The bZIP Transcription Factor Nrl Stimulates Rhodopsin Promoter Activity in Primary Retinal Cell Cultures*

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Rajan Kumar‡, Shiming Chen‡, David Scheurer‡, Qing-Liang Wang‡, Elia Duh‡, Ching-Hwa Sung‡, Alnawaz Rehemtulla, Anand Swaroop**, Ruben Adler‡‡‡, and Donald J. Zack‡‡‡§§

From the Departments of Ophthalmology, §§Neuroscience, and §§Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287-9277, Departments of Ophthalmology, Cell Biology, and Anatomy, Cornell University School of Medicine, New York, New York 10021, and Departments of Radiation Oncology, and Ophthalmology and Human Genetics, University of Michigan, Ann Arbor, Michigan 48105

In vitro DNA binding assays and transient transfection analysis with monkey kidney cells have implicated Nrl, a member of the Maf-Nrl subfamily of bZIP transcription factors, and the Nrl response element (NRE) in the regulation of rhodopsin expression. We have now further explored the role of the NRE and surrounding promoter elements. Using the yeast one-hybrid screen with integrated NRE and flanking DNA as bait, the predominant clone obtained was bovine Nrl. Recovery of truncated clones in the screen demonstrated that the carboxyl-terminal half of Nrl, which contains the basic and leucine zipper domains, is sufficient for DNA binding. To functionally dissect the rhodopsin promoter, transient expression studies with primary chick retinal cell cultures were performed. Deletion and mutation analyses identified two positive regulatory sequences: one between -40 and -84 base pairs (bp) and another between -84 and -130 bp. Activity of the -40 to -84 region was shown to be largely due to the NRE. On co-transfection with an NRL expression vector, there were 3-5-fold increases in the activity of rhodopsin promoter constructs containing an intact NRE but little or no effect with rhodopsin promoters containing a mutated or deleted NRE. Nrl was more effective than the related bZIP proteins, c-Fos and c-Jun, in stimulating rhodopsin promoter activity. The -84 to -130-bp region acted synergistically with the NRE to enhance both the level of basal expression and the degree of Nrl-mediated trans-activation. These studies support Nrl as a regulator of rhodopsin expression in vivo, identify an additional regulatory region just upstream of the NRE, and demonstrate the utility of primary retinal cell cultures for characterizing both the cis-acting response elements and trans-acting factors that regulate photoreceptor gene expression.

Rhodopsin is the visual pigment of vertebrate rods, and its activation by light initiates the phototransduction process (1). In recent years there has been increasing interest in understanding the mechanisms regulating rhodopsin gene expression, motivated both by the fundamental importance of the protein in visual transduction and by its role as a prototype for the study of photoreceptor-specific gene regulation (2). In addition, since mutations in the rhodopsin gene can lead to retinal degeneration (3, 4), understanding of its regulation will have implications for designing effective retinal gene therapy strategies (5).

Nuclear runoff experiments have demonstrated that the expression of rhodopsin is primarily regulated at the transcriptional level (6, 7). Transgenic studies have defined two regulatory regions, a rhodopsin proximal promoter region (RPR) and a more distal rhodopsin enhancer region (RER) (1). The rhodopsin proximal promoter region, located within -176 to +70 and -400 to +80 bp in the bovine (34) and murine (8) genes, respectively, provides low level, yet photoreceptor-specific, expression. The RER, a 100-bp sequence located approximately 2 kilobases upstream from the mRNA initiation site, acts as an enhancer (9). Biochemical analysis has identified a number of putative cis-acting DNA regulatory elements; they include the Ret1-PE1 (10, 11), Ret2, Ret3 (12), Ret4 (13), and Mash-1 (14) binding sites and a site homologous to the Drosophila glass response element (15).

