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Retinoic Acid Regulates the Expression of Photoreceptor Transcription Factor NRL*

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Abstract

NRL (neural retina leucine zipper) is a key basic motif-leucine zipper (bZIP) transcription factor, which orchestrates rod photoreceptor differentiation by activating the expression of rod-specific genes. The deletion of *Nrl* in mice results in functional cones that are derived from rod precursors. However, signaling pathways modulating the expression or activity of NRL have not been elucidated. Here, we show that retinoic acid (RA), a diffusible factor implicated in rod development, activates the expression of NRL in serum-deprived Y79 human retinoblastoma cells and in primary cultures of rat and porcine photoreceptors. The effect of RA is mimicked by TTNPB, a RA receptor agonist, and requires new protein synthesis. DNaseI footprinting and electrophoretic mobility shift assays (EMSA) using bovine retinal nuclear extract demonstrate that RA response elements (RAREs) identified within the *Nrl* promoter bind to RA receptors. Furthermore, in transiently transfected Y79 and HEK293 cells the activity of *Nrl*-promoter driving a luciferase reporter gene is induced by RA, and this activation is mediated by RAREs. Our data suggest that signaling by RA via RA receptors regulates the expression of NRL, providing a framework for delineating early steps in photoreceptor cell fate determination.

The vertebrate retina is a convenient and relatively less complex model to investigate gene regulatory networks during development of the central nervous system. It consists of seven major cell types (six neurons and one glia) that are generated in a conserved histogenic order from common pool(s) of retinal progenitors (1). Given the multipotency of retinal progenitors, one can predict that differentiation of distinct cell types depends upon precisely timed expression of cell type-specific genes under the coordinated and combinatorial influence of signaling molecules and transcription factors (1–4). Similar regulatory networks are also responsible for maintaining appropriate expression levels of phototransduction proteins in adult retina (5).

Photoreceptors (rods and cones) account for over 70% of all cells in the mammalian retina, and in many species rods greatly outnumber cones (6). A number of transcription regulatory

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factors are implicated during photoreceptor development; these include the homeodomain transcription factors CRX (7–9) and OTX2 (10), the retinoblastoma protein RB (11), thyroid hormone receptor TRβ2 (12,13), and rod-specific orphan nuclear receptor NR2E3 (14–18). Consistent with their roles in photoreceptor gene regulation, mutations in human *CRX* and *NR2E3* result in retinopathies (19–21).

NRL[3] is a bZIP transcription factor of the Maf subfamily (22). NRL is conserved in vertebrates and is specifically expressed in photoreceptors and pineal gland (23–26). Loss of *Nrl* in mice results in functional S-cones that are derived from post-mitotic precursors normally fated to be rods (25,27). Mutations in *NRL* are associated with retinal degenerative diseases in humans (28,29). NRL acts synergistically (or antagonistically) with CRX, NR2E3, FIZ1, and other transcription factors to regulate the expression of rhodopsin, cGMP-phosphodiesterase-α and -β, and many other rod genes (15,30–36). Hence, NRL is a crucial intrinsic regulator of photoreceptor development and function.

Extrinsic factors are thought to influence the timing, ratio, and functioning of different cell types during retinal differentiation (1–3). Soluble factors in the local microenvironment are expected to modify the competence of retinal progenitor cells to generate cone or rod photoreceptors (4,37–39). The vitamin A derivative, retinoic acid (RA), is an important morphogen that acts through its receptors (RAR and RXR), which are members of steroid-thyroid hormone nuclear receptor subfamily (2,40). RA is involved in the development of eye as well as other tissues; its deficiency causes microphthalmia and other defects (41,42). RA promotes rod differentiation both *in vitro* and *in vivo* (2,41,43,44). RA also modulates the expression of several photoreceptor-specific genes, including arrestin and *CRX* (45–47).

Given the complex networks of gene regulation during photoreceptor differentiation, the mechanism(s) by which extrinsic factors influence cell type-specific gene networks are not completely understood. In this report, we have used the regulation of NRL expression as a paradigm to gain insights into signaling pathways that control photoreceptor development. Using serum-deprived Y79 human retinoblastoma cells and cultured rat and porcine photoreceptors, we show that expression of NRL is induced by serum as well as RA. We demonstrate that RA acts on the RAREs identified within the *NRL* promoter to induce its expression. Our studies reveal a possible regulatory mechanism by which RA influences photoreceptor differentiation and rod-specific gene expression.

EXPERIMENTAL PROCEDURES

Reagents

Tissue culture media and serum were obtained from Invitrogen (Carlsbad, CA). Retinoic acids, growth factors, and other reagents were procured from Sigma. Stock solutions of RA and growth factors were prepared in 1% ethanol and/or dimethyl sulfoxide.

Cell Culture

Y79 human retinoblastoma cells (ATCC HTB 18) and HEK293 (ATCC CRL-1573) were maintained in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, under standard conditions with 15% (v/v) fetal bovine serum (FBS), penicillin G (100 units/ml), and streptomycin (100 μg/ml) at 37 °C and 5% CO_2. For serum starvation and RA treatment experiments, Y79 cells (5×10^4) were cultured in the presence or absence of the serum (same batch of serum was used in all the experiments), *at*RA, *9-cis*-RA, cycloheximide (CHX), and

[3]The abbreviations used are: NRL, neural retina leucine zipper; RA, retinoic acid; FBS, fetal bovine serum; CHX, cycloheximide; PBS, phosphate-buffered saline; EMSA, electrophoretic mobility shift assay; DAPI, 4,6-di-amino-phenyl-indolamine; RNE, retinal nuclear extract; HEK, human embryonic kidney; RARE, RA response elements.

