Previous transgenic mouse experiments localized the mammalian rhodopsin gene promoter to a region just upstream of the mRNA start site, and also suggested the existence of a second more distal regulatory region. A highly conserved 100-base pair (bp) sequence which is homologous to the red and green opsin locus control region is located 1.5–2 kilobases upstream of the rhodopsin gene (depending on the species). In order to test the activity of this 100-bp region, transgenic mice were generated with bovine rhodopsin promoter/\textit{lacZ} constructs which differed only by the presence or absence of the sequence. Of 11 lines generated, all demonstrated photoreceptor-specific expression of the transgene, but the lines with the putative regulatory region showed significantly higher expression. Additional transgenic lines in which the region was fused to a minimal heterologous promoter did not show transgene expression in the retina.

Gel mobility shift and DNase I footprint assays demonstrated that bovine retinal nuclear extracts contain retina-specific as well as ubiquitously expressed factors that interact with the putative regulatory region in a sequence-specific manner. These results indicate that the 100-bp sequence can indeed function in vivo as a rhodopsin enhancer region.

The neural retina is a specialized part of the central nervous system which both transduces light energy into neurochemical signals and begins initial information processing. It has a complex laminar structure in which there is segregation of form and function. Morphological and thymidine labeling studies have demonstrated that the different types of neuronal and glial cells that make up the retina are born and differentiate in a defined temporal and spatial sequence (1). Cell lineage studies, utilizing both retroviral (2, 3) and fluorescent dextran (4) markers, indicate that most, if not all, of these cells arise from common progenitors. However, despite these and other important advances in the cell biology of retinal development (5), the actual molecular mechanisms which regulate cell fate determination and the development of committed progenitors into mature retina cells remain poorly understood.

Since development and differentiation of the retina are thought to involve a cascade of events in which different genes are turned on and off in a precisely regulated manner, one approach to studying retinal development is to analyze the mechanisms that control gene expression within the retina. Identification of transcription factors which regulate cell type and lineage-specific gene expression could, for example, lead to the discovery of master regulatory factors analogous to those controlling other lineages, such as the MyoD/myogenin/myf-5 family involved in muscle development (6).

Efforts to define the cis-acting DNA elements and trans-acting factors which regulate retina-specific gene expression have so far focused primarily on photoreceptor-specific gene products such as rhodopsin (7–13), red and green opsins (14, 15), blue opsin (16, 17, 18), interphotoreceptor retinoid-binding protein (19, 20), S-antigen (21, 22), arrestin (23), and \(\alpha\)-transducin (24). Rhodopsin provides a particularly attractive model system for these studies because: 1) both the gene and the protein are well characterized (25); 2) its expression is tightly regulated both in terms of cell-type specificity and developmental timing, and it shows diurnal modulation (26); 3) it is expressed at high levels; and 4) its similarity with the color opsins allows useful homology comparisons (27). Moreover, approximately 30% of cases of autosomal dominant retinitis pigmentosa, a currently untreatable disease in which photoreceptor degeneration leads to blindness, are due to mutations in the rhodopsin gene (28–30). Development of effective gene therapy for autosomal dominant retinitis pigmentosa will require thorough understanding of rhodopsin regulation (31), particularly since even wild type rhodopsin can lead to retinal degeneration when abnormally expressed (32, 33).

The induction and regulated increase in rhodopsin expression seen during rod development is largely controlled at the transcriptional level (34–36). Transgenic mouse studies utilizing overlapping sets of promoter-\textit{lacZ} fusion constructs have identified some of the DNA elements that regulate photoreceptor-specific expression of rhodopsin (37). Bovine upstream fragments from −2174 to +70 bp, from −735 to +70 bp, from −222 to +70 bp, and from −176 to +70 bp (relative to the mRNA start site) (7) as well as murine 4.4 kb and 0.5 kb constructs (8) all direct photoreceptor-specific expression. There are, however, important differences between the various constructs. Although position effects can cause considerable variation, the level of transgene expression is generally higher with the larger constructs than with the smaller ones. In addition, a superior-temporal to inferior-nasal transgene expression gradient is seen with the longer than with the shorter

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† To whom correspondence should be addressed: Johns Hopkins University School of Medicine, 809 Maumenee, 600 North Wolfe St., Baltimore, MD 21287-9289. Tel.: 410-550-5230; Fax: 410-550-5382; E-mail: don_zack@mail.bjh.edu.

