Vol. 191, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 26, 1993 Pages 76-82

SHARED NUCLEAR PROTEIN BINDING SITES IN THE UPSTREAM REGION OF THE RAT OPSIN GENE

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Received January 5, 1993

DNase I protection and gel retardation assays have identified two sequences 5' to the rat opsin gene that interact with nuclear proteins from retina but not from a number of neuronal and non-neuronal tissues. These sites, Ret 2 and Ret 3, are over 1200 base pairs apart but seem to interact with the same protein(s). Synthetic oligonucleotides corresponding to each site were able to inhibit complex formation in a gel retardation assay using an oligonucleotide corresponding to the other site. The proteins binding to the Ret 2 and Ret 3 sites co-eluted in both ion exchange and gel filtration chromatography. The protein(s) were also present at adult levels at birth, suggesting that they may represent differentiation products expressed in the proliferating retinal epithelium.

Rod photoreceptors are among the last neuronal cell types to form in the mammalian retina. In rats, the major period of rod photoreceptor generation begins at about E19 (birth = E22), reaches a peak at postnatal day 1 (PN1) to PN2 and is over by PN8 (1, 2, 3). Lineage studies using retroviral markers have shown that rod photoreceptors originate from the same neuroepithelial progenitors as other retinal neurons and glia, and that the decision to become a rod is not made until or just after the final mitosis (4, 5).

Expression of the rod visual pigment protein opsin can be detected immunologically on rods within approximately 48 hr. after the cells become postmitotic (6, 7, 8). Opsin RNA has been detected at birth in rats by both transcription run off and filter hybridization experiments, indicating that the developmental expression of opsin is regulated primarily at the transcriptional level (9).

Production of transgenic mice using the mouse or bovine opsin gene promoter region and a LacZ reporter gene has shown that the major *cis*-acting elements regulating the developmental and tissue specific expression of opsin are located in the 2.0kb region 5' to the transcription start site (10, 11). Sequence comparisons among opsin genes of several mammals reveal two highly conserved regions in the 5' untranscribed regions. One consists of approximately 300bp immediately upstream of the transcription start site and the other is a ~100bp region approximately 1.5kb upstream. The first 300bp upstream are both necessary and sufficient for the correct tissue and developmental expression of the marker gene. However, sequences in more upstream regions appear to be needed for higher and more uniform levels of expression, and more precise temporal expression.

0006-291X/93 $4.00

We have previously reported the identification of a DNA-binding protein complex that binds to a specific site, termed Ret 1, in the proximal promoter of the rat opsin gene (12). This binding activity is both tissue-specific and developmentally regulated, making it a strong candidate for controlling opsin expression. Here we report the characterization of two additional binding sites in the proximal and distal regions of the opsin upstream sequence that are distinct from the Ret 1 site and we present evidence that the two sites can interact with the same nuclear protein(s).

MATERIALS AND METHODS

Isolation of rat opsin upstream sequences. Clones containing the rat opsin gene were isolated from a genomic library as described before (12) a 2.1kb SacI/ECoRI fragment containing the transcription start site and 2020bp of upstream sequence was subcloned into a pBS M13+ plasmid. This subclone was sequenced on both strands using the dideoxy chain termination method (13).

Preparation of nuclear extracts. Nuclear extracts were prepared from fresh tissue homogenates by the method of Dignam et al (14). Leupeptin, pepstatin A, aprotinin and phenylmethyl sulfonyl fluoride were added to inhibit protease activity. Protein concentrations were determined using the Bio-Rad protein assay system with γ-globulin heavy chain as protein standard.

DNase I footprinting analysis. DNase I footprinting analyses were performed as described (12).

Synthesis and labeling of oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystem 391 DNA synthesizer, purified by SDS-polyacrylamide electrophoresis (SDS-PAGE) and annealed. The two complementary oligonucleotide strands were synthesized to have one extra nucleotide at the 3' end after annealing. This was used for labeling with [^{32}P]dNTP using Klenow fragment of DNA polymerase I.