4-(E-2- (5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl) benzoic acid (TTNPB) at indicated concentrations. Me_2SO or ethanol was added to Y79 cells in lieu of the soluble factors as negative control.

For protein synthesis inhibition experiments, Y79 cells were serum-starved for 24 h, and then simultaneously treated with RA and CHX for 8 or 24 h. NRL expression was analyzed by immunoblotting. In another set of experiments, serum-starved Y79 cells were first incubated with RA alone for 8 or 24 h and then CHX was added. Cell extracts were then analyzed 24 h later for examining NRL expression by immunoblotting.

Primary cultures of new-born rat retinal cells and enriched adult porcine photoreceptors were prepared according to published procedures (48). For newborn rat retinal cultures, rat pups were anesthetized and decapitated, the retinas dissected into CO_2-independent Dulbecco's modified Eagle's medium and chopped into small fragments. The fragments were washed twice in Ca/Mg-free PBS and then digested in PBS containing 0.1% papain for 25 min at 37 °C. Tissue was dissociated by repeated passage through flame polished Pasteur pipettes, then seeded into tissue culture plates precoated with laminin, in Neurobasal A medium (Invitrogen) containing 2% FBS. After 48 h, medium was changed to a chemically defined formula (Neurobasal A supplemented with B27) for a further 48 h, and then treated according to the different experiments (below).

For pig photoreceptor cultures, eyes were obtained from freshly slaughtered adult pigs, the retinas removed and dissected under sterile conditions. Tissue was minced, digested with papain, and dissociated by mild mechanical trituration. Cells obtained from the first two supernatants were pooled and seeded at $5 \times 10^5/cm^2$ into 6 × 35 well tissue culture plates as above. Cells were cultured as outlined above (48 h Neurobasal A/2% FBS, then 48 h Neurobasal A with B27).

Experimental Treatments and Immunochemistry

After the 4-day culture period, both primary cell models were treated as follows. RA was added to test wells (1, 5, 10, 20, and 40 μM, stock solution prepared in Me_2SO, 10 μl/well). Negative control wells received Me_2SO alone, and positive control wells were treated with Neurobasal containing 2% FBS. For immunoblotting, the medium was removed after 24 h; cells were rinsed in PBS and processed as indicated.

For immunocytochemical studies, medium was removed after 24 h, and cells were fixed in 4% paraformaldehyde in PBS for 15 min. Cells were permeabilized for 5 min using 0.1% Triton X-100, then preincubated in blocking buffer (PBS containing 0.1% bovine serum albumin, 0.1% Tween 20 and 0.1% sodium azide) for 30 min. Cells were incubated overnight in affinity-purified anti-NRL antiserum (1:1000 dilution), and monoclonal anti-rhodopsin antibody rho-4D2 (45), rinsed thoroughly, and incubated with secondary antibodies (anti-rabbit IgG-Alexa594 and anti-mouse IgG-Alexa488) combined with 4,6-di-amino-phenyl-indolamine (DAPI) (all from Molecular Probes Inc., Eugene, OR) for 2 h. Cells were washed, mounted in PBS/glycerol, and examined under a Nikon Optiphot 2 fluorescence microscope. All images were captured using a CCD camera and transferred to a dedicated PC. The same capture parameters were used for each stain, and final panels were made using untreated images for direct comparison of staining intensities.

Protein Expression Analysis

Y79 and newborn rat retinal cells were sonicated in PBS and clarified supernatant was used for further analysis. Protein concentration was determined using Bio-Rad protein assay reagent. Equal amounts of proteins were analyzed by SDS-PAGE followed by immunoblotting.

Proteins were detected using anti-NRL polyclonal antibody as described (15,23). Immunoblots from three independent experiments for rat and pig retinal cultures were analyzed by densitometric scanning, and normalized to serum-supplemented control levels in each case. Statistical analysis of data were performed using the one-tailed Student's *t* test, with $p < 0.05$ accepted as level of significance.

Plasmid Constructs

DNA fragments of 2.5 kb (Nl), 1.2 kb (Nm), and 200 bp (Ns) from the 5′-flanking region of the mouse *Nrl* promoter (GenBank™: AY526079; (25) were amplified and cloned into pGL3-basic vector (Madison, WI) in-frame with the luciferase reporter gene. The following site-directed mutants of the *Nrl* promoter were generated from pGL3-Nl using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and sequence-verified: pGL3-Nl-mutIII-1, pGL3-Nl-mutIII-2, and pGL3-Nl-mutII-1, containing deletion of the putative RAREs at positions −781 to −767, −709 to −700, and −453 to −443, respectively.