\# The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; RER, rhodopsin enhancer region; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-(3-cholamidopropl)dimethylammonio)-1-propanesulfonic acid; LCR, locus control region; X-gal, 5-bromo-4-chloro-3-indoly\(\beta\)-galactosidase; EMSA, electrophoretic mobility shift assays.

\$ S. Chen and D. Zadik, unpublished results.
constructs, which show either a spotty or diffuse pattern of expression.

These results suggested that there may be at least two classes of elements regulating rhodopsin expression: a "proximal region" in the vicinity of the mRNA start site (within ~176 to ~70 bp in the bovine gene) that serves as a minimal promoter capable of directing photoreceptor-specific expression, and a "distal region," located further upstream, which serves as an enhancer. In addition, the finding of a gradient of expression which is unique to mice with the longer constructs raised the possibility that either the putative enhancer or a different distal sequence might function as a topological element controlling spatial expression across the retina.

In this paper we directly address the identity of the putative rhodopsin enhancer. Although the deletion series employed in the initial bovine transgenic experiments suggested that the enhancer and topological regulatory elements were located between ~2174 and ~734 bp, the mapping was not detailed enough to define a specific location. Based on sequence comparison of the mouse, cow, and human rhodopsin upstream regions, we hypothesized that the enhancer activity might be contained within a highly conserved 100-bp region and have generated transgenic mice that contain promoter-reporter fusion constructs that differ only by the presence or absence of this candidate region. Characterization of these mice indicates that the 100-bp candidate region displays many of the properties of an enhancer. It is not, however, required to establish an expression gradient across the retina. We also present biochemical evidence that bovine nuclear extracts contain both retina-specific and ubiquitously expressed proteins which bind to areas within the enhancer region in a sequence specific manner.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—To generate the rhodopsin promoter/lacZ fusion construct that contained the rhodopsin enhancer region (rho-2145), PCR was carried out using a plasmid which contains the region from 2174 to 734 bp as template and the primers 5′-ACGAAGTTGACCTGGCACCAGGGGCTG-3′ (which contains 6 bp of 5′ spacer DNA, a KpnI site, and spans the sequence from ~2145 to ~2128) and 5′-CAGGAAGCTCTCTCTAGGAG-3′ (which spans the sequence from ~1599 to ~1616). The PCR product was then digested with KpnI and Xhol (which cuts at ~1623 bp, gel purifies), and directionally cloned into the previously described hsp70/lacZ vector. A total of 1895 bp upstream of the bovine rhodopsin gene (43). The 5′ primer used in the reaction, 5′-AGCTCAGCTGAGCGCCATAGTTGGG-3′, contained 6 bp of 5′ spacer DNA, a XhoI site, and spans the sequence from ~1923 to ~1906. Each construct was then cut with HindIII and KpnI and the purified fragment was used for microinjection.

The bovine RER/ heterologous promoter fusion construct was generated using the 0.3-kb BamHI/NcoI fragment from plasmid pE2Dz (kindly provided by Jerey Nathans and Yanshu Wang, J ohns Hopkins University, Baltimore, MD) which contains the region from ~88 to ~230 bp from the hsp70 A1 gene (38). The fragment, which was gel purified after filling-in the BamHI site with Klenow DNA polymerase, was directionally cloned into the plasmid Rho-2174/plaCf which had been previously described (47). The resulting plasmid was used for microinjection.