Sequence analysis also disclosed the presence of a putative binding site for the Maf/Nrl family of transcription factors in the rhodopsin promoter. Nrl is an evolutionarily conserved basic motif-leucine zipper (bZIP)-containing DNA binding protein, which is homologous to the v-Maf oncoprotein (16). The Maf-Nrl subfamily has been implicated in cell type-specific regulation of genes in tissues as diverse as the hematopoietic system (17, 18), cerebellum (19), and developing hindbrain (20–22). Maf and Nrl proteins bind an extended AP-1-like sequence, and form both homodimers and heterodimers with other bZIP family members (23–27). Nrl itself is specifically expressed in retinal cells, including photoreceptors, in which its expression precedes that of rhodopsin during development (28). Recently, we have shown by electrophoretic mobility shift analysis that Nrl can bind in vitro to an oligomer containing the Nrl response element (NRE) present in the rhodopsin promoter, and that cotransfection of CV-1 monkey kidney cells with an Nrl-expression vector can stimulate expression of rho-
Nrl Stimulates Rhodopsin Promoter Activity

In this report we provide more direct evidence supporting a role for Nrl in the regulation of rhodopsin gene expression. In the first part of the study, using the yeast one-hybrid system, we demonstrate that Nrl can bind to the NRE in vivo and regulate expression of a reporter gene. We then use transient expression analysis of primary chick retinal cultures (30) to further analyze Nrl, the NRE, and related cis-elements in the rhodopsin promoter. The cells in these primary cultures, unlike those in transformed lines, express a highly differentiated phenotype resembling closely their in vivo counterparts (31, 32).

Using transfection and cotransfection studies, we show that Nrl can activate rhodopsin expression in retinal cells in an NRE-dependent manner and identify a positive regulatory region that is located just upstream of the NRE and acts synergistically with it.

EXPERIMENTAL PROCEDURES

**Yeast One-hybrid Screening**—A retinal cDNA fusion library for one-hybrid screening was generated from poly(A)+ bovine retinal RNA. Oligo(dT)-primed cDNA was directionally inserted into the EcoRI and Xhol sites of plasmid pACTII (Stive Elledge, Baylor College of Medicine) just downstream of the GAL4 activation domain. The ligation products were electroporated into Escherichia coli strain DH10B and plated onto LB/Gallicillin plates. The colonies of such a transformation efficiency was 10^15 transformants/μg of DNA. Approximately 2 × 10^6 independent colonies were obtained. Testing of individual colonies revealed that the average insert size was 2 kilobases. Bacteria from the plates was harvested by scraping, and DNA was prepared using Qiagen Maxi columns according to the manufacturer’s directions. The resulting plasmid library was amplified twice before use. Bait constructs containing tetramers of the 73- to 30-bp sequence from the bovine rhodopsin promoter in the vectors pPHIS-I and pLaZ were prepared (Clontech). The pPHIS-I and pLaZ bait constructs were then linearized with Xhol and NcoI, respectively, and integrated into the genome of YM4271. The resulting strain with the integrated pPHIS-I-bait construct was used for library screening according to Clontech’s protocol. Positive library clones were defined as those that, on retransformation, led to those in transformed lines, express a highly differentiated phenotype resembling closely their in vivo counterparts.

**Preparation of Constructs**—Reporter constructs were generated by cloning appropriate DNA fragments into the multiple cloning site of the pGL2-basic luciferase-containing plasmid (Promega, Madison, WI). For the generation of hRho-2174-bait construct (32, 33) rhodopsin promoter sequence was amplified twice with ethanol, resuspended in 2–4 ml of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and filtered through a Microcon-100 column (Amicon, Beverly, MA).

**Transfections**—Embryonic day 8 (E8) and E17 chick retinal cultures were prepared as described previously (32, 38). Photoreceptors in these cultures express polarized, phagocyte-rich processes. The isolated photoreceptor segment processes, express a number of photoreceptor-specific genes, including rhodopsin, undergo photomechanical movements in response to light, and respond to neurotransmitters and retinoids (31, 32). Conditions for transient expression were optimized using E8 retinal cultures. Pilot studies revealed that the calcium phosphate method yielded higher transfection efficiencies than transfection with Transfectam (Promega Corp., Madison, WI), LipofectAMINE (Life Technologies), or calcium phosphate with glycercol shock. The calcium phosphate procedure was then optimized with respect to the amount of reporter and carrier DNA, the volume of calcium phosphate precipitate, cell density, the number of days in vitro prior to transfection, and the interval between transfection and reading. These studies results described below. Using this protocol and a Ras sarcoma virus long terminal repeat-luciferase reporter construct, transfection efficiencies as high as 15% could be obtained (data not shown). E8 cultures were transfected after 8 days in vitro. E17 cultures were transfected after 5–5 days in vitro. DNA in 80 μl of solution I (0.25 mM CaCl2) was added dropwise to 80 μl of solution II (25 mM HEPES, pH 7.1, 140 mM NaCl, and 14 mM Na2HPO4) while vortexing at a slow speed. The mixture was incubated at room temperature for 15 min and then added dropwise to the appropriate cell culture dish. For cotransfection assays, all plasmids were added to solution I before mixing with solution II. hRho-4907 was transfected at 9 μg of DNA/plate; for all other reporter constructs, equivalent molar amounts of DNA were used. Each transfection included 1 μg of CMV-lacZ as an internal control, to measure and correct for differences in transfection efficiency. Transfections were performed in duplicate during each experiment, and all experiments were done three independent times.