DNaseI Footprinting and Electrophoretic Mobility Shift Assays (EMSA)

Bovine retinal nuclear extract (RNE) was prepared as described (49). Solid phase DNaseI footprinting was performed as described (50), using 100 μg of RNE, and various fragments from the upstream conserved regions of the mouse *Nrl* promoter were used as template. For EMSA, oligonucleotides containing the wild-type mouse *Nrl* promoter sequence(oligo III-2 nucleotides −726 to −686: 5′-<ACGGGGAAAAGGTGAGAGGAAGC>-3′, oligo II-1 nucleotides −469 to −427: 5′-<GCAGGGGCTGAAATGTGAGGA>-3′) or deletion of the putative RAREs (mt-Oligo III-2: 5′-<CTGAGACACCGCACGGGGAGGAAGCTGAGGGC>-3′; and mt-Oligo II-1: 5′-<GGTGAAGGTAGGGCAGTGAGGATGCTTGAAAA>-3′) were end-labeled using [γ-^{32}P] ATP (Amersham Biosciences) and incubated in binding buffer (20 mM HEPES pH 7.5, 60 mM KCl, 0.5 mM dithiothreitol, 1 mM $MgCl_2$, 12% glycerol) with RNE (20 μg) and poly(dI-dC) (50 μg/ml) for 30 min at room temperature. In competition experiments, a non-radiolabeled oligonucleotide was used in molar excess of the labeled oligonucleotide. In some gel-shift experiments, antibodies were added after the incubation of ^{32}P-labeled oligonucleotides with RNE. Samples were loaded on 7.5% non-denaturing polyacrylamide gel. After electrophoresis, the gels were dried and exposed to x-ray film.

Transient Transfection and Luciferase Assay

Transient transfection of Y79 cells was performed using FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). Prior to transfection, cells were serum-starved 24 h in Opti-MEM (Invitrogen), diluted to 1.5×10^5 cells in 250 μl and seeded into 24-well plates. Transfection was performed with 0.5 μg of promoter-luciferase construct and 1.5 μl of FuGENE 6. One hour after transfection, 10 μM RA or 1% ethanol was added to each well. Transfected cells were cultured for additional 24 h and harvested. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI). Experiments were repeated at least three times, and the luciferase activity was calculated as a fold change from the base line luciferase activity obtained in the presence of vector only.

Transient transfection of HEK293 (ATCC CRL-1573) cells was performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The wild type and mutant *Nrl* promoter-luciferase constructs, and pCMV-β-gal were added to the cells at a concentration of 0.1 μg and 0.05 μg, respectively. After 3 h, 100 μl of Dulbecco's modified Eagle's medium with 0 or 500 nM *at*RA was added to each well. Cells were harvested after 24 h in 100 μl of Glo lysis buffer (Promega), and luciferase activity was measured.

RESULTS

Serum-deprivation of Y79 Cells

We had shown earlier that NRL is expressed in Y79 cells but not in other tested cell lines (22). To generate an efficient *in vitro* model system to study regulation of NRL expression, we carried out serum deprivation of Y79 cells. Northern blot analysis and RT-PCR failed to detect *NRL* transcripts within 24 h after serum deprivation (data not shown). Immunoblot analysis showed that NRL expression in Y79 cells decreased 8 h after serum depletion and was undetectable by 24 h (Fig. 1*A*). No cell death was detected because of serum deprivation within the time span of the experiments (data not shown). When serum was supplied to these cells, NRL expression was detectable in 2 h and completely restored within 8 h (Fig. 1*B*). Multiple immunoreactive bands in 29–35 kDa range represent different phosphorylated isoforms of NRL that are detected by affinity-purified anti-NRL antibody (23). Additional bands observed in immunoblots may represent unrelated cross-reactive proteins, and their levels did not change after serum deprivation.

RA Effect on NRL Expression

To identify some of the possible activators in serum, we tested the effect of a number of soluble factors on NRL expression. We detected a dose-dependent increase in NRL expression following incubation with *at*RA and its isomer, *9-cis* RA (Fig. 2*A*). The effect of RA was mimicked by a RAR-specific agonist, TTNPB (Fig. 2*B*). Northern blot analysis of RNA from the treated cells also showed RA induction of *NRL* transcripts (data not shown).

We then evaluated the time course of NRL induction by RA. An increase in NRL protein was observed in serum-starved Y79 cells after 8 h of incubation with *at*RA (Fig. 2*C*). A similar effect was observed with *9-cis* RA (data not shown). Treatment of cells with *at*RA and CHX (20 μg/ml), an inhibitor of protein synthesis (51), blocked NRL induction when both were added simultaneously (Fig. 2*D*). This suggests that intermediate protein synthesis is necessary for RA-mediated induction of NRL expression. However, when cells were pretreated with RA for 8 or 24 h, CHX had no detectable effect on NRL expression (Fig. 2*D*). These results suggest that synthesis of intermediary factors necessary for NRL induction occurs within 8 h of RA treatment.

RA Stimulation of NRL Expression in Rat and Porcine Photoreceptors

To investigate the effect of RA on the expression of NRL in photoreceptors *in vitro*, we utilized two different culture models. Immunoblotting of proteins isolated from monolayer cultures of newborn rat retina revealed that maintenance of cells in chemically defined conditions for 24 h led to moderate but reproducible decreases in NRL expression levels, and that either re-addition of serum or increasing doses of RA increased the NRL band intensity (Fig. 3*A*). Only a single NRL-immunoreactive band was visible using the newborn rat retinal cells (Fig. 3*A*). Similar induction in NRL expression was observed using highly enriched photoreceptor cultures prepared from adult pig retina, which however showed two NRL-immunoreactive bands (Fig. 3*B*). In both rat and pig cultures, maximal effects were observed with 5–20 μM RA, and higher doses led to some toxicity especially in cells from new-born rat retina. Immunocytochemical studies of pig photoreceptor cultures revealed that NRL was confined to rod nuclei in all cases, and that signal was relatively strong in serum- or RA-supplemented conditions. The serum-free photoreceptor culture displayed a modest but reproducible decrease in NRL-specific signal in the nuclei, as seen in immunoblots (Fig. 3*C*). Expression levels in newborn rat retinal cultures were too low to be detected by immunocytochemistry (data not shown).