Gel retardation analysis. Nuclear extracts were incubated with $2x10^4$ cpm (specific activity 10^8 cpm/μg) end-labeled oligonucleotide in 50 mM Tris-HCl (pH7.6), 50 mM NaCl, 10% glycerol, 1 mM DTT, 200 μg/ml poly(dI:dC) in a final volume of 25 μl for 30 minutes on ice and resolved by PAGE and autoradiographed. The X-ray film densities were digitized using a Hoefer G370 densitometer for quantitative comparisons. For competition experiments unlabeled and labeled oligonucleotides were added together. Since all oligonucleotides used were of similar length, excesses were calculated on the basis of weight.

Ion exchange chromatography. 2.5 mg bovine retina nuclear extract (in 20 mM HEPES (pH7.9), 100mM KCL, 20% glycerol, 1mM EDTA, 12.5 mM $MgCL_2$ and 1mM DTT) was loaded on a mono Q column in a sample volume of 0.5 ml. Proteins were eluted in the loading buffer with a linear salt gradient of 0.1 to 1.0 M KCl at a flowrate of 0.5 ml/min. Fractions of 1 ml each were collected and 20 μl from each fraction was used for gel retardation assays as above except in a final volume of 50 μl.

Gel filtration chromatography. About 2.5 mg bovine retina nuclear extract was loaded onto a 30 ml Superose 12 column in a sample volume of 0.5 ml. Proteins were eluted in the loading buffer at a flowrate of 0.5ml/min. Fractions of 1 ml each were collected and 15 μl from each fraction was taken for gel retardation assays. The column was calibrated with molecular weight marker proteins thyroglobulin, bovine serum albumin dimer and monomer, and ovalbumin.

RESULTS

The sequence of the 2.1kb ECoRI/Sac I subclone is shown in figure 1. The transcription start site, the TATA box and the Ret 1 site have all been defined previously (12). The shaded areas

