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Spatial and Temporal Expression of AP-1 Responsive Rod Photoreceptor Genes and bZIP Transcription Factors During Development of the Rat Retina

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Purpose: The promoter region of the rod-specific β subunit of cGMP PDE (β-PDE) and opsin genes contains highly conserved cis-acting elements, which include an AP-1 and/or *Nrl* response element (NRE: An extended AP-1 like sequence). Transactivation of AP-1 or NRE appears necessary to drive expression of these rod-specific genes during adulthood, however, their role during development is relatively unknown. Therefore, we determined the spatial and temporal relationships between rod morphological and functional development, rod-specific gene expression, and expression of the bZIP transcription factors *c-fos*, *junD* and *Nrl*.

Methods: Retinas from 0-45 day old (PN0-45) dark- and light-adapted Long-Evans rats were used. Morphological development was monitored by light and electron microscopy. Whole retinal trypsin-activated cGMP-PDE activity and rhodopsin content were measured biochemically. The expression of opsin, β-PDE, *c-fos*, *junD* and *Nrl* mRNAs were determined by Northern blot analysis. The cellular localization of *Nrl* was examined with in situ hybridization.

Results: The mRNAs for opsin, β-PDE and c-*fos* were observed at PN0-2, while cGMP-PDE activity and rhodopsin were detected first at PN5: coincident with rod outer segment development. The developmental pattern of cGMP-PDE activity and rhodopsin accumulation paralleled the expression of β-PDE and opsin mRNA and all reached their maximal levels by PN45. *Nrl* expression, for all three transcripts found in the rat retina, was low on PN2 and reached its maximal level at PN14. The *c-fos* and *Nrl* expression preceded β-PDE and opsin mRNA expression by 1-2 days. *Nrl* expression was detected first in the distal post-mitotic retina at PN5 and then in all nuclear layers during retinal development. Maximal expression shifted from the ganglion cells to the outer nuclear layer as the neural retina matured. In contrast, *junD* expression was highest at PN0 and declined to a stable level by PN10.

Conclusions: Colocalization of Nrl and c-Fos suggests that expression of rod-specific genes, which utilize AP-1 or NRE sites in their promoter, could be regulated through the formation of Nrl-Fos dimers. We hypothesize that Nrl and c-Fos play a fundamental role in the initiation and regulation of the rod-specific gene expression in developing and adult rod photoreceptors.

Biochemical and molecular analyses of mammalian retinal development have revealed that both gene-specific and cell type-specific regulatory factors are involved in rod cell differentiation, proliferation, and growth [1-6]. The identification of extrinsic factors responsible for rod cell fate is progressing rapidly [3,5-7]. However, the molecular mechanisms underlying rod photoreceptor, and especially rod outer segment (ROS), differentiation and growth are less well understood. During rod development, there is a coordinate expression of several rod-specific genes that encode proteins of the phototransduction cascade: rhodopsin, cGMP-PDE, and transducin [1,8,9]. The coordinated temporal expression of rhodopsin, the rod α-subunit of cGMP-PDE, and the rod α-subunit of transducin is regulated at the transcriptional level [1,2,10].

Several conserved cis-regulatory elements are located in the promoter regions of the genes encoding rhodopsin, rod beta-subunit of cGMP-PDE (β-PDE), and rod γ subunit of transducin (γ-T) [4,11-17]. Several transcription regulatory proteins have been identified to bind to these elements. The first transcription factor shown to bind to a conserved sequence element (an extended AP-1 like sequence called Nrl-response element: NRE) in the rhodopsin promoter was Nrl [14], which earlier was isolated by subtraction cloning [15]. Nrl also was able to transactivate rhodopsin promoter activity in cultured cells [14,18]. Recently, Crx (cone-rod homeobox), which is expressed predominantly in photoreceptors and pineal glands in adult mouse, was demonstrated to bind to several sequences in promoter regions of various rod-specific genes and activate promoter activity [16,17]. Importantly, Nrl and Crx synergistically transactivate rhodopsin promoter activity [16].

Promoters of several photoreceptor-specific genes contain an AP-1 binding site, which is similar to NRE [11,13,14]. Results from several laboratories suggest that the AP-1 and/or NRE sites are functionally relevant and necessary for transcription of the rod-specific β-PDE gene [19] and opsin gene [14,18]. The AP-1 complex consist of dimers of the *jun* and *fos* family members but not homodimers of fos. Therefore, AP-1 complexes in the retina may contain Nrl in addition to Jun and Fos proteins. These basic motif-leucine zipper (bZIP) proteins are expressed in a large number of different tissues and regulate the expression of a wide variety of genes [6,20-22]. It should be noted that bZIP proteins like Nrl bind to DNA

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as homodimers or heterodimers and that heterodimerization enhances the sequence site specificity [20-22]. The identity of Nrl's partner in rod-specific gene regulation is unknown, although it can form dimers with several bZIP proteins (e.g., Fos and Jun) in vitro [21]. In vivo, however, the heterodimerization of Nrl will depend on the availability and consequently expression of specific bZIP proteins. *Nrl*, c-*fos*, and *junD* are expressed in vertebrate photoreceptors as well as other retinal cells [23-25]. For example, in dark-adapted neonatal and adult rat retinas, c-*fos*expression is localized almost exclusively to the outer nuclear layer (ONL), whereas *junD* expression is observed throughout all retinal nuclear layers [23,25]. The distribution of *Nrl* expression in dark-adapted retinas is not known. In the light-adapted neonatal and adult rat retinas, c-*fos* and *junD* expression are observed in all retinal nuclear layers [23,25]. Similarly, in light-adapted adult mouse retina *Nrl* expression is detected in all retinal nuclear layers [24].