Role of RA Receptors

We next examined whether RA acts directly on the *Nrl* promoter. DNaseI footprinting analysis of conserved sequences upstream of the transcription start site of the mouse *Nrl* gene identified putative RAREs (regions III-1, III-2, and II-1), in addition to other transcription factor binding elements (Fig. 4, *A* and *B*; data not shown). Oligonucleotides encompassing these protected sequences were radiolabeled and used for EMSA analysis (Fig. 4*C*). We observed mobility shift of the radiolabeled oligonucleotides in the presence of bovine retinal nuclear extracts (Fig. 4*D*). The intensity of the shifted bands was reduced or eliminated by molar excess of the same non-radiolabeled oligonucleotide, but not by a mutant oligonucleotide carrying a deletion of the putative RAREs. The shifted bands were also diminished when anti-RARα, anti-RXRα, or anti-RXRγ but not RARβ, RARγ, or RXRβ-specific antibodies were added (Fig. 4*D*).

To investigate the functional relevance of the binding of RA receptors to the *Nrl* promoter, we performed transient transfection experiments in serum-deprived Y79 cells using *Nrl* promoter-luciferase constructs containing the 2.5-kb fragment (pGL3-Nl) as well as deletion variants encompassing the footprinted regions III and II (pGL3-Nm and pGL3-Ns) (Fig. 5*A*). Addition of *at*RA showed over a 2-fold increase in luciferase activity with pGL3-Nl and pGL3-Nm constructs, which included the putative RAREs (Fig. 5*B*). The pGL3-Ns construct did not show a detectable increase in the reporter activity in the presence of RA. All three constructs induced luciferase reporter activity when transiently transfected into Y79 cells in the presence of serum (data not shown).

To further ascertain the involvement of putative RAREs in RA-mediated up-regulation of *Nrl* promoter activity, we performed site-directed mutagenesis and deleted the putative RAREs from the pGL3-Nl promoter-luciferase construct. As predicted, the pGL3-Nl construct showed a dose-dependent response to RA treatment in HEK293 cells with maximum effect in the presence of 500 nM *at*RA (Fig. 5*C*). However, deletions encompassing the region III-1 (pGL3-Nl-mutIII-1 and pGL3-Nl-mutIII-2) resulted in a reduction in luciferase activity in the presence of 500 nM *at*RA (Fig. 5*C*). Although we observed binding of RXRα and RXRγ on *Nrl* promoter, deletion of the putative RXR binding site (pGL3-Nl-mutII-1) did not have any appreciable effect on the luciferase activity. This might reflect heterodimerization between RARs and RXRs at other sites (potentially footprint III-2) on the promoter, which compensates for the lack of binding of RXRs to footprint II-1.

DISCUSSION

A coordinated interplay of intrinsic factors and extrinsic cues dictates the generation of retinal neurons. Extracellular signaling molecules modulate the synergistic (or antagonistic) action of a limited number of transcription factors that guide the expression of cell-type specific genes (52). NRL is the key transcriptional regulatory protein, essential for rod photoreceptor differentiation (27). NRL expression in cone photoreceptor precursors transforms their fate to functional rods, suggesting that NRL initiates the cascade of molecular events required for rod differentiation.[4] It is however unclear as to how NRL expression is initiated in specific neuroepithelial progenitors when they are exiting cell cycle. RA has previously been implicated as a mediator of rod differentiation (37). In this study, we provide evidence in support of RA being one of the signaling molecules that can induce NRL expression. Our data come from studies in Y79 cells and dissociated rod photoreceptors of newborn rat and adult porcine retina. We also show that the effect of RA is mediated by RA receptors and cis-sequence elements present within the *Nrl* promoter.

[4]E. Oh and A. Swaroop, unpublished data.

The lack or reduction of NRL transcripts and protein in the absence of serum suggests that one or more soluble factors regulate its expression at the level of transcription. Serum contains a complex mixture of growth factors, cytokines and other signaling molecules that stimulate the expression of several genes including c-*fos*, c-*myc*, *cyclin D1*, and *VEGF*, in cultured cells (53). While we have identified RA as one of the molecules, it is likely that additional pathways exist. Although NRL levels are decreased in normal rod photoreceptor *in vitro* upon withdrawal of serum, they remain detectable. Additionally, NRL contains a number of consensus phosphorylation sites; hence, it is possible that growth factor signaling through the extracellular signal-related kinase (ERK) pathway plays an important role in modulating NRL activity and/or stability. Induction of NRL expression occurs within 2 h of treatment with serum, whereas a gradual increase in NRL expression was observed when cells were treated with RA. This suggests that RA-mediated effect requires *de novo* protein synthesis, a phenomenon observed previously for the expression of human cone-arrestin gene (45). Treatment of Y79 cells with RA is reported to cause an increase in the levels of RARs and RXRs (45). Therefore, we propose that RA stimulates the expression of its own receptors, which in turn act on the *Nrl* promoter, leading to a time delay in inducing NRL expression.

The amount and activity of transcription factors is critical for regulation of their downstream targets (54). Vertebrate rod photoreceptors are highly metabolically active post-mitotic neurons; ~9 billion opsin molecules are synthesized every second in each human retina and transported to the outer segments, the site where phototransduction occurs (55). The expression of opsins and other phototransduction proteins must be stringently controlled because over- or underexpression of rhodopsin leads to photoreceptor degeneration (56,57). The expression of NRL has to be continuously maintained at transcriptional and/or post-transcriptional levels; missense mutations that affect the activity of NRL lead to photoreceptor degeneration (28, 29). It is therefore expected that amount and activity of NRL are critical determinants of normal rod photoreceptor function. Our serum-depletion data suggest that NRL has a relatively short half-life. In this respect, RA could be a critical signaling molecule in up-regulating NRL expression.