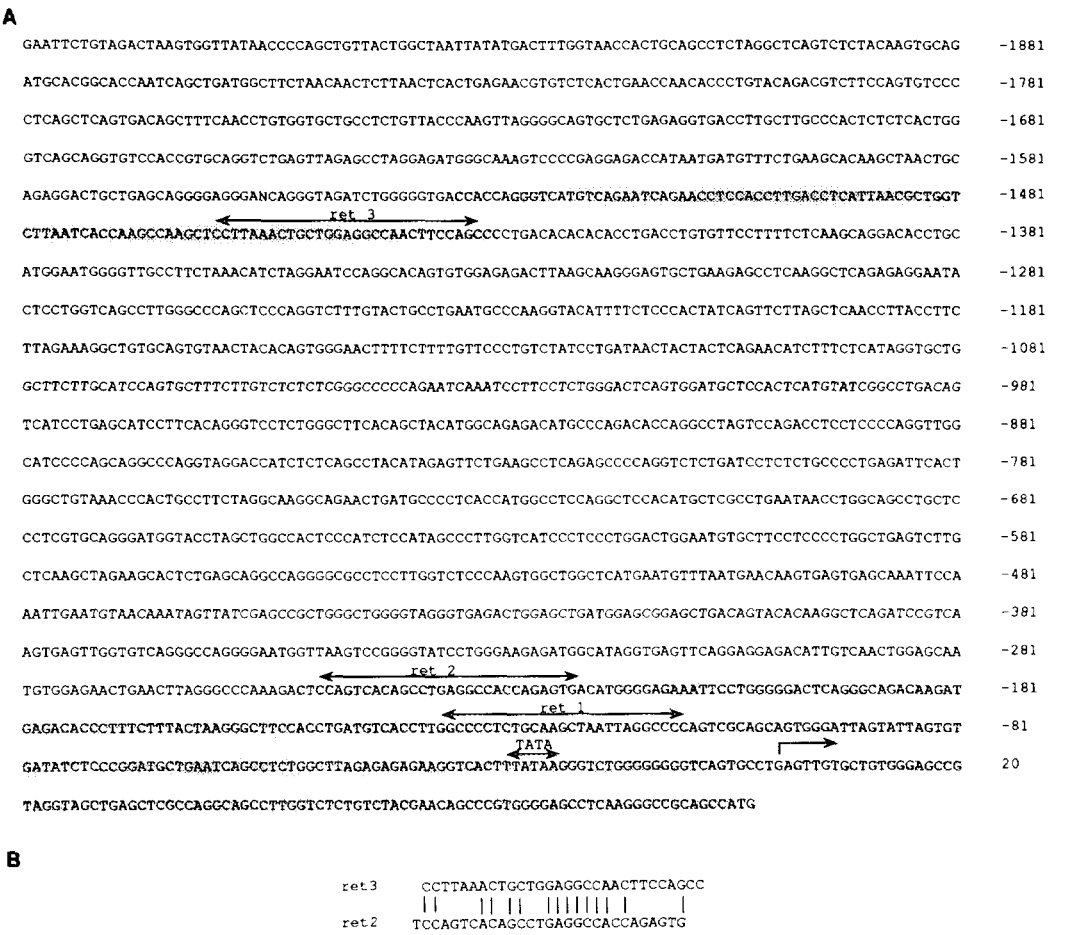

Figure 1. A. 5' upstream sequence of rat opsin gene. Arrowed bars show the sequences of characterized protein binding sites and the TATAA sequence. The bent arrow shows the transcription start site. The sequence ends with the translation start codon. Numbers refer to positions from the transcription start site. Shaded areas are those with high homologies between rat, mouse, human and bovine sequences. **B.** Sequences of Ret 2 and Ret 3, with the parallel vertical bars showing identical nucleotides.

represent regions of homology between the rat sequence and those of cow, mouse and human (10). DNase I protection assays were performed using fragments of this subclone. Among the regions protected by retinal nuclear proteins were the two shown in figure 2 which have been named Ret 2 and Ret 3. Ret 2 is in the proximal region and extends from -255bp to -222bp. Ret 3 is in the distal region of homology and extends from -1460bp to -1432bp. As shown in figure 1B, the sequences of the two protected regions show 50% homology overall, with six identical base pairs in the middle.

Oligonucleotides corresponding to the Ret 2 and Ret 3 regions were synthesized and used in gel retardation experiments. As shown in figure 3, each oligonucleotide formed a specific DNA-protein complex with nuclear extract from retina but not from any other tissues (s in figure 3). In

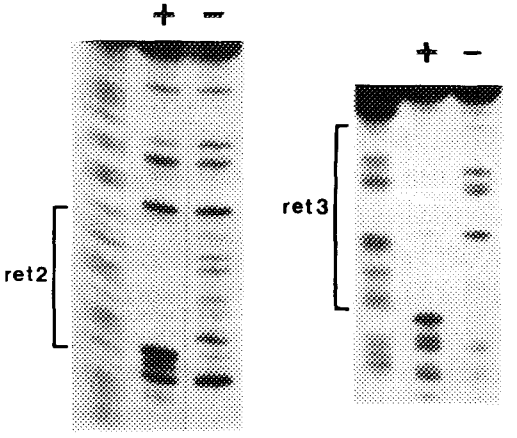

Figure 2. DNase I footprinting of Ret 2 and Ret 3. The first lane shows the G+A reactions from Maxam and Gilbert sequencing of the coding strand. "+" shows protection with retina nuclear extract and "-" shows protection with BSA as control. The regions protected by the retina extract are marked with black bars.

addition, a faster moving complex was formed in retina extracts as well as other tissue extracts (**ns** in figure 3), although it is not detected in all experiments with all tissue extracts. This band is judged to be non-specific because it could not be eliminated even with large excess of unlabeled oligonucleotides (see below). In addition, for Ret 3, another binding complex (* in figure 3) is detected sometimes in tissue extracts other than rat retina. The retina-specific binding activities detected with each oligo-nucleotide were present at birth and showed little change during postnatal development, as shown for Ret 3 in figure 3, right panel.

