NRL protein and truncated NRL proteins missing part of the leucine zipper domain (NRL<sup>210</sup>) and the entire leucine zipper domain (NRL<sup>190</sup>) were prepared in vitro and incubated with GST-CRX-HD. Full-length NRL interacted strongly with the GST-CRX-HD fusion protein, whereas NRL<sup>210</sup> and NRL<sup>190</sup> (having a deletion of 27 and 47 C-terminal residues, respectively) showed decreased binding. The GST protein showed no interaction with any of the translated NRL proteins.

**FIG. 6. NRL leucine zipper is required for interaction with the CRX homeodomain. **
that NRL-ZIP bait exists as a homodimer and that the surface with leucine residues in the zipper is unavailable for interaction. We suggest that bCRX interacts with the NRL-ZIP homodimer and that the NRL interaction surface is the outside surface of the dimer (positions a and d around the helical wheel) (53). Additional experiments will be necessary to test this hypothesis.

Homeodomains bind to DNA, and this is true for CRX as well (31, 38, 40). Based on the published structure of the paired homeodomain (54), we hypothesize that CRX-HD has three helix motifs: h1, from residues 50–59; h2, residues 69–76; and h3, residues 80–89. Helix-3 should be important for making contacts with DNA. It would then appear that the helices h1 and h2 are more accessible for interaction with NRL, consistent with the yeast two-hybrid screening results and CRX deletion analysis in GST pull-down assays. Both h1 and h2 are rich in ionic and polar amino acids that can make salt-bridge and hydrogen bonds with the NRL interaction surface. The conclusion that CRX-HD is involved in both DNA binding and protein–protein interaction is strengthened by the GST pull-down experiments with mutant CRX proteins (see Fig. 7). The glutamine-rich plus basic region that follows the homeodomain could provide a surface for additional interaction, since it enhanced the binding in pull-down assays.

Homeodomain proteins appear to have a broad influence on gene expression, since they bind to a wide range of DNA sequences with similar affinity and this binding can be positively correlated to transcriptional activity (55, 56). CRX may have a similar global influence on photoreceptor-specific gene expression, since it can bind to and transactivate from regulatory elements in several photoreceptor-specific genes, including rhodopsin, interphotoreceptor retinoid-binding protein, arrestin, and β-phosphodiesterase (31, 57). In contrast, nonhomeodomain transcription factors bind to promoters in fewer genes with greater sequence specificity (58). Such transcription factors, like NRL, may influence the specificity of gene transcription while homeodomain proteins, such as CRX, may facilitate structural control of larger chromatin regions and help facilitate the effects of other transcription factors upon the enhanceosome (56).

Interaction of NRL with CRX in the two test systems (yeast two-hybrid and GST pull-down) did not require the presence of RPRR sequence elements. The addition of micrococal nuclease to the GST pull-down reactions (in order to remove DNA template) did not significantly alter the interaction of CRX with NRL. This is different from the interaction of AP1 and NFAT, which occurs only when their cognate DNA binding region is present to form a quartenary complex (47, 59). Our studies raise the possibility that the two activator proteins (NRL and CRX) form a stable complex by directly interacting with each other and probably with other proteins prior to their binding to their cognate cis-sequence elements in RPRR. CRX is shown to bind to Ret-4, BAT-1, and Ret1 elements in RPRR in vitro (31). We hypothesize that a stable NRL-CRX complex would influence the recognition of Ret-4 and BAT-1 flanking the NRE and provide binding sequence specificity during the organization of the rhodopsin enhanceosome. In addition, this interaction may result in cooperative and efficient DNA binding and explain their synergistic transactivation of the rhodopsin promoter. Mobility shift and DNase I experiments using the NRL and CRX proteins may help to confirm if the distribution of CRX on RPRR is influenced by NRL.

The ability of NRL and CRX to physically interact with each other correlates well with their functional synergistic interaction at the rhodopsin promoter and illustrates one possible mechanism of context-dependent transcriptional regulation.

2 K. P. Mitton and A. Swaroop, unpublished data.
(60). This interaction also points to cross-talk among different signal transduction pathways that activate or modulate rhodopsin expression in developing and mature photoreceptors. Further investigations are in progress to identify and characterize other activator proteins that are also involved in the formation of the rhodopsin enhanceosome. Elucidation of extracellular factors that influence the expression and activity of NRL, CRX, and these other factors should provide important new insights into the regulation of rhodopsin expression.

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