RA-mediated signal transduction occurs through its interaction with two classes of nuclear receptors: retinoic acid receptor (RARα, RARβ, and RARγ) and retinoid X receptor (RXRα, RXRβ, and RXRγ). *9-cis* RA is a ligand for RXRs, whereas the RAR subtype binds both *at*RA and *9-cis* RA (44). Given that RARα, RXRα, and RXRγ are expressed in the outer nuclear layer of the developing mouse retina (58,59), our results suggest that RA receptors play a significant role in activating NRL expression during retinal development. Because RXRs form heterodimers with RARs we cannot rule out the possibility of the binding of such heterodimers on the *Nrl* promoter.

We observe high induction of endogenous levels of NRL by RA; however, transient transfection experiments using a 2.5-kb fragment of *Nrl* promoter show a relatively weaker (2–2.5-fold) effect of RA. These data indicate that whereas RAREs are important in mediating RA-dependent up-regulation of the *Nrl* promoter, the 2.5-kb promoter fragment is not in the right context of chromatin in Y79 cells and therefore, may not be able to bind to or recruit other transcription factors necessary for NRL expression. Furthermore, RA may not be the only soluble factor that can affect NRL expression. A number of other factors have been shown to influence rod photoreceptor differentiation; these include taurine and FGF (60,61). We have observed an increase in NRL expression in the presence of FGF,[5] whereas taurine had no detectable effect in the same experiment (data not shown). These results reveal that either a combination of some of these factors is required for optimal activity, or their effect on rod differentiation is mediated by a pathway distinct from the one studied here.

[5]S. Siffroi-Fernandez, H. Khanna, A. Swaroop, and D. Hicks, manuscript in preparation.

Although our studies have been performed using cell culture models to demonstrate RA-mediated regulation of NRL expression, the data obtained using Y79 retinoblastoma cells and cultured photoreceptors can be extrapolated to the *in vivo* situation. Y79 cells are childhood intraocular tumors of photoreceptor origin and express a number of photoreceptor-specific genes, including *NRL*, all RA receptors, and can be maintained under standard conditions with serum (22,62). Our studies offer convenient *in vitro* model systems of using serum-deprived cells to study the role of soluble factors in photoreceptor development and maintenance.

In summary, we demonstrate a previously undescribed functional link between an environmental factor involved in rod development (RA) and a key transcriptional regulator (NRL). Given that retinal progenitors express RA receptors throughout rod development (59), we hypothesize that RA directs these cells toward photoreceptor cell fate and influences rod differentiation by up-regulating NRL. A detailed analysis of NRL expression in RA receptor knock-out mice (63) may facilitate understanding of the role of RA receptors in rod photoreceptor development.

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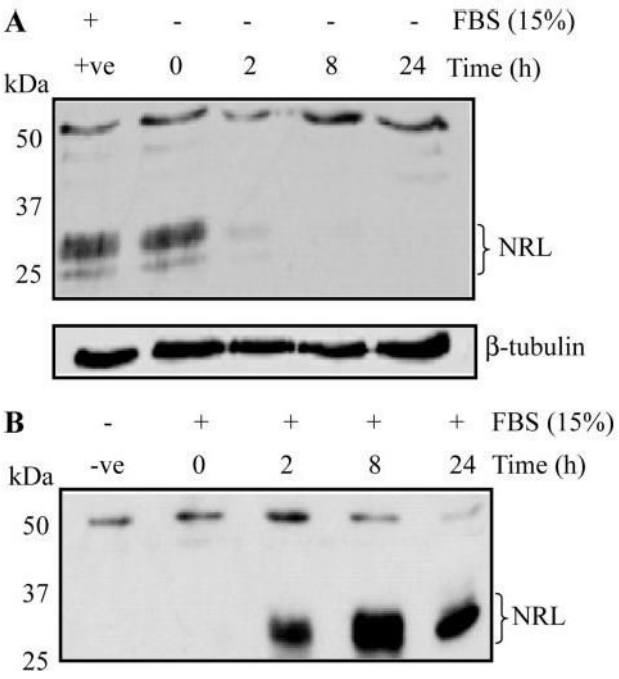

FIGURE 1. Serum induces NRL expression in Y79 cells

Y79 cells were grown in RPMI media without (*A*) or with (*B*) FBS (15%) for indicated time intervals, and protein extracts were analyzed by immunoblotting using anti-NRL antibody. Multiple isoforms of NRL are indicated by a *bracket*. Lanes are as indicated. *Lower panel* in *A* shows that the same blot was probed with anti-β-tubulin antibody, which served as a loading control. Molecular masses of markers are shown in kDa. The positive control (*+ve*) represents Y79 cells grown in 15% FBS.