Transgenic mice were generated at the J ohns Hopkins University School of Medicine Transgenic Mouse Facility by pronuclear microinjection of 26AF1 (female) × C57BL/Jm (male) embryos using established techniques (39), as described previously (7). Animals were screened by PCR as described previously (7, 37) except that 30-bp primers (5′-GATGTTGGCAGATGCTTAGCTTCGTA3′, 5′-CAAGCGCAAACCTGATGGCAAAAAGGCTCACC-3′, and 5′-AGTACGGCCCCGGCTGACATTACCATCAGT−3′) were used instead of the previ-
of 12.5 mM HEPES, pH 7.6, 100 mM KCl, 5 mM ZnSO$_4$, 0.5 mM dithiothreitol, 2% (w/v) polyvinyl alcohol, 10% glycerol, and 1 μg of poly(dI-dC). For method 2, binding buffer consisted of 12.5 mM HEPES, pH 7.6, 60 mM KCl, 5 mM MgCl$_2$, 0.5 mM dithiothreitol, 10% glycerol, and 1 μg of poly(dI-dC). After 15 min incubation on ice, the reaction tubes were transferred to room temperature, incubated for 1 min, MgCl$_2$, and CaCl$_2$ were added to give final concentrations of 5 and 2.5 mM, respectively, and then DNase I (Worthington) at the appropriate concentration (see Figs. 7 and 8) was added. Digestion was carried out for the times indicated and then terminated by the addition of 90 μl of stop solution (20 mM EDTA, pH 8.0, 1% (w/v) SDS, 0.2 mM NaCl, and 250 μg/ml glycogen) and 10 μl of 2.5 mg/ml proteinase K (Sigma). After incubation at room temperature for 5 min, samples were extracted with phenol/chloroform, precipitated with ethanol, and washed with 75% ethanol. Samples were resolved on standard 6% sequencing gels.

**RESULTS**

Evolutionary Conservation of the Putative Rhodopsin Enhancer Region and Homology to the Red and Green Opsin Locus Control Region

The 102-bp distal sequence corresponding to -2044 to -1943 bp in the bovine rhodopsin upstream sequence shows 64% sequence identity when compared with the homologous regions upstream of the human, mouse, and rat rhodopsin genes (Fig. 1). The identity is 77% in the central, more conserved region (-2024 to -1963 bp in the bovine sequence), but decreases at both ends of the sequence. Note that despite the high degree of sequence conservation, the actual position of the distal region relative to the mRNA start site varies significantly between the different species, from 1.5 to 2 kb upstream (human, -1906 to -1805; mouse, -1575 to -1477 bp; and rat, -1537 to -1434 bp). For ease of reference, and based on the data presented below, the distal region will henceforth be referred to as the "rhodopsin enhancer region" (RER).

The RER also shows homology to the highly conserved 37-bp sequence in the color opsin locus control region (LCR), an element involved in regulation of the red and green visual pigment gene cluster (15) (Fig. 1). This area of homology contains a sequence, CTAAT (-1985 to -1981 bp in the bovine sequence), that is similar to the homeodomain consensus binding sequence (45), and henceforth will be referred to as "rhodopsin homeodomain binding site-1" (RHBS-1). The LCR sequence has a 6-bp deletion, relative to the RER, that is located just upstream of the putative homeodomain binding site. This 6-bp sequence and the putative homeodomain site both appear to be involved in sequence-specific DNA-protein interactions (see below).

**RER Contains Enhancer Activity**

In order to explore the function of the RER in vivo, transgenic mice were generated with two similar constructs that both contained bovine rhodopsin upstream DNA fused to a lacZ reporter gene, but differed by the presence or absence of the RER (Fig. 2). The construct containing the RER (rho-2045) extended from -2045 to -70 bp, while the construct without the RER (rho-1923) extended from -1923 to +70 bp. The constructs were designed so that rho-2045 would extend slightly beyond the 5' end of the RER and rho-1923 would start slightly downstream of the 3' end of the RER.