**Luciferase Assays**—Cells lysates were prepared 40–48 h after transfection by scraping the cells from individual plates into 200 μl of lysis buffer following the manufacturer’s instructions (Promega). The lysates were either used immediately for luciferase and β-galactosidase assays or stored on ice for a few hours. Twenty μl of cell lysate was mixed with 50 μl of reconstituted luciferin (Promega). Luciferase activity was measured with a TD-20e luminometer (Turner Designs, Inc., Mountain View, CA) with an integration time of 10 s.

**β-Galactosidase Assays**—Fifty μl of cell lysate was mixed with an equal volume of 8 mg/ml chlorophenyl red-β-n-galactopyranoside (Boehringer Mannheim) in duplicate in a 96-well plate format, and reactions were measured using an EL312e enzyme-linked immunosorbent assay reader (Bio-Tek Instruments, Inc., Winooski, VT). Serial dilution of a β-galactosidase enzyme standard on the same plate was used to determine the linearity of the reaction and to calculate concentrations based on the rate of reaction (Kinetic software; Bio-Tek Instruments). The resulting β-galactosidase values were used to correct the luciferase values for transfection efficiency.

**RESULTS**

**Nrl Binds to Chromosomal DNA Containing the NRE**—Sequence comparison of the mouse (34), rat (10), Chinese hamster (39), cow (40), and human (35) rhodopsin proximal promoter regions demonstrate that the NRE and flanking DNA are highly conserved (Fig. 1). The NRE itself, spanning the bovine sequence between –68 and –56 bp, is conserved in 12 of its 13 nucleotide positions. We have previously shown that an oligomer pair containing the rhodopsin NRE can bind in vitro to proteins in a bovine retinal nuclear extract and that Nrl is one of the proteins in the bound complex (29). To identify transcription factors that can bind to the rhodopsin NRE and its flanking DNA in vivo, in the context of chromatin structure, we used the yeast one-hybrid system (19, 41, 42). A bovine retinal cDNA-GAL4 activation domain fusion library was generated and transformed into yeast cells containing as bait a stably integrated tetramer of the bovine rhodopsin promoter sequence from –73 to –30 bp cloned upstream of a HIS3 reporter construct. A total of 1.7 × 10^6 yeast transformants were screened. Thirty-one positive colonies that grew on His− plates were identified in the first round. On retransformation, plasmids from 11 of these 31 clones were still positive with both the HIS3 and lacZ reporter assays. Sequence analysis revealed that seven of the clones contained full-length Nrl open reading
frames, whereas three others contained 5'-Nrl truncations. The 11th clone was highly homologous to aspartate aminotransferase and probably represented a false positive. In a second round of library screening, involving 3.2 \( \times 10^6 \) transformants, of 44 confirmed positives that have been characterized, 42 represent Nrl clones. The smallest 5'-truncation, present in 310 of 44 confirmed positives that have been characterized, 42 represent Nrl clones. The smallest 5'-truncation, present in four identical clones, encodes a fusion protein in which the Nrl sequence begins at residue 129 (see Fig. 2), demonstrating that residues 129–236 are sufficient for DNA binding.

Fig. 2 shows the predicted amino acid sequence of the bovine Nrl protein, as determined by the sequence of one of the full-length library clones and its comparison with the previously determined human and murine Nrl sequences (16, 43). The bovine sequence is 92 and 85% identical to the human and murine sequences, respectively. Interestingly, the majority of the sequence differences are present in the immediate carboxy-terminal region, just beyond the leucine zipper motif.