Transcription of rod-specific genes is determined by specific cis-regulatory elements in the promoter region and the availability of their cognate binding proteins [10,12,14,16,17,18]. Although Nrl has been suggested to regulate expression of rhodopsin and other genes [14,18], it has not been determined whether AP-1 transcriptional complexes in different rod-specific promoters contain Nrl as a homodimer or a heterodimer. To clarify the role of Nrl and other bZIP transcription factors in regulating expression of AP-1 responsive rod-specific genes, we undertook a comprehensive expression analysis to determine their spatial and temporal relationship during the development of rat retina. Specifically, our aims were to determine: (i) the temporal relationships between the differentiation and development of ROS and the onset and increase in retinal cGMP-PDE activity, rhodopsin concentration, and rod-specific β-PDE and opsin gene expression, (ii) the temporal and spatial patterns of expression of *c-fos*, *junD* and *Nrl* genes in developing dark-adapted rat retinas, and (iii) the temporal relationships between the developmental expression of β-PDE, opsin and c-*fos*, *junD* and *Nrl*.

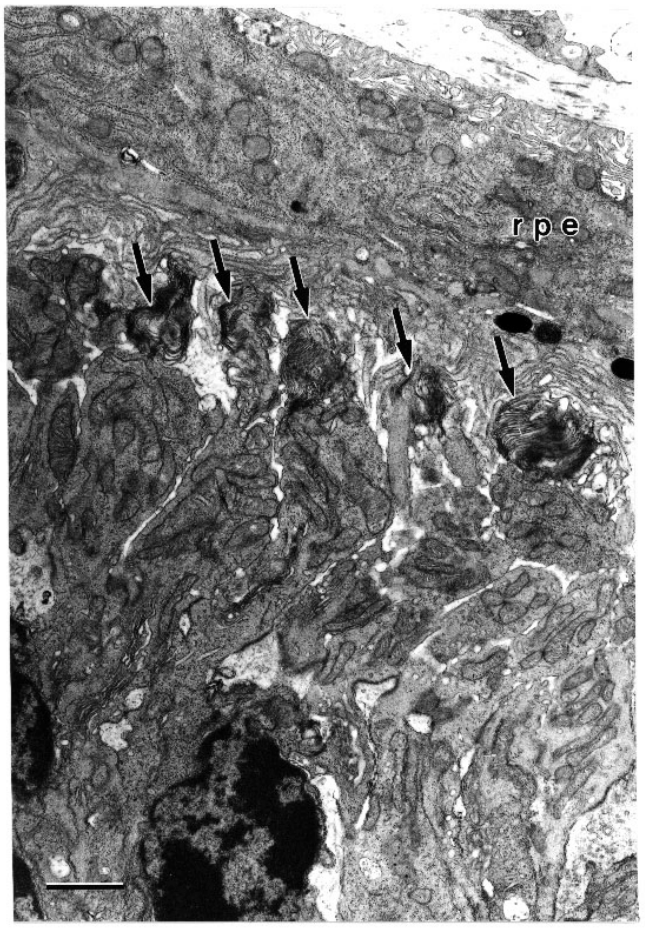

Figure 1. Representative electron micrograph of pigmented rat retina at postnatal day 6. At PN6, the photoreceptor inner segments with their numerous well-preserved mitochondria are well developed. In contrast, the photoreceptor outer segments are just beginning to form at PN6 (arrows). A cilium extending towards the retinal pigment epithelium (rpe) is evident. (x11,500; bar = 1 μm)

METHODS

Materials: All chemicals were of analytical or molecular biological grade and were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless otherwise noted. The β-PDE cDNA (provided by Dr. Debora Farber at Jules Stein Eye Institute at UCLA, Los Angeles, CA) was isolated from murine retina and cloned into pBLUESCRIPT KS (-) at the EcoR I cloning site. The mouse opsin cDNA was provided by Dr. Wolfgang Baehr (University of Utah Health Science Center, Salt Lake City, UT). The *c-fos* probe was derived from a 40 base single stranded synthetic oligonucleotide, based on the sequence from exon 1 of rat *c-fos* (Calbiochem-Novabiochem Corp., San Diego, CA). The *Nrl*probe for Northern blot analysis was a 1.9 kb fragment of the mouse *Nrl* cDNA (MR5) in pBLUESCRIPT KS (-) [26], comprising 131 bp of 5' untranslated region, the coding region and 1 kb of 3' untranslated sequences. The mouse

Table 1. Postnatal age of onset or appearance, fifty percent of maximal level and maximal level for various rat rod photoreceptor, and retinal measures obtained during development and adulthood[a]

Measure	Age of Onset	Age at 50% of Maximal Level	Age at Maximal Level
ROS length	PN6	PN14	PN45
Rhodopsin concentration	PN5	PN14	PN45
cGMP PDE activity	PN5	PN15-16	PN45
opsin expression	PN1	PN12-14	PN45
b-PDE expression	PN2	PN12-14	PN45
c-*fos* expression	PN0	PN14	PN45
Nrl expression (2.0 kb)	PN0	PN7	PN14
junD expression	PN0	PN8	PN0
Nrl in situ expression	PN5	PN10	PN15

[a] Except for the *Nrl* in situ expression data, the ages are values determined from graphs presented in this manuscript. The ages for the in situ expression were obtained by visual inspection of semi-quantitative analyses as described in the Methods.

junD cDNA (provided by Dr. Daniel Nathans at The Johns Hopkins University School of Medicine, Baltimore, MD) was cloned into pGEM3 at the EcoR I cloning site. The 18S ribosomal RNA (rRNA) oligo probe was provided by Dr. Cheryl Craft (USC School of Medicine, Los Angeles, CA). All cDNAs were labeled with [α-^{32}P] dCTP using random primed DNA labeling kit (Boehringer Mannheim Corp., Indianapolis, IN), and the probes were purified using Sephadex G-50 columns (Boehringer Mannheim Corp.). The *c-fos* and 18S oligonucleotide probes were labeled with [γ-^{32}P] ATP using T4 kinase (Promega) and purified using Sephadex G-25 columns (Boehringer Mannheim). For in situ hybridization, the 5'-biotin-labeled *Nrl*oligonucleotide (5'-AAGATGAGAC AGAACAGGATG-3') was derived from the 3' UTR region in exon 3 [26], and was synthesized by Midland Certified Reagent Co. (Midland, TX).