Six independent lines were obtained with construct rho-2045 (2045-2, -15, -19, -21, -35, -65) and five with rho-1923 (1923-8, -15, -21, -39, -45). Fig. 3 shows the results of solution assays for β-galactosidase activity on eyes from each of the lines at 22-26 days of age. Eyes from the lines containing the RER had on the average 10-fold higher activity than eyes from the lines that did not contain the RER; this difference is statistically significant (p = 0.026, Wilcoxon Rank sum test).

Analysis of each of the transgenic lines by quantitative Southern analysis did not show evidence of a correlation of copy number with expression level. Copy number for lines 2045-2, -15, -19, -21, -35, -65 were approximately 1, 2, 2, 5, 40, and 15, respectively. Copy number for lines 1923-8, -15, -21, -39, -45 were approximately 50, 25, 1, 7, and 30, respectively (data not shown). The variation in β-galactosidase activity between the lines which contain the same construct presumably reflects position effects related to the transgene integration site.

**RER Is Not Necessary to Generate a Superior-temporal to Inferior-nasal Expression Gradient**

X-gal staining of retinal sections showed that all 11 transgenic lines express the transgene in a photoreceptor cell-specific manner (data not shown). The X-gal staining patterns seen in retinal whole mounts were similar to the superior-temporal to inferior-nasal gradient pattern seen previously with the -2174 to +70 lines (7) (Fig. 4). The gradient was seen in lines with the RER (Fig. 4, A-C) as well as in lines without the RER (Fig. 4, D and E). Although there is some variation in the gradient patterns seen (with some lines expression in the superior-temporal retina is spotty (Fig. 4, B and E) while with other lines it is more continuous (Fig. 4, A, C, and D)), there is...
which in turn is fused to the minegene in order to provide an intron and poly(A) addition site (7, 63).

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lines were constructed. The retinas from 15–28 animals from

Schematic diagram of the constructs used in this study. A, construct rho-2045, which consists of bovine rhodopsin sequences extending from −2045 to +70 fused to the lacZ cassette from placF. The positions of the RER and proximal promoter region are indicated. The lacZ cassette from placF contains the 3′-untranslated region from the mouse protamine gene in order to provide an intron and poly(A) addition site (7, 63). B, construct rho-1923, which consists of bovine rhodopsin sequences extending from −1923 to +70 fused to the lacZ cassette from placF. The RER is not included in this construct. C, construct RER-hsp70, which consists of bovine rhodopsin sequences extending from −2174 to −1620 fused to the −88 to +230 bp promoter fragment from the hsp70 A1 gene, which in turn is fused to the lacZ cassette from placF.

no simple correlation between the expression pattern and the presence or absence of the RER.

X-gal staining, a function of β-galactosidase enzyme activity, cannot per se elucidate whether the observed expression gradients reflect mechanisms operating at the protein level, such as translational control or differences in protein stability, or differences in transgene mRNA levels. We therefore performed whole mount in situ hybridization in order to visualize lacZ mRNA directly. The resulting patterns were essentially identical to those seen with X-gal staining. Fig. 4F shows a histological section through an in situ whole mount demonstrating an area of spotty transgene expression.

Whole mount in situ hybridization was also performed with a rhodopsin probe to determine whether there were any developmental stages in which the endogenous rhodopsin gene was expressed in a gradient pattern similar to that seen with the transgenes. Eyes were examined daily or every other day from postnatal day 1, before rhodopsin mRNA could be detected, to postnatal day 20, at which time there was strong and uniform expression throughout the retina. Gradients in expression patterns similar to those seen with the transgenes were not observed at any of the developmental stages studied (data not shown).

RER Does Not Activate Retinal Expression of a Minimal Heterologous Promoter

The RER was tested for its ability to activate a minimal heterologous promoter. Lines of transgenic mice were generated containing a fusion gene in which the −2174 to −1620 bp fragment, which contains the RER, was ligated 5′ of a 0.3-kb DNA fragment from the hsp70 A1 heat shock gene which contains a minimal promoter but is devoid of any heat shock response elements (38) (Fig. 2). Four independent transgenic lines were constructed. The retinas from 15–28 animals from each line were tested both by β-galactosidase solution assay and by staining with X-gal. In no case was transgene activity above background detected (data not shown).