**NRE Acts as a Positive Regulatory Element in Retinal Cells**—Procedures for transient expression analysis using primary chick retinal cultures were developed (see “Experimental Procedures”) to examine whether Nrl can trans-activate rhodopsin promoter activity in retinal cells and to explore the ability of DNA elements flanking the NRE to modulate Nrl activity. To determine possible differences in promoter activity associated with the differentiation stage of the retinal cells, transfections were performed with chick retinal cultures derived from both E8 and E17. E8-derived cultures are free of glial and retinal pigment epithelial cells. They contain a mixture of photoreceptors and nonphotoreceptor neurons; the photoreceptors, which constitute approximately 10–20% of the cell population, differentiate largely in vitro (31, 44). In E17 cultures, photoreceptors represent 30–60% of the population, and many aspects of their differentiation occur in vivo, prior to their isolation for culture. Chick retinal cells were transfected with a series of luciferase fusion constructs containing DNA from the region upstream of the bovine (bRho) and murine (mRho) rhodopsin.

Chick retinal cells were transfected with a series of luciferase fusion constructs containing DNA from the region upstream of the bovine (bRho) and murine (mRho) rhodopsin. (The nomenclature used for reporter constructs includes abbreviations for the species and gene name, followed by a number that refers to the position of the 5'-end of the construct relative to the mRNA start site.) The bRho-2174, bRho-225, bRho-176, mRho-4907, mRho-1609, mRho-1488, and mRho-270 constructs demonstrated similar levels of activity, ranging between 66 and 107 relative light units (Fig. 3A). These results demonstrate that the bovine and murine rhodopsin proximal promoter regions, consisting of less than 300 bp of upstream DNA, are as active in chick retinal cultures as longer constructs containing the RER. (The RER spans the region from −2044 to −1943 and from −1575 to −1477 bp in the cow and mouse genes, respectively (9).)

To look specifically at the activity of the NRE and flanking DNA, the additional constructs bRho-38, bRho-84, bRho-130, mRho-40, mRho-84, mRho-130, mRho-176, and mRho-222 were generated. As shown in Fig. 3, B and C, the bovine (murine) constructs showed evidence of positive regulatory elements between −38 (−40) and −84 (−85) bp, which contain the NRE, and between −84 (−85) and −130 bp. The activity of the −84 to −130-bp region, particularly for the bovine constructs, is significantly greater than the NRE-containing region. The results also suggest the possibility of a negative element located between −130 and −176 bp. The importance of the NRE within the −38 to −84-bp sequence was confirmed by the finding that bRho-225 (mRho-225), which contains a mutated NRE, demonstrated significantly reduced reporter activity (Fig. 3B).

**Nrl Activates Expression of Transfected Rhodopsin Promoters via the NRE**—To further investigate the functional role of Nrl in rhodopsin regulation, E8 and E17 retinal cell cultures were transfected with a series of luciferase fusion constructs containing DNA from the region upstream of the bovine (bRho) and murine (mRho) rhodopsin.
were cotransfected with bRho constructs together with an expression vector containing the human NRL cDNA (pMT-NRL). Cotransfection of pMT-NRL resulted in induction of reporter gene activity with constructs that contained an intact NRE but had little effect with constructs that lacked the NRE or contained a mutated NRE, in both E8 (Fig. 4A) and E17 (Fig. 4B) cells. In E8 cultures, but not in E17 cultures, the Rho-130 constructs showed substantially greater stimulation with pMT-NRL cotransfection than did the Rho-84 constructs, even though there are no NRE-like sequences between −84 and −130 bp. This suggested that in the E8 cultures there are factors that can interact with sequences in the −84- to −130-bp region and increase NRL-mediated trans-activation. The human reporter construct hRho-85, which contains a homologous NRE, also showed approximately 3-fold stimulation on cotransfection with pMT-NRL (Fig. 4B), as did analogous murine rhodopsin constructs (data not shown).