Experimental animals: All experimental and animal care procedures were in compliance with the principles of the American Physiological Society, the NIH Guide for the Care and Use of Laboratory Animals and Maintenance (NIH publication No. 85-123, 1985) and were approved by the Institutional Animal Care Committee of the University of Houston. Rats were housed in a room maintained at 22±1 °C with a 12:12 hr light dark cycle and cage illumination of 5-10 lux as described [27]. Pregnant female Long-Evans hooded rats (Harlan Sprague Dawley, Indianapolis, IN) were monitored daily for birth. Upon giving birth, postnatal day 0 (PN0), litters were culled to eight pups. Only female rats were used in experiments.

Light and electron microscopy procedures: The histological procedures were conducted essentially as described [28]. Briefly, the animals were sacrificed by decapitation 2 h after light onset. The eyes were removed and fixed by immersion fixation using 3% glutaraldehyde, 2% paraformaldehyde and 0.1% $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4). The tissue was embedded in Spurr's epoxy medium. Thin sections were stained with Toluidine Blue and examined using a BH2 Olympus microscope (Leeds Instrument, Inc., Irving, TX) while ultra-thin sections were stained with 3.5% uranyl acetate and Reynold's lead citrate and examined using a JEOL 100-C transmission electron microscope (Tokyo, Japan).

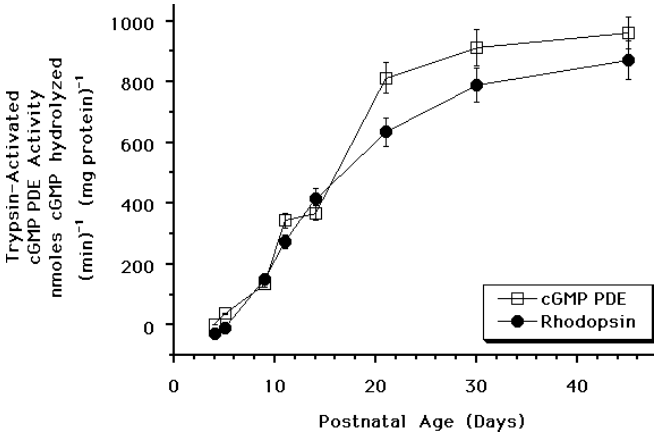

Figure 2. Trypsin-activated cGMP PDE activity and rhodopsin content during rat retinal development. Trypsin-activated cGMP PDE activity and rhodopsin concentration were determined as described in Methods. The values for trypsin-activated PDE, assayed in triplicate, represent the mean±SEM for 4-7 pairs of retinas per age and are expressed as nmoles cGMP hydrolyzed per minute per mg protein. The rhodopsin values represent the mean±SEM for 5-7 retinas per age and are expressed as nmole rhodopsin per mg dry weight.

Whole retinal cGMP PDE activity assay: Trypsin-activated cGMP PDE activity was measured according to the procedure described by Srivastava et al [29]. Briefly, aliquots of the retinal homogenate were treated with trypsin (0.1 mg/ml) for 5 min at 4 °C to remove the two inhibitory γ subunits of the PDE [30]. The reaction was terminated with soybean trypsin inhibitor. Aliquots of homogenates containing 2 µg total protein were assayed in the presence of 500 µM cGMP at 30 °C. All rates of hydrolysis were linear with respect to time. Assays were terminated by boiling and then treated with Crotalus atrox venom for 15 minutes at 30 °C to convert the 5'-nucleotide to the nucleoside. Samples were chromatographed, non-adsorbed nucleoside was collected, scintillation cocktail was added, and samples were counted. Protein concentration was determined using the Bradford assay. The values for trypsin-activated PDE, assayed in triplicate, represent the mean±SEM (standard error of the mean) for 4-7 pairs of retinas per age and are expressed as nmoles cGMP hydrolyzed per minute per mg protein.

Rhodopsin measurements: All rats were dark-adapted overnight and sacrificed by decapitation. The neural retinas were removed rapidly and assayed for rhodopsin content. The entire procedure was carried out under dim red light (wavelength >650 nm) according to the procedures described by Fox and Rubinstein [31]. Briefly, rhodopsin was extracted from each of 4-8 neuroretinas per age with 2% Emulphogene BC-720 (Gaf Corp., Wayne, NJ). The pre-bleach and post-bleach spectra were taken from 350 to 700 nm. The absorbance of rhodopsin at its peak wavelength (497-500 nm) was obtained. A separate group of rats were utilized to measure retinal dry weight. The rhodopsin values represent the mean±SEM for 5-7 retinas per age and are expressed as nmole rhodopsin per mg dry weight.

RNA preparation and Northern blot analysis: All rats were dark-adapted overnight and sacrificed by decapitation just at light onset for the following reason. In dark-adapted, compared to light-adapted, retinas: (i) the *c-fos* expression is almost exclusively in photoreceptors, (ii) *junD* is shown to be expressed in photoreceptors, (iii) opsin expression is maximal, and (iv) minimal variations are observed due to diurnal expression of opsin and *c-fos* mRNA [8,23,25,32]. Retinas (n = 2-8 retinas per age per blot) were excised rapidly, frozen in liquid nitrogen and stored at -80 °C until used. Total RNA was prepared according to the published procedure [33]. Samples of total retinal RNA (10 µg) were denatured and separated on a 1% agarose gel containing 10% formaldehyde. After electrophoresis, RNA was transferred to Hybond-N nylon membranes (Amersham Life Science Inc., Arlington Heights, IL) by electroblotting and cross-linked to the membrane with UV

light. Blots were prehybridized and hybridized according to standard methods [34] and exposed to Fuji RX film with an intensifying screen at -80 °C. After probing for different cDNAs, the blots were stripped and rehybridized with the 18S rRNA oligonucleotide probe.