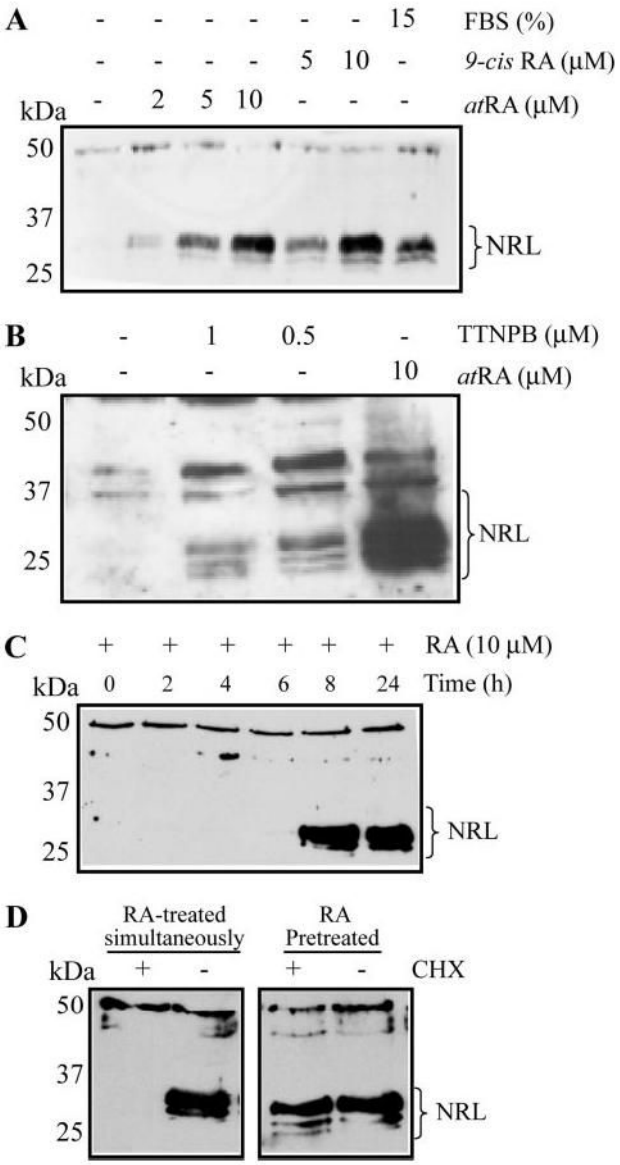

FIGURE 2. RA stimulates expression of NRL protein in Y79 cells

Serum-starved Y79 cells were incubated with indicated concentrations of 9-*cis atRA*, 15% FBS (*A*) or TTNPB (*B*) for 24 h. Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-NRL antibody. Negative controls included 1% ethanol or Me_2SO in lieu of the soluble factors. A *bracket* indicates multiple phosphorylated NRL isoforms. Lanes are as indicated. Molecular mass markers are indicated on the *left*. Additional bands in the higher molecular mass range may represent cross-reacting proteins (23). *C*, time-dependent effect of RA: serum-deprived Y79 cells were incubated with medium containing 10 μM RA for indicated time intervals. At the end of incubation, cells extract was analyzed by SDS-PAGE and immunoblotting using anti-NRL antibody. Lanes are as indicated. *D*, effect of protein synthesis inhibitor CHX on RA-mediated NRL induction was studied by incubating serum-starved Y79 cells with media containing *at*RA (10 μM) and CHX (20 μg/ml) (*left panel*; RA-treated simultaneously). In a similar experiment, cells were pretreated with RA for 24 h

followed by addition of CHX (*right panel*). Cell extracts were analyzed by SDS-PAGE and immunoblotting using the anti-NRL antibody.

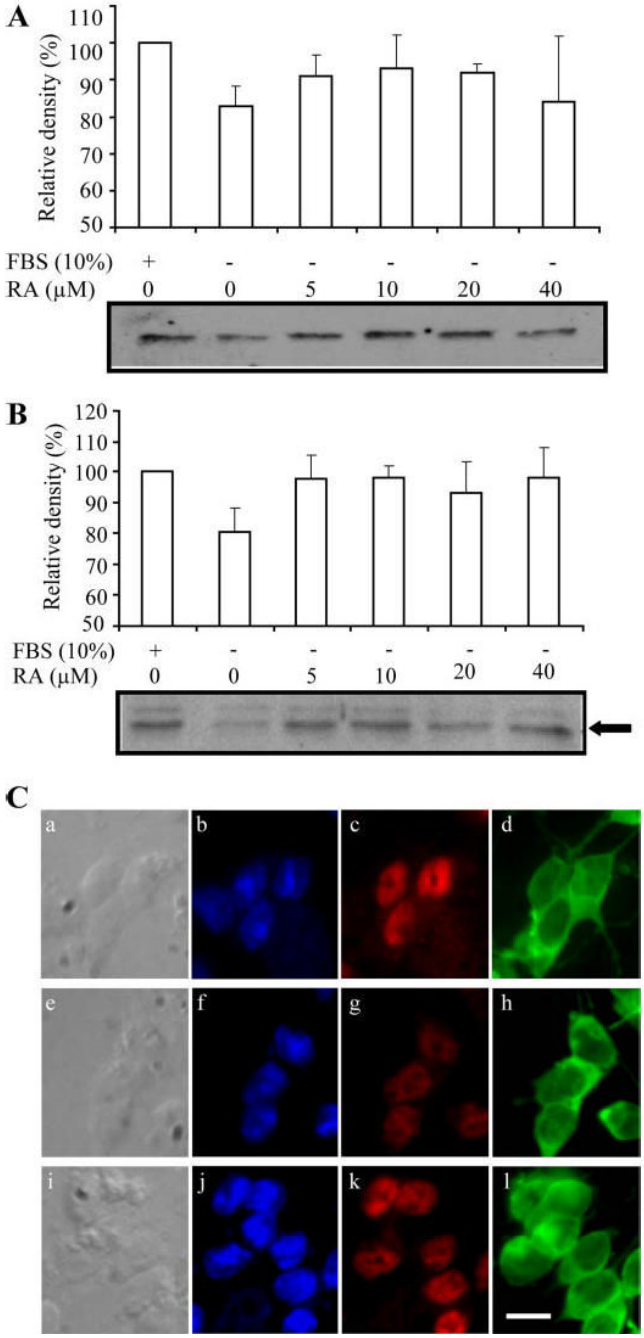

FIGURE 3. RA increases NRL protein levels in cultured rat and porcine photoreceptors