DNA-Protein Interactions Involving the RER

Gel Mobility Shift Analysis—Electrophoretic mobility shift assays (EMSA) and DNase I footprinting were performed to look for evidence of specific DNA-protein interactions involving the RER. Probe for the EMSA assays was generated using PCR to divide the bovine RER and immediately surrounding DNA (−2140 to −1894 bp) into five 64-bp overlapping fragments. (The overlap between adjacent fragments was 18 bp.) Several of the PCR fragments gave strong mobility shifts with bovine nuclear extract. We chose to concentrate on the region from −2049 to −1986 bp because it showed a particularly interesting pattern of shifts and because it overlapped with the region homologous to the red/green opsin LCR. DNA oligomers which subdivide this region were used in order to more precisely define the DNA binding sites. Oligomer pair A, containing the region −2005 to −1986 bp plus a SalI site at the 3′ end (Fig. 1), was found to give a shift pattern that was essentially identical to that obtained with the entire −2049 to −1986 bp PCR fragment (Fig. 5, lane 2).

The sequence specificity of the DNA-protein interactions with the −2049 to −1986 bp sequence was explored using direct binding and cold oligomer competition with a series of oligomers containing site-specific mutations. The entire sequence was first scanned with oligomers in which successive groups of 3 bp were mutated one group at a time. The sequence CGATGG was identified by this analysis as an important core sequence that was required to generate the wild-type mobility shift pattern. Mutations in the sequence flanking this core did not significantly affect the shift pattern (data not shown). To further analyze the CGATGG region, each of the 6 bp in the core sequence was mutated individually and used as a cold competitor (Fig. 5, lanes 3–23). Wild-type oligomer efficiently inhibited all bands, except for band F which was only partially inhibited at the concentration used (lanes 3–5). The oligomers containing single base changes dramatically altered the shift patterns, demonstrating a high degree of sequence specificity in protein interactions with the CGATGG core. Moreover, the specificity of interaction was present at the level of individual shifted bands. For example, the oligomer with a C to A mutation at position 1 (A1) showed nearly wild-type ability to inhibit all bands (lanes 6–8). In contrast, the oligomer with a G to T mutation at position 5 (T5) showed essentially no ability to inhibit bands D, E, and F, although it still inhibited bands A, B, and C effectively (lanes 18–20). The oligomer with a G to T mutation at position 6 (T6) behaved similarly to T5, except that it was less effective at inhibiting the B and C bands and, at high concentrations, it slightly inhibited bands D, E, and F (lanes 21–23). The oligomer with an A to C mutation at position 3 (C3) was less effective at inhibiting the B, C, D, E, and F bands, but was essentially as effective as the wild-type oligomer in inhibiting band A (lanes 12–14). Since the C3 mutation makes the bovine core sequence identical to that of the mouse and rat sequences (Fig. 1), this result suggests that the putative rat and mouse core binding proteins may display binding preferences that are distinct from the bovine protein(s).

The tissue specificity of the proteins which interact with the −2005 to −1986 probe was examined by comparing EMSA patterns generated with bovine retina, cerebellum, cerebral cortex, kidney, and liver extracts (Fig. 5, lanes 2 and 24–27). Band D appears to be neuron-specific since it is observed with retina, cerebellum, and cerebral cortex extracts but not with kidney or liver extracts. Bands A–C appear restricted to retina.
and perhaps cerebellum, but since they are weaker the differences between the tissues may be less significant.