The optical densities of the β-PDE, 1.7 kb opsin, *c-fos*, *junD* and the three *Nrl* mRNA transcripts and of 18S rRNA on the blot were determined with the BioImage Scanner (Millipore Corp., Bedford, MA). The mRNA expression was normalized for loading variance by comparing the respective mRNA tran-

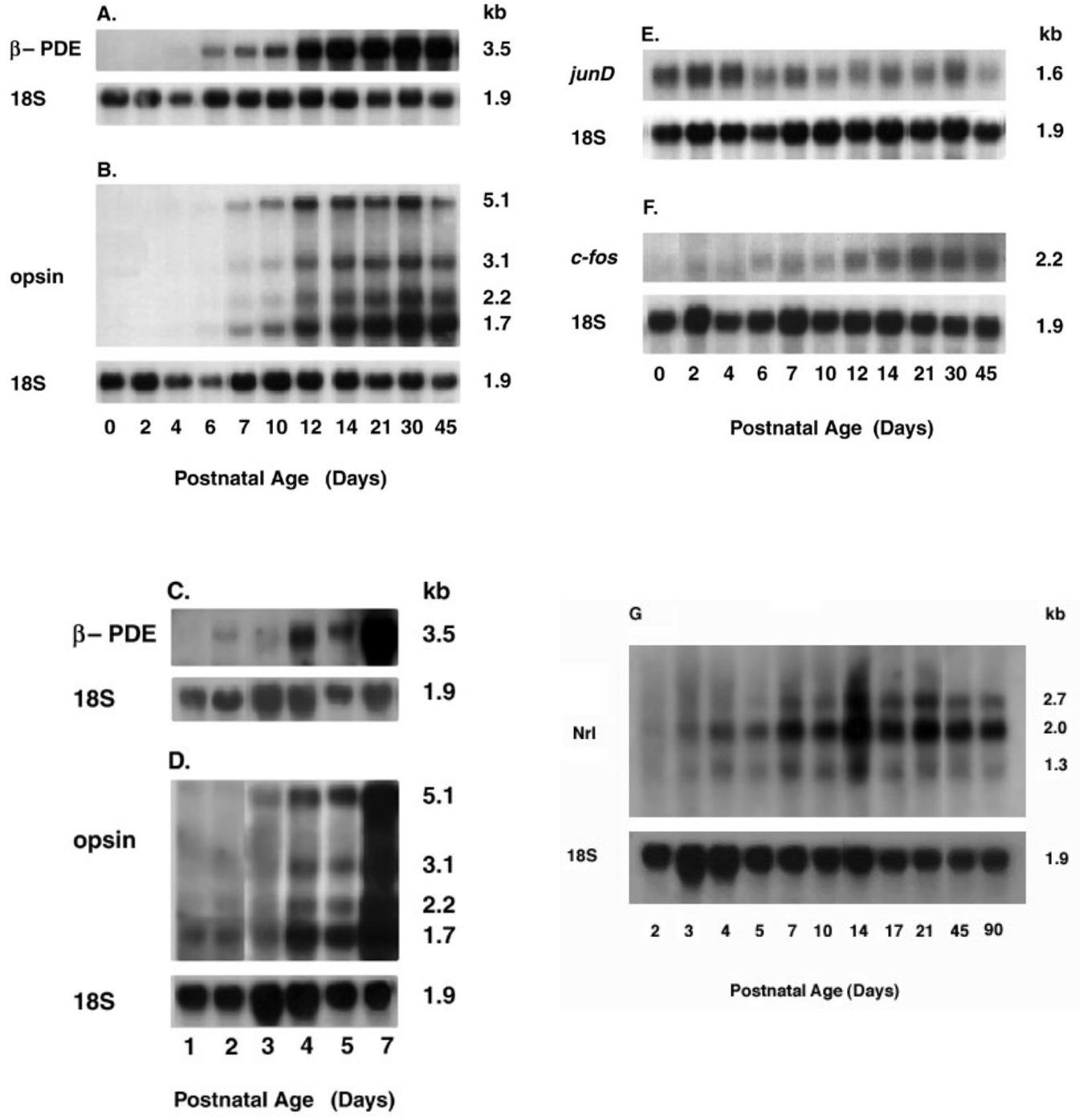

Figure 3. Developmental expression of mRNAs for rod-specific β subunit of cGMP PDE, opsin, *junD*, c-*fos* and *Nrl* in rat retinas. Total retinal RNA from PN0-45 dark-adapted rats was isolated and steady-state levels of (A and C) β-PDE (B and D) opsin, (E) *junD*, (F) *c-fos*, (G) *Nrl* mRNA were determined using Northern blot hybridization and image analysis as described in Methods. 10 µg of RNA was loaded per well. To detect and quantify the transcripts at earlier ages, however, it was necessary to increase the exposure times for (C) β-PDE and (D) opsin. Five to seven blots, from independent samples, were analyzed at each age. Compared to the expression in dark-adapted developing and adult retinas, the expression of β-PDE, *junD*, and the three *Nrl* transcripts in light-adapted retinas was not different while opsin and c-*fos* expression were significantly decreased (data not shown).

script optical density values with that of 18S rRNA. Five to seven blots, from independent samples, were analyzed at each age. The mean±SEM optical densities were determined for each age, normalized to the maximal level of gene expression and then plotted as percent of maximal expression per gene±SEM.