Nrl, c-Fos, and c-Jun Differentially Trans-activate the Rhodopsin Promoter—Since the NRE is an extended AP-1 element, which binds to c-Fos-c-Jun heterodimers, we investigated whether c-Fos and c-Jun could also trans-activate the rhodopsin promoter. As shown in Fig. 5A, cotransfected Nrl, c-Fos, and c-Jun expression plasmids stimulated reporter gene activity from the bRho-130 construct by 3.2-, 1.9-, and 0.9-fold, respectively. When the expression constructs were cotransfected as pairs, Nrl-c-Fos and Nrl-c-Jun were both more potent than c-Fos-c-Jun. In contrast, in control experiments with a collagenase reporter construct (hCol-71), which contains a canonical AP-1 site but lacks an NRE, the maximal stimulation was seen with c-Fos, and Nrl was only minimally effective (Fig. 5B). Taken together, these results indicate that in primary chick retinal cells NRL preferentially trans-activates the rhodopsin NRE compared with c-Fos and c-Jun. The results are also consistent with in vitro studies that have shown that the central cytidine in the consensus Maf binding site (TGCTGACT-CAGCA) is important in determining the specificity of DNA interaction with bZIP proteins (25). Maf, Jun homodimers, and Fos-Jun heterodimers were all found to bind equally well to the consensus sequence; however, when the central cytidine nucleotide was altered (TGCTGATTCAGCA), as occurs in the rhodopsin NRE, Maf still bound strongly to the oligomer, whereas the binding of both Jun homodimers and Fos-Jun heterodimers was abolished.

**DISCUSSION**

Using the yeast one-hybrid system together with transient expression analysis of primary chick retinal cultures, we have obtained evidence supporting an important in vivo role for the NRL transcription factor Nrl in the regulation of rhodopsin expression. Independent one-hybrid assays consistently demonstrated that Nrl is the predominant gene obtained using the rhodopsin −73- to −30-bp sequence as bait, thus suggesting that Nrl may indeed be the primary transcription factor bind-

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**Fig. 3.** Expression of murine and bovine rhodopsin constructs in E17 cultures. E17 chick retinal cultures were transfected with equal molar amounts of the indicated constructs by the calcium phosphate method as described under “Experimental Procedures.” The constructs in A were designed to assess the activity of the NRE and other upstream regions, whereas the bovine and murine constructs shown in B and C, respectively, were designed to dissect the rhodopsin proximal promoter region (RPPR). In B, the construct bRho225(m62–66), which contains a mutated NRE in which the central 5 bp (CTGAT) have been mutated to GTCGAG, is also included. Relative light units (R.L.U.) were corrected for transfection efficiency with a β-galactosidase internal control and represent the ratio of the activity of each construct relative to the activity of the promotorless construct pGL2-basic. The values are means of three independent experiments performed in duplicate. Bars, 1 S.E.
The observation that Nrl can bind to the NRE and flanking DNA in the one-hybrid system adds to our previous demonstration that Nrl can bind to the rhodopsin NRE in an in vitro electrophoretic mobility shift assay (29) by demonstrating that the interaction can take place in an in vivo situation in the context of chromatin structure, which is often more stringent than naked DNA. In addition, since in some of the full-length constructs the Nrl coding region is out of frame with the GAL4 activation domain, the activation of reporter gene expression is presumably due to the activity of the Nrl trans-activation domain itself. In an analogous analysis of c-Maf, it was similarly found that the exogenous activation domain was not needed when the full-length coding region was used (19).

The one-hybrid analysis also provided a deletion analysis of the regions of Nrl required for DNA binding. The finding that the smallest 5’-truncations obtained in the screen corresponded to amino acid residues 129–236 is consistent with the structure of Nrl and previous biochemical studies with the Maf-Nrl family, which indicate that in addition to the basic region and leucine zipper motifs, a highly conserved sequence just amino-terminal to the basic region (extended homology domain; see Fig. 2) is also required for specific DNA binding (19, 23, 24, 45). Consistent with the model that the trans-activating activity is associated with the amino-terminal part of the molecule, the Nrl coding region in the deletions was in frame with the GAL4 activation domain.

In a complementary set of studies, transient expression analyses were carried out to explore the rhodopsin promoter regulatory interactions in the context of retinal cells. Primary chick retinal cell cultures were chosen for these studies, because they allow transfection efficiencies as high as 15% and contain photoreceptor cells that exhibit many of the molecular, structural, and functional properties of their in vivo counterparts (31). These characteristics are particularly appealing, since they allow analysis of the results of transient expression studies in the context of other differentiated cell behaviors, which is seldom possible using transformed cell lines. Mouse retinal cells would theoretically provide another attractive system for
transfection analysis, since they also undergo extensive differ-
entiation in culture (46); however, pilot studies with mouse
cells demonstrated transfection efficiencies that were so low as
to preclude meaningful promoter analysis.