EMSA was also performed with an overlapping sequence, oligomer pair B, which spans the region from \(21995\) to \(21973\) bp (Fig. 1), to analyze the putative CTAAT homeodomain binding site, RHBS-1, together with surrounding DNA (Fig. 6). Five shifted bands were observed (A-E, lane 2), which were effectively competed by unlabeled oligomer B (lanes 3–5) but not by an unrelated cold oligomer (lanes 6–8). Comparison of the shift pattern generated with retina, cerebral cortex, cerebellum, liver, and kidney nuclear extracts suggested that bands B and C might be retina-specific (lanes 2 and 9–12). Band D, or a band

Fig. 3. \(\beta\)-Galactosidase activity of eyes from transgenic mice that differ by the presence or absence of the rhodopsin RER. Eyes from 22–26-day-old transgenic animals from the indicated lines were enucleated and tested for total \(\beta\)-galactosidase activity. Activity is expressed in milliunits (1 milliunit will hydrolyze 1.0 nmol of \(o\)-nitrophenol-\(\beta\)-\(D\)-galactopyranoside to \(o\)-nitrophenol and galactose per min). The rho-2045 lines contain the RER and the rho-1923 lines do not. The previously described 2174–31 line, which includes the RER, is included as a positive control for a high expressing line (7). The copy number for lines 2045–2, -15, -19, -21, -35, -65, and 1923–8, -15, -21, -39, -45 were approximately 1, 2, 2, 5, 40, 15, 50, 25, 1, 7, and 30, respectively.

Fig. 4. Comparison of transgene expression patterns in rho-2045 and rho-1923 mice. A–C, retinal whole-mount preparations from animal lines 2045-2, 2045-19, and 2045-65, respectively, stained with X-gal. D and E, retinal whole-mount preparation from animal lines 1923-39 and 1923-45, respectively, stained with X-gal. All eyes were from animals that were approximately 1 month old. The retinas in A, D, and E are from right eyes and the retinas in B and C are from left eyes. F, in situ hybridization of retinal whole-mount preparation from line 2045-65 using a lacZ antisense riboprobe. After hybridization the eye whole-mount was embedded in JB-4 and sectioned at 10 \(\mu\)m.
DNase I Footprint Analysis—Pilot studies, designed to compare different DNase I footprint protocols and different salt and divalent cation concentrations, revealed that the conditions which were optimal for demonstrating a footprint over one particular sequence were often not optimal for showing a footprint over a different sequence. Footprints with method 1, which utilized a restriction fragment including the region from −2143 to −1895 bp as template and in which the binding reaction contained 100 mM KCl and no Mg²⁺, demonstrated four areas of protection (Figs. 1 and 7, A and B). Region I, which spans the sequence GTCTGGCCACCAGGGGCCG, showed the strongest protection. A hypersensitive site was present just 3′ of the protected area. The protection over region III was also strong and spanned the sequence ACCTAATCACA, which includes the RHBS-1 site. Region II (CTTTACCTTTGACCTCTTT) showed a weaker but still consistent footprint. Region IV (CCTACACCCGGCCACACCTG), which was clearly seen with the “top” strand labeled but not with the “bottom” labeled, overlaps with the ret-3 site described in the rat rhodopsin gene (10) (Fig. 1). Method 2, which utilized a PCR generated template which spanned the sequence from −2143 to −1895 bp and a binding buffer which contained 60 mM KCl and 5 mM MgCl₂, showed significant protection over regions II and III (Fig. 7C). However, no significant protection was observed with this method over region I, although hypersensitivity sites were present nearby, nor over region IV. Comparison of footprinting patterns with extracts from bovine retina, cerebellum, cerebral cortex, liver, and skeletal muscle suggested that the binding activity for region III was retina-specific, the activity for region II was neural tissue-specific, and the activity for region I was weakly present in liver as well as retina (Fig. 8).

Due to the weakness and variability of protection over region IV, its tissue distribution is unclear.

DISCUSSION

RER Has Enhancer Activity—The transgenic mouse data presented in this paper indicate that the RER, which was identified by sequence comparison of the bovine, murine, and human rhodopsin gene upstream regions, has enhancer-like activity. Five of the six transgenic lines containing the RER expressed significantly more transgene activity than any of the five lines without the region. Analysis of transgene copy number indicates that these differences in expression level are not due to differences in copy number.