In situ Hybridization of Nrl*:* For in situ hybridization, whole eyes from dark- and light-adapted rats were removed and fixed in 4% paraformaldehyde (in 0.1 M sodium cacodylate buffer, pH 7.2) for 1 h at 4 °C. The eyecups were fixed for an additional hour after removing the lens and vitreous. The cornea and surrounding peripheral sclera were detached and the eyecups were cryoprotected in 30% sucrose overnight at 4 °C before quick-freezing in Tissue Freezing Medium (Electron Microscopy Sciences, Washington, PA) using liquid nitrogen. Cryostat sections of 10 µm thick at each age were mounted on slides and stored at -80 °C until they were used.

The sections from all examined ages were processed together in batches such that all slides were treated identically. Prehybridization and hybridization procedures were carried out according to Protocol 2 from Amersham Life Science, Inc (In situ hybridization manual). The sections were hydrolyzed in 0.02 M HCl, treated with 0.5 µg/µl proteinase K solution at 37 °C and prehybridized for 1 h at room temperature in 2X SSC, 0.05% Tween-20, 2% non-fat milk, 4% normal goat serum (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA) and 1% BSA. Each series of sections was dehydrated in graded alcohol and then hybridized overnight at 55 °C in the solution containing 2X SSC, 5% dextran sulfate, 0.2% non-fat milk and 50% formamide and 25-200 ng/ml of the 5'-biotinylated *Nrl* probe. After hybridization, slides were washed sequentially and incubated at 4 °C overnight with 4 nm (LM grade) colloidal gold-streptavidin (Jackson ImmunoResearch Labs, Inc.) diluted in PBS-Tween to a final absorbance at 520 nm of 0.01-0.10. Sections were stained with silver enhancement reagent (Amersham Life Science, Inc.) according to manufacture's instructions, washed in water, dehydrated in graded alcohol and mounted. Since a serial dilution of the *Nrl* probe was utilized, a semi-quantitative comparison of retinal expression of *Nrl* at each age was obtained for each batch of identically treated slides.

Statistical analysis: All data are presented as means±SEM (standard error of the mean). Data were analyzed using the appropriate analysis of variance and Fisher's Protected Least Significant Difference posthoc comparisons, according to procedures provided by StatView statistical package (Abacus Concepts, Inc., Berkeley, CA). All statistical analyses were performed on untransformed data and the difference between groups was regarded as significant if $p < 0.05$.

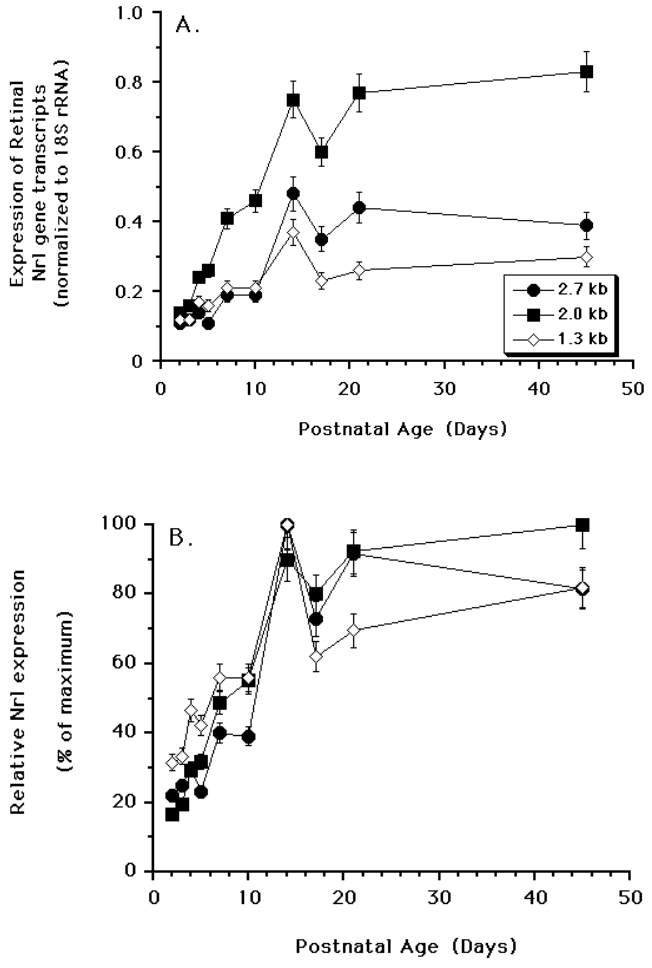

Figure 4. Quantitative analysis of developmental expression of mRNAs for *Nrl* transcripts in rat retinas. The optical density of the 2.7, 2.0, and 1.3 kb *Nrl* mRNA transcripts were determined and normalized for equal loading by comparison with 18S rRNA. The optical densities were determined at each age for each transcript and (A) plotted as relative percent of gene expression±SEM and (B) normalized to the maximal level of gene expression, then plotted as maximal expression per transcript±SEM. Five to seven blots, from independent samples, were analyzed at each age.

RESULTS

Light and electron microscopy of the developing rat retina: The morphological development of Long-Evans hooded rat retinas, especially the ROS, was examined using light and electron microscopy. On PN4, no ROS were observed in the rat retina. On PN5, the beginning of rudimentary ROS discs were detected with the electron microscope (data not shown) while on PN6 numerous ROS discs were clearly visible (Figure 1). By PN7, the ROS were visible at the light microscopic level. At PN14 and PN21, ROS were significantly increased in length such that they were approximately 50% and 80% of their final adult length obtained by PN45, respectively (Table 1). A similar pattern of ROS development was observed in the peripheral retina: albeit delayed by two days. This pattern of retinal development we observed is similar to that reported for the albino rat [35,36].