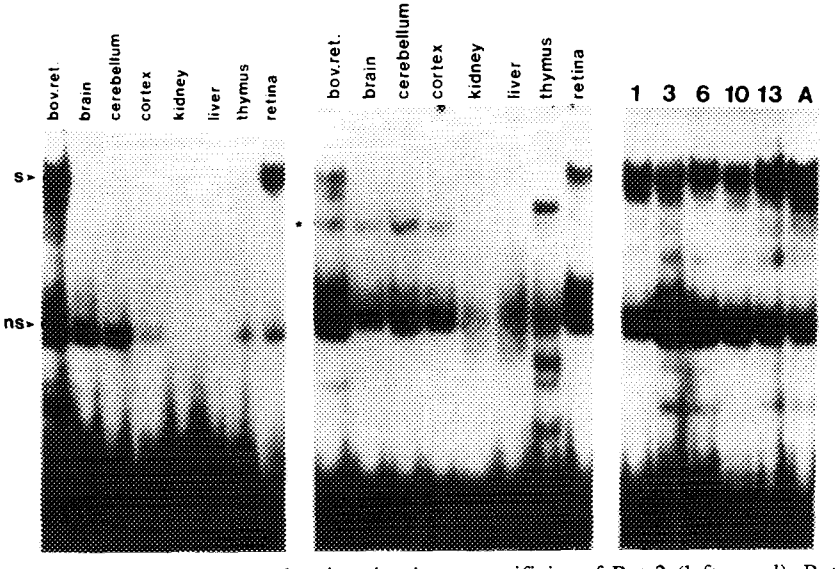

Figure 3. Gel retardation assays showing the tissue specificity of Ret 2 (left panel), Ret 3 (middle panel) and the binding activity of Ret 3 at postnatal days 1, 3, 6, 10, 13 and adult (right panel). All lanes were loaded with equal amounts of nuclear extract from the different tissue sources and developmental stages.

Because of the similarities in the Ret 2 and Ret 3 sequences, and the similar pattern seen in the gel retardation, the possibility that the same protein(s) bind to the two sites was tested directly. Gel retardation assays were performed in which the interaction of radiolabeled Ret 2 or Ret 3 with rat retinal nuclear extract was inhibited by unlabeled Ret 2, Ret 3 or other oligonucleotides. The upper panels of figure 4A and 4B show individual experiments with Ret 2 and Ret 3 respectively. The combined results from multiple experiments are plotted in the lower panels of figure 4A and 4B. A ten-fold excess of Ret 2 was sufficient to reduce binding to label Ret 2 by 50% and all binding was abolished with a 100-fold excess. Unlabeled Ret 3 also inhibited binding to labeled Ret 2 with a similar potency. Neither Ret 1 nor AP3 significantly reduced binding even with 100 fold excess. Similarly, interaction of labeled Ret 3 with retinal nuclear proteins was inhibited by both unlabeled Ret 3 and Ret 2 but not by Ret 1 or AP3 (figure 4B).