Analyses of rat (*A*) and porcine (*B*) retinal cultures after incubation with indicated concentrations of RA or FBS. Newborn rat retinal cells and adult pig photoreceptors were cultured *in vitro*, as described under "Experimental Procedures." Cell extracts were analyzed by SDS-PAGE and immunoblotting using anti-NRL antibody. In both panels, the intensity of the NRL immunoreactive band was reduced in serum-free culture compared with +FBS, and was partially restored by increasing doses of RA. This reduction was significantly different ($p < 0.05$) compared with serum-supplemented controls (*). For rat cultures, this reduction was also significantly different from 20 μM RA, but not for other values. 40 μM RA was toxic for cell survival in newborn rat retina. For pig cultures, the decrease was significantly different

compared with all RA concentrations, except 20 μM. The *arrow* in *B* indicates the major NRL immunoreactive band used for scanning. Histograms show densitometric scan of representative blots for each culture model. Experiments were performed three times on independent cultures with similar results. *C*, adult pig photoreceptor cultures were prepared and immuno-stained as described under "Experimental Procedures." Nomarski differential contrast images of cells are depicted in *panels a*, *e*, and *i*; DAPI staining (*blue*) of the nuclei in the same fields is shown in *panels b*, *f*, and *j*; NRL immunolabeling (*red*) of the same fields is shown in *panels c*, *g*, and *k*; and anti-rhodopsin immunolabeling (*green*) of the same fields is shown in *panels d*, *h*, and *l*. Positive control cultures, maintained in chemically defined medium to which serum-supplemented medium was added for 24 h, revealed strong nuclear NRL immunoreactivity (*panel c*), as did cells treated with RA (10 μM) for 24 h (*panel k*); however cells maintained in chemically defined medium demonstrated less intense nuclear staining (*panel g*). In all cases, rhodopsin staining was not detectably different. Scale bar in *panel l* is 4 μm for all panels. Experiments were repeated using three independent cultures with similar results.