Development of cGMP PDE enzyme activity and rhodopsin concentration: Since cGMP-PDE and rhodopsin are good markers for rod photoreceptor differentiation, we examined the trypsin-activated cGMP PDE activity and rhodopsin concentration during retinal development. Both trypsin-activated cGMP PDE activity and rhodopsin were first detected on PN5, increased relatively linearly until PN30, and reached their maximal levels by PN45 (Figure 2, Table 1). The onset of

basal cGMP PDE activity also occurred at PN5 and followed a similar pattern of development, although its activity was markedly lower at all ages relative to the trypsin-activated enzyme (Srivastava and Fox, unpublished results). The rhodopsin concentration reached one-half of its maximal level of 1.25±0.09 nmoles rhodopsin per mg dry weight on PN14 (Figure 2). Trypsin-activated cGMP PDE activity reached one-half of its maximal level of 960±53 nmoles cGMP hydrolyzed per min per mg protein between PN15-16 (Figure 2).

Developmental expression patterns of β-PDE, opsin, junD, c-fos *and* Nrl*:* To demonstrate the temporal relationships between rod-specific genes and their cognate regulatory transcription factors, we determined the expression patterns of two rod-specific genes, β-PDE and opsin, and three AP-1 family genes that are expressed in the ONL: *c-fos*, *junD*, and *Nrl* [23-25,32]. Figure 3 presents representative Northern blots of RNA isolated from PN0-45 dark-adapted rat retinas for these genes as well as 18S rRNA (used as control for RNA loading). A 3.4 kb transcript was the only β-PDE species of mRNA detected (Figure 3A). Four transcripts of opsin, varying in size from 1.7 to 5.1 kb, were identified in PN7-45 rats after 3 h of exposure with intensifying screens (Figure 3B). After longer exposure times, signals above background level were detected at PN1 and PN2 for opsin and β-PDE, respectively, though the levels were low compared to the adult levels (Figure 3C and Figure 3D). Similar to the mouse [37], the relative intensity of all the opsin transcripts was relatively constant throughout development. Therefore, the most abundant 1.7 kb transcript was used for all quantitative measurements. The expression of 1.6 kb *junD* transcript that was detected at all ages examined decreased during development (Figure 3E). A 2.2 kb transcript of *c-fos* was detected first at PN1 and its expression increased during retinal development (Figure 3F).

Three *Nrl* transcripts of 2.7 kb, 2.0 kb, and 1.3 kb were found in rat retinas (Figure 3G). All three transcripts were expressed at low but detectable levels on PN0-2 (data for PN0 not shown) and their expression increased rapidly during development (Figure 4). As illustrated in Figure 4A, the 2.0 kb transcript was approximately twice as abundant as either the 2.7 or 1.3 kb transcripts. Relative to their maximal level of expression (90-100%) that occurred initially on PN14, the transcripts from dark-adapted rats were at ~20-30% on PN3 and 40-60% on PN5-7 (Figure 4B; Table 1). The expression of the three *Nrl* transcripts in light-adapted developing and adult rats (data not shown) were similar to those in the dark-adapted rats (Figure 3G and Figure 4).

β-PDE and opsin expression were low at PN2 (<1% of adult maximum) and increased over 2.0 log units during development (Figure 5). Relative to their maximal level of expression at PN45, expression for both genes was at ~10% on PN7, at 50% between PN12-14 and at 80-90% on PN21 (Figure 4; Table 1). The mRNA expression for both genes preceded the detection of their respective proteins by 2-3 days (compare Figure 2 and Figure 5). The expression of *junD* was highest at PN0-2, decreased rapidly during the next ten days and reached a steady-state level of ~30% of its maximum on PN14. In marked contrast, *c-fos* expression was low during early postnatal development and increased to a maximal level of expression at PN45. Relative to its maximal level of expression, *c-fos* expression was at ~6% on PN0-2, at 50% on PN14 and at 60-70% on PN17 (Table 1). Thus, the expression of *c-fos* and *Nrl* preceded the expression of both rod-specific