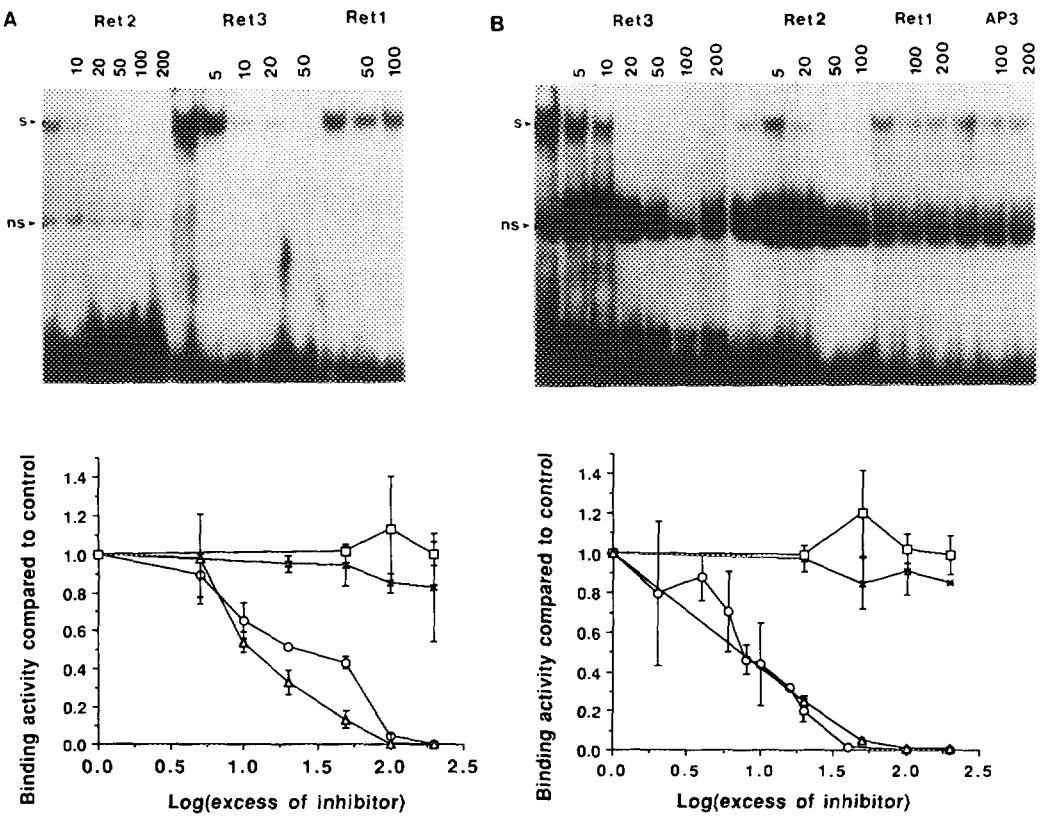

Figure 4. Gel retardation assays of Ret 2 and Ret 3 with different unlabeled oligonucleotides as inhibitors. **A.** *Upper panel* shows a gel retardation assay with labeled Ret 2 oligonucleotide; unlabeled Ret 2, Ret 3 or Ret 1 oligonucleotides were added as inhibitors. The amounts (fold excess) of inhibitors added are indicated by numbers above each lane; the unlabeled lanes are without inhibitors. *Lower panel* shows competition curves from several experiments including the one in the upper panel. The intensity of the specific band relative to the lane with no inhibitor is plotted as a function of the amount of inhibitor added. The competing oligonucleotides are Ret 2 (O), Ret 3 (Δ), Ret 1 (✖), and AP3 (❑). Error bars show standard deviations of at least three experiments. **B.** Similar experiments with Ret 3 as the labeled probe. Competing oligonucleotides are Ret 3 (O), Ret 2 (Δ), Ret 1 (✖), and AP3 (❑).

Bovine retinal nuclear proteins were fractionated by FPLC using a MonoQ ion exchange column. When fractions were assayed for the ability to interact with Ret 2 or Ret 3 oligonucleotides, peak activity in each case was eluted with a KCl concentration of 0.56 M (figure 5A). The binding activities also co-eluted from a Superose 12 gel filtration column at a position corresponding to a molecular size of 115 kD relative to the elution position of known globular proteins (figure 5B).