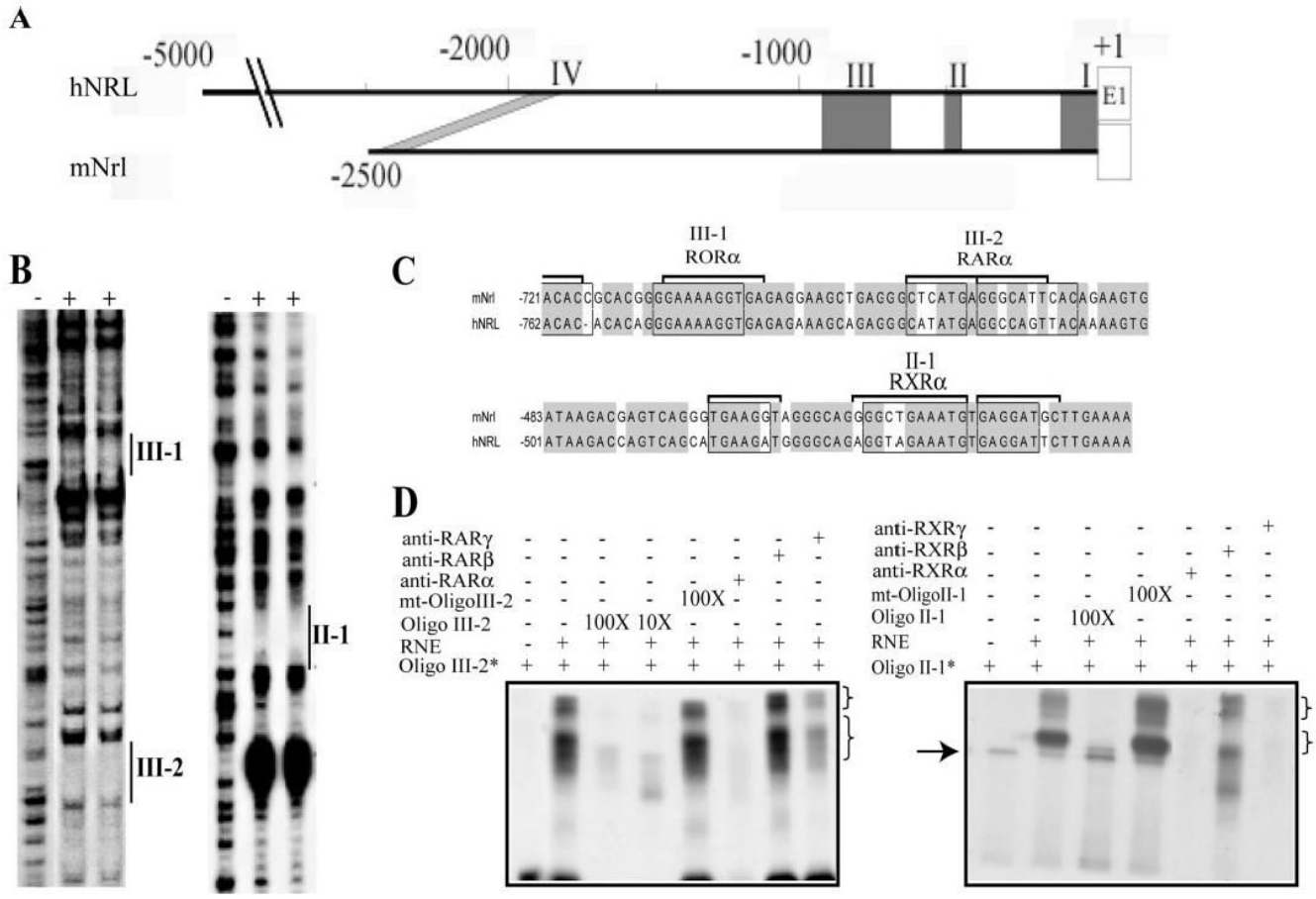

FIGURE 4. Putative RAREs within the *Nrl* promoter are protected by retinal nuclear proteins

A, schematic representation of the *Nrl* promoter showing regions of homology (I, II, III, and IV) between human (*h*) and mouse (*m*) *Nrl*. E1 denotes exon 1 of the *Nrl* gene. *B*, DNaseI footprinting using bovine RNE was performed as described under "Experimental Procedures." Footprints corresponding to regions II and III are shown. *Vertical lines* indicate footprinted regions. (−) denotes footprint in the absence of RNE whereas (+) indicates the experiment in the presence of RNE. Footprints containing the putative RAREs are indicated by III-1, III-2, and II-1. *C*, sequence of the putative RAREs in the footprints (II and III) of both mouse and human *Nrl* promoter region. Regions III-1 and III-2 contain putative ROR (orphan receptor) and RAR response elements whereas region II-1 contains a putative RXR binding element. *D*, *EMSA*, oligonucleotides corresponding to the regions III-2 (Oligo III-2) and II-1 (Oligo II-1) were radiolabeled using [γ-^{32}P]dATP and incubated with bovine retinal nuclear extract followed by analysis using non-denaturing PAGE, as described under "Experimental Procedures." Competition experiments were performed with unlabeled oligonucleotides to validate the specificity of the band shift. Experiments in the presence of antibody against various receptor ligands showed the presence or absence of the specific proteins. *Arrow* indicates a nonspecific band shift. * indicates radiolabeled oligo used in the experiment; *mt-Oligo* represents mutant oligonucleotide from which the putative RAREs have been deleted. Lanes are as indicated. *Brackets* indicate specific gel-shifted bands.

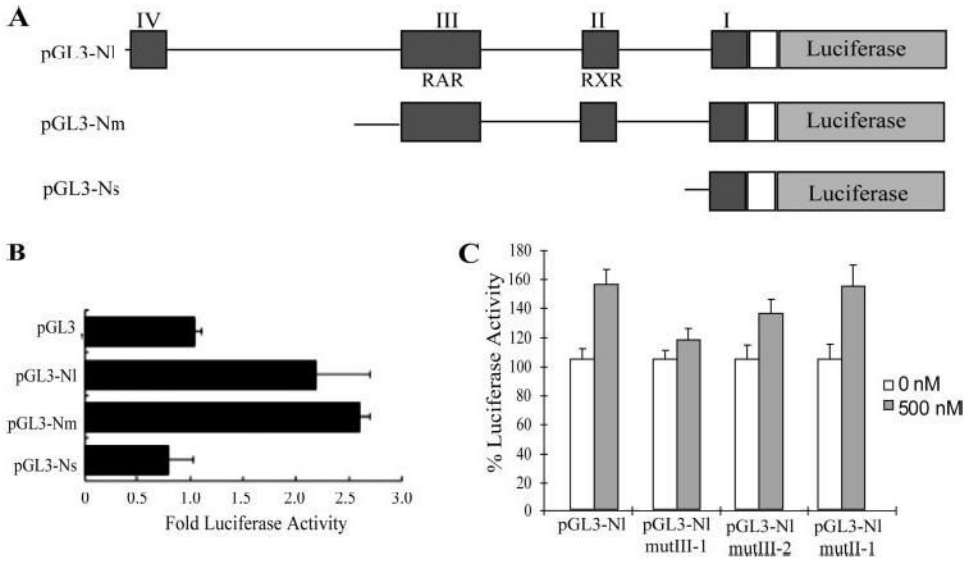

FIGURE 5. RA receptors bind to and activate *Nrl* promoter

A, schematic representation of the mouse *Nrl* promoter-luciferase constructs used to study the response to RA. The deletion fragments were cloned into pGL3-basic plasmid in-frame with the luciferase reporter gene. RAR and RXR response elements in regions III and II, respectively are depicted. These constructs were used in a separate assay to check for intrinsic promoter activity (data not shown). *B*, *Nrl* promoter-luciferase constructs were transfected into Y79 cells as described under "Experimental Procedures." Promoterless vector, pGL3 vector was used as negative control and the value of luciferase activity was set to 1. Results are expressed as a ratio of luciferase values obtained in the presence or absence of RA and represent an average of three independent experiments. *C*, site-directed mutants of the pGL3-Nl construct (pGL3-Nl-mut III-1, III-2, or II-1), containing deletions of the putative RAREs, were used to transfect HEK293 cells in the presence of indicated concentrations of *at*RA. The value of the control (transfected with the wild-type pGL3-Nl with no *at*RA) was set at 100% luciferase activity. Results are expressed as percent luciferase activity as compared with the control.