The positive regulatory activity of enhancers is generally position independent. Although the position independence of the RER was not directly tested, it is suggested by phylogenetic analysis which shows that despite the high sequence conservation between the mouse, rat, cow, and human RERs, there is significant variation in their position relative to the mRNA start site (Fig. 1). Furthermore, comparison of the bovine RER with those of the other species revealed a conserved 25-bp sequence that appears to have been inverted and transposed downstream (7). The 37-bp core sequence in the red/green opsin LCR, which shows sequence homology with the RER (Fig. 1),
also exhibits similar variation in position and orientation (15). The striking sequence homology between the RER and the 37-bp conserved sequence in the red/green opsin LCR probably reflects evolution of the rod and cone opsins from a common visual pigment progenitor gene. The lack of correlation of expression level with copy number in the rho-2045 mice argues that in the rhodopsin gene the RER does not function as a LCR, and suggests that the acquisition of such activity took place after the divergence of the genes. Whether the red/green LCR has the ability to act as a rhodopsin enhancer remains to be determined.

RER-Protein Interactions Demonstrate Sequence and Tissue Specificity—Like many enhancers and other regulatory regions (46, 47), the RER contains multiple sites for DNA-protein interaction, as shown by both EMSA and DNase I footprinting. As is consistent with a combinatorial model of transcriptional regulation (48–50), some of the DNA binding activities appear to be preferentially expressed in the retina (e.g. bands B and C in Fig. 6), some are specific to neuronal tissue (e.g. band D in Fig. 5, and others are more ubiquitously expressed. Mobility...
Tissue specificity of RER DNase I footprint activity. A and B, comparison of RER footprint patterns with nuclear extracts from bovine retina (R), cerebellum (C), cerebral cortex (CC), liver (L), and skeletal muscle (M). The templates in A and B were labeled on the top and bottom strands, respectively. All lanes contained 50 μg of the indicated extract except for lane 1 which did not contain any extract. The nomenclature for labeling protected areas is the same as in Fig. 7. The protocol employed was method I (see “Experimental Procedures”). The amounts of DNase I used per 50-μl reaction in lanes 1–12 were 3.3, 80, 80, 80, 80, 40, 3.3, 80, 80, 80, 80, and 40 ng, respectively, and the digestion time was 1 min.

The DNase I footprint experiments provide data that is complementary to but not identical with that obtained with the EMSAs. Protected regions I, II, and III all correspond to highly conserved sequences (Fig. 1). Protected region IV is less highly conserved. The protection and mobility shift assays both provide evidence for protein interaction with RHBS-1. Protection regions II and III flank both sides of the CGATGG core sequence, and correspond to areas showing significant homology to the red/green LCR 37-bp sequence; however, there is no significant protection over the CGATGG sequence itself. This may partially result from difficulty in detecting a footprint over the region due to the relative lack of bands corresponding to the CGATGG sequence in the absence of nuclear extract, a reflection of the non-random nature of DNase I cleavage. It may also reflect a low abundance of the CGATGG binding protein(s), since EMSAs are generally more sensitive than footprint assays because a detectable signal in an EMSA requires a shift of only a small fraction of the labeled probe whereas in a footprint assay a large fraction of the labeled template needs to be protected. Alternatively, variation in the binding conditions in the two assays or the greater complexity of protein-DNA interactions involved in the footprint assay may account for the differences.

Other binding regions within the RER include the ret-3 site (10), which overlaps region IV (Fig. 1), and a sequence that is homologous to the proposed chick homologue of the Drosophila glass binding site (11). The binding site for the putative transcription factor B0, TGACCT, which was identified upstream of the arrestin gene (23), is also present within the RER, in footprint region II. However, although these in vitro studies of DNA-protein interaction are suggestive, they do not demonstrate that the individual interactions are biologically significant. Future functional analyses, such as additional transgenic, retinal cell culture, and retinal in vitro transcription assays, as well as cloning of the factors involved will be required to establish and characterize the biological role of the individual DNA elements within the RER.