DISCUSSION

In the opsin gene, transgenic mouse studies and sequence homologies between species suggest that two regions of the promoter are important for transcriptional regulation. A 300bp region immediately 5' to the transcription start site is probably responsible for correct tissue and developmental distribution of the opsin gene product and a distal region 1.5 kb upstream has roles

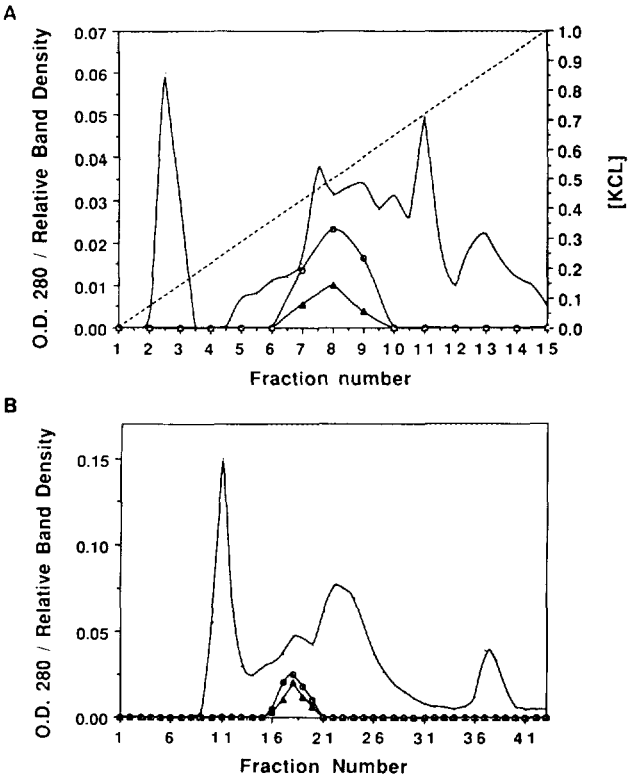

Figure 5. A. Ion exchange chromatography. Autoradiograph of a gel retardation assay using fractions from an ion exchange chromatogram was digitized with a densitometer (Hoefer GS370). The density of each fraction was compared to the preloading material and the ratio was used as the measure for binding activity. The specific binding activity for both Ret 2 and Ret 3 eluted in fractions 7-9. The bell-shaped peaks in this figure show the position and relative binding activity of the active fractions of Ret 2 (O) and Ret 3 (▲). **B.** Gel filtration chromatography. The autoradiograph of a gel retardation assay using the fractions from a gel filtration chromatogram was analysed and the results were plotted the same way as for the ion exchange chromatography. The binding activity for both Ret 2 (O) and Ret 3 (▲) eluted in fractions 16-20, as shown by the bell-shaped peaks.

in increasing transcription level and fine tuning the spatial and temporal distribution. We have now described three nuclear protein binding sites in these regions. We reported earlier the Ret 1 site in the proximal region with a possible role in tissue and developmental regulation of the opsin gene. Here we report two other binding sites which can interact with common protein(s) in vitro. One site is located in the proximal region at -255bp to -222bp, the other is in the distal region at -1460bp to -1432bp. Whether similar interactions can take place in vivo remains to be shown.

It is possible that the two sites we have defined act as enhancer elements. Such elements typically are located far from the promoter and enhancer binding proteins interact with promoter binding proteins by looping out the intervening sequences. It is conceivable that the same DNA binding protein(s) bind to both Ret 2 and Ret 3 sites and interact by forming a homodimer or multimer. If this were the case, then the position of the Ret 2 site in the proximal promoter might be important. Alternatively, the proteins binding to each sequence could interact with other proteins independently, having different or similar regulatory effects.

The activity binding to Ret 2 and Ret 3 is tissue restricted but unlike Ret 1, is not developmentally regulated. This suggests that the temporal and spatial determination of opsin gene expression are, at least in part, separately regulated.

ACKNOWLEDGMENTS

We thank A. LaRue for excellent technical assistance and R. Brown and K. Field for help with the manuscript. This work was supported by NIH grants EY00785 (Core) and NS20483, and by the Darien Lions (Darien, CT) and Research to Prevent Blindness, Inc. The nucleotide sequence presented here was submitted to GenBank. The accession number is L01537.

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