The deletion analysis did not demonstrate any activity asso-
ciated with the rhodopsin RER. Although the reason for this is
unclear, it is perhaps not surprising, given that presently there
is no evidence to suggest the existence of a chick version of the
NRE because it leads to an increase in the trans-activating
activity of another protein that binds to and acts directly
through the NRE-containing region. It appears to act synergistically with
the NRE because it can stimulate rhodopsin promoter activity
and regulatory element(s) within the

The deletion analysis did provide evidence of the presence of
 cis-acting elements located between −130 and −40 bp, a region
that is highly conserved among the mouse, rat, Chinese ham-
er, cow, and human rhodopsin genes. Within this region, two
separate domains could be defined, one between −40 and −84
bp and another between −85 and −130 bp, that contain posi-
tive regulatory elements. A third domain, located between
 −130 and −176 bp, may possess a negative regulatory element.

At least part of the activity of the −40 to −84 bp region derives
from the NRE present between −56 and −68 bp. The −85 to
−130 bp region, which contains part of the rhodopsin ret1-
PCE1 element (10), is particularly active, stronger than the
NRE-containing region. It appears to act synergistically with
the NRE because it leads to an increase in the trans-activating
activity of co-transfected Nrl, even though it lacks a binding
site for Nrl. The synergism could be mediated by a regulatory
protein that binds to a sequence between −85 and −130 bp and
stabilizes the interaction between Nrl and the NRE or interacts
with bound Nrl and increases its trans-activating activity.

Alternatively Nrl could independently stimulate the synthesis or
activity of another protein that binds to and acts directly through
the −85 to −130 bp sequence. Regardless of its nature, the
synergetic activity appears to be developmentally regulated,
since it is seen with E8 but not with E17 cultures (Fig. 4,
A and B). Future studies will better define the mode of inter-
action and regulatory element(s) within the −85 to −130 bp
region.

The experiments presented here indicate that Nrl can stimu-
late rhodopsin expression in an NRE-dependent manner in
yeast cells and chick retinal cell cultures, respectively, but
they do not by themselves demonstrate that Nrl plays a similar role
in vitro. Several lines of evidence, however, are consistent with
this possibility and favor the involvement of Nrl compared with
other bZIP proteins: 1) in the adult, Nrl is specifically ex-
pressed in the retina, including photoreceptors (16); 2) Nrl expres-
sion precedes rhodopsin expression during development (28); 3) the position and sequence of the rhodopsin NRE is
evolutionarily conserved; 4) supershift experiments with reti-
nal nuclear extracts indicate that the activity that binds to a
rhodopsin NRE oligomer contains Nrl, or an immunologically
similar molecule, but not Fos or Jun (29); 5) although Fos and
Jun have been shown to be expressed in the retina, they have
not been identified in photoreceptors (47, 48); 6) Nrl is more
specific than either Fos or Jun for the rhodopsin promoter; and
7) neither Fos nor Jun was detected in the one-hybrid screen.

It is also clear that Nrl by itself is not sufficient to turn on
rhodopsin expression in vitro. Rhodopsin expression is limited
to rod photoreceptors, but Nrl is expressed in a number of
different retinal and neuronal cell types (16). Additionally,
retinoblastoma cell lines express Nrl but not rhodopsin (16, 49).

Like other genes that are regulated in a cell type- and devel-
omentally restricted manner, control of rhodopsin expression
is likely to be mediated by a combinatorial array of transcription
factors, some of which are retina- and/or cell type-specific,
whereas others are more ubiquitously expressed (2). On the
other hand, Nrl may also be involved in the regulation of other
photoreceptor-specific genes that contain NRE-like sites, such as
the human red and green opsin (50) and bovine interphotore-
ceptor retinoid-binding protein genes (3) and nonphotoreceptor
genes, such as the quail gene QR1, a developmentally
restricted gene expressed in retinal Muller cells (51). Future
experiments will be necessary to define more precisely this
apparently complex role of Nrl in vivo.

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