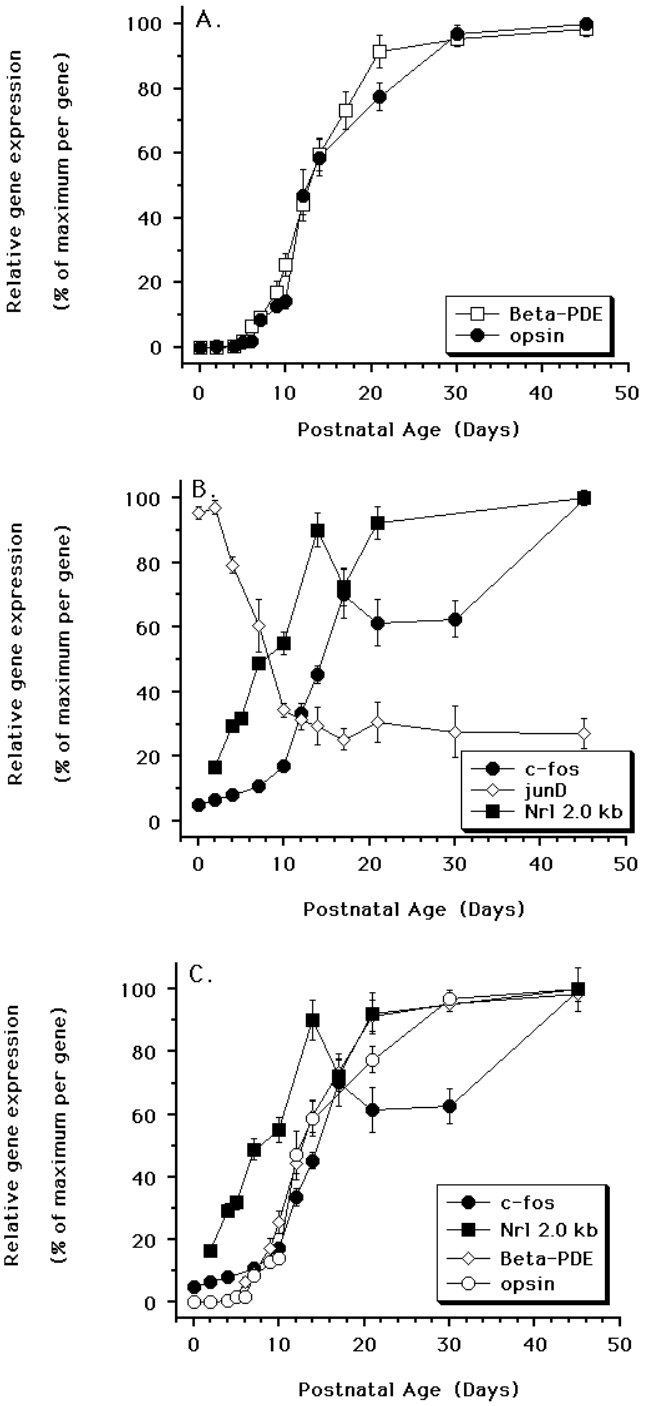

Figure 5. Quantitative analysis of developmental expression of mRNAs for rod-specific β-subunit of cGMP PDE, opsin, *junD*, c-*fos*and 2.0 kb *Nrl* in rat retinas. The optical density of the β-subunit of PDE, 1.7 kb opsin, *c-fos* and *junD*, and 2.0 kb *Nrl* mRNA transcripts were determined and normalized for equal loading by comparison with 18S rRNA. The optical densities were determined for each age, normalized to the maximal level of gene expression, then plotted as percent of maximal expression per gene±SEM. Five to seven blots, from independent samples, were analyzed at each age.

genes by 1-2 days (compare Figure 3, Figure 4, and Figure 5; only data for the 2.0 kb *Nrl* transcript is presented). Interestingly, using in situ hybridization, Ohki et al. [25] did not detect *c-fos* expression in rat retina until after PN10. Compared to the expression in dark-adapted retinas (Figure 3 and Figure 5), the expression of β-PDE and *junD* in light-adapted retinas was not different while opsin and *c-fos* expression were significantly decreased (data not shown).

Spatiotemporal Expression Pattern of Nrl *During Retinal Development: Nrl* has previously been implicated in regulation of rhodopsin expression [14,18]. Therefore, we studied the spatiotemporal expression pattern of the *Nrl* gene in developing and adult dark-adapted and light-adapted rat retinas using in situ hybridization. Figure 6 presents representative micrographs from light-adapted rat retinas from a series of slides whose sections were all incubated in the same concentration of Nrl probe. At PN5, *Nrl* expression was just above the background level of detection and it localized to the inner nuclear layer (INL) and ganglion cell layer (GCL) (Figure 6A). Expression in all nuclear layers increased during development such that, at PN10 (Figure 6B) and PN15 (Figure 6C) expression appeared equal in all nuclear layers. The maximal level of *Nrl* expression was reached at PN15 (Figure 6C; Table 1). During retinal development, the highest level of *Nrl* expression shifted from the GCL and INL to the ONL (Compare Figures 6A, B with Figures 6C, D and E). Figure 6F is a PN45 retina hybridized with sense probe, which showed no signal at all. Similarly, no signal was observed on slides incubated in the absence of *Nrl*, colloidal gold-streptavidin or silver enhancement reagent (data not shown). There were no differences in retinal *Nrl* expression between light-adapted and dark-adapted retinas at any age (data not shown).

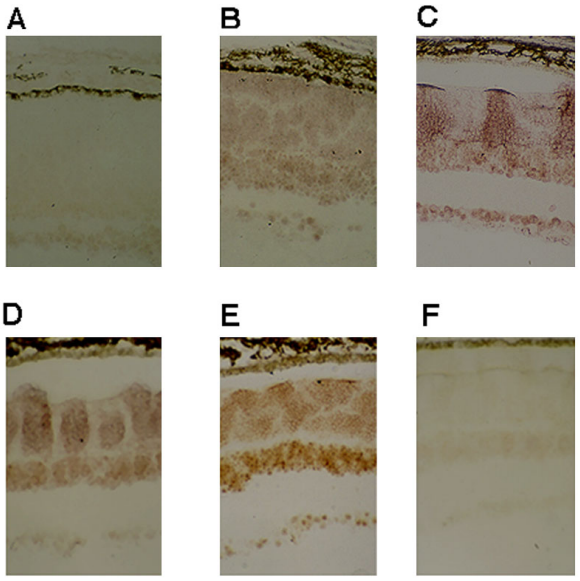

Figure 6. Determination of the spatiotemporal expression of the *Nrl* gene in developing rat retinas using in situ hybridization. The expression of *Nrl* gene in light-adapted rat retinas at (A) PN5, (B) PN10, (C) PN15, (D) PN21, and (E) PN45 was detected using biotin-labeled oligo probes and immunogold-silver enhancement techniques. The *Nrl* gene expression was detectable at PN5, mostly in the inner nuclear (INL) and ganglion cell (GCL) layers. The expression of *Nrl* increased during development, reaching a peak at PN15. Note that the pattern of *Nrl* expression shifted from the proximal to distal or outer retina throughout development. Figure 6F is a PN45 retina hybridized with sense probe, which showed no signal at all. Similarly, no signal was observed on slides incubated in the absence of *Nrl*, colloidal gold-streptavidin or silver enhancement reagent (data not shown). The expression of *Nrl* in light-adapted developing and adult retinas was not different from that in dark-adapted retinas (data not shown).

DISCUSSION

The objective of this study was to determine the temporal relationships between rod morphological and functional development, rod-specific gene expression, and expression of the bZIP transcription factor genes, *c-fos, junD* and *Nrl*, in pigmented rat retinas. There are three major conclusions. First, the developmental pattern of cGMP-PDE activity and rhodopsin accumulation paralleled the expression of β-PDE and opsin mRNA, and the onset of gene expression preceded the appearance of the proteins by 2-3 days. Second, the *c-fos* and *Nrl* expression preceded β-PDE and opsin mRNA expression by 1-2 days. Thereafter, the transcripts for these four genes were detected continuously and in all the developmental stages examined. In contrast, the expression of *junD* was highest at birth and decreased to a steady-state level during neonatal development. Third, *Nrl*expression was detected first in the distal post-mitotic retina and then in all nuclear layers during retinal development. Maximal expression shifted from the GCL to ONL as the neural retina matured.

This is the first study to determine the developmental pattern of trypsin-activated retinal cGMP-PDE activity in any species and of rod-specific β-PDE mRNA in the rat. Rat retinal basal and trypsin-activated cGMP PDE activity were detected first on PN5. This is coincident with the appearance of rod outer segments (ROS) but 2-3 days after the initial expression of β-PDE mRNA. The expression of β-PDE mRNA, cGMP-PDE activity, and ROS length reached their adult and maximal values by PN45. The rod cGMP-PDE immunoreactivity was first detected at PN5, with maximum staining reached at PN10 [9]. This apparent discrepancy in the timing of maximal activity and expression may be due to the use of a single high concentration of antibody in the immunohistochemistry study. The ability of trypsin to activate basal cGMP-PDE activity at PN5 suggested that both γ subunits of this heterotetrameric protein were bound to the catalytic α and β subunits when its initial activity was detected [30]. A similar increasing pattern of basal retinal cGMP-PDE activity was observed in developing mice [38,39]. The onset and developmental profile of ROS elongation, rhodopsin accumulation and increase in opsin mRNA in our pigmented rat retinas are similar to those found for albino rat and mouse retinas [37,40,41].

The tissue- and cell type-specific expression of genes requires the interaction of various DNA-binding regulatory proteins to their cognate cis-sequence elements in the promoter region [42,43]. The presence of conserved AP-1 and/or NRE as well as other DNA-sequence elements in the promoter regions of β-PDE, opsin and γ-transducin suggests that multiple

transcription factors are involved in the regulation of these rod-specific genes during rod development and maintenance of rod function. Significant regulatory activity associated with AP-1 and/or NRE sites is observed for β-PDE [18] and rhodopsin [12].

The activity of the rhodopsin promoter is modulated by *Nrl*[14,18] and Crx in a synergistic manner [16]. Crx appears to bind to three different sites, Ret 1, Ret 4 and BAT-1, in the rhodopsin promoter [12,16]. These sites are close to NRE in rhodopsin promoter [12,16]. bZIP proteins like Nrl bind to DNA as homodimers or heterodimers. Moreover, heterodimerization enhances the sequence site specificity [20-22]. The identity of Nrl's partner in rod-specific gene regulation is unknown, although it forms dimers with several bZIP proteins in vitro [21]. The heterodimerization of Nrl in vivo will depend on the availability and expression of specific bZIP proteins. In addition to Nrl, *c-fos* and *junD* are expressed in developing and adult rat photoreceptors [23-25], but *c-jun* is not expressed in ONL [23]. The expression pattern of *junD* was opposite to the expression of rod-specific genes and of *c-fos* and *Nrl*, indicating that it may not be involved in the activation of rod specific genes at AP-1 and/or NRE sites. Our results show that the onset and developmental pattern of *c-fos* and *Nrl* expression parallels that of rod-specific genes, β-PDE and opsin. Using dark-adapted rats, *c-fos* expression was localized almost exclusively to the photoreceptors [25,32]. Moreover, and importantly, the early expression of *c-fos* should be independent of light since the first electroretinographic (ERG) response is not detectable until PN12-13, the age at eye opening [35, 40]. Our studies in the developing and adult retina are the first to demonstrate that *Nrl* and junD retinal expression are not regulated by light. These results also confirm that *c-fos* expression in developing and adult retinas is light-regulated, while *junD* expression in the adult retina is not influenced by light [23,25,32,44].

Interestingly, adult *c-fos* deficient mice (*c-fos*-/-), compared to either *c-fos*+/+ littermates or control mice, have a 22% loss of rod photoreceptor cells in the central and peripheral retina while the number of cones is unchanged (He, Campbell and Fox; unpublished data). A search of the literature and Genbank revealed that no AP-1 like sites are present in the promoter region of cone-specific opsins or PDEs [45-49]. This may explain a normal number of cones in the *c-fos* -/- mice. Our morphometric data on *c-fos*-/- mice is consistent with the results presented by Hafezi et al. [50] and with the observations of Reme and co-workers (personal communications) that adult *c-fos*-/- mice have a 20% decrease in rhodopsin content as well as decreased and delayed ERG a-waves and b-waves.

In summary, our findings suggest that Nrl and c-Fos play an important role in the regulation of the rod-specific gene expression in developing and adult rod photoreceptors. Since c-Fos homodimers can not bind DNA, we hypothesize that AP-1 like sequence elements in promoters of rod-specific genes bind to heterodimers of c-Fos and Nrl and/or to Nrl homodimers. Our observation that not all the rod photoreceptor cells are lost in *c-fos*-/- mice and that *c-fos* expression is decreased in ONL during light onset suggests that other bZIP proteins may also form heterodimers with Nrl to transactivate gene expression at AP-1 sites. Further investigations are in progress to identify retinal Nrl-interacting proteins that specify expression of genes in rod photoreceptors.

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