Regulation of Retinal Spatial Expression Patterns—The superior-temporal to inferior-nasal transgene expression gradient demonstrated by some of the rhodopsin upstream region/ lacZ transgenic lines does not accurately depict the expression pattern of the endogenous rhodopsin gene. One possibility is that the transgene gradient reflects the chance creation of a regulatory region that partially mimics or responds to a gene which serves as a positional marker within the retina. Such positional markers have been proposed as being important in maintaining spatial information and determining neural connectivity, and a number of retinal gradients have been identified (51–54). Based on an analogous finding with myosin light chain hybrid promoters in which the transgene was expressed as a gradient while the endogenous gene was expressed uniformly, it was suggested that the gradient expression pattern might be due to the fortuitous ability of the transgenic promoter to interact with a regionally expressed regulatory molecule or morphogen (55–57). Consistent with such a model is the finding that retinoic acid and some of the enzymes involved in its metabolism are expressed in a superior-temporal to inferior-nasal gradient across the retina (54) and retinoids have been implicated in the regulation of Drosophila opsin expression (58).

The finding that superior-temporal to inferior-nasal expression gradients occur only in transgenic lines carrying longer upstream fragments suggested the hypothesis that the RER might also function as a topological element regulating retinal spatial expression patterns. Our results, however, argue against a simple model in which the RER is necessary and sufficient for gradient expression. The rho-2045 and the rho-1923 lines both exhibit superior-temporal to inferior-nasal gradients. Although variations of the gradient between lines are seen, there is no correlation between a particular type of pattern and the presence or absence of the RER. Essentially identical superior-temporal to inferior-nasal gradients can be seen with both rho-2045 and rho-1923 mice. It therefore appears that the DNA sequences required for the gradient pattern are located downstream of the RER.

Model for Regulation of Rhodopsin Transcription—The results presented in this paper, together with previous work, suggest a model for mammalian rhodopsin regulation in which: 1) a proximal regulatory region acts as a minimal promoter and determines photoreceptor-specificity, and 2) a distal regulatory region acts as an enhancer to increase the level of expression. We propose to refer to the proximal regulatory region as the “rhodopsin promoter” and, as noted above, propose to refer to the distal regulatory region as the RER. As the DNA elements within these regions are defined in more detail, the nomenclature can be adjusted accordingly. In the cow, the rhodopsin promoter is located within the sequence between −176 to +70 bp (7) and the RER is located within the sequence from −2045 to −1923 bp. This arrangement of an upstream promoter and a more distal enhancer is similar to that seen with the Drosophila major rhodopsin gene, NinaE, except that with NinaE there are two redundant enhancer elements and they are located...
closer to the core promoter (59).

Although the rhodopsin promoter and RER appear to account for most aspects of rhodopsin transcriptional regulation, they do not account for all aspects. Transgenic lines containing 2.2 kb of bovine rhodopsin upstream DNA as well as lines carrying 4.4 kb of murine upstream DNA show low level leaky expression in cones (60, 61). Since endogenous rhodopsin is not thought to be expressed in cones, this finding suggests that the 2.2- and 4.4-kb constructs, both of which contain the promoter and the RER, may be missing a negative regulatory element which binds to a factor which "silences" expression in cones. The finding that transgene expression in mice carrying a 11-kb BamHI genomic mouse rhodopsin fragment, which contains 5' upstream sequence, is rod-specific is consistent with such a hypothesis and suggests that the putative silencer element may be located between 4.4 and 5 kb upstream, within intron sequence, or in 3' DNA (60). It is interesting to speculate that the putative silencer protein may repress a number of rod-specific proteins in non-rods and thus may be analogous to the putative silencer protein may repress a number of rod-specific proteins in non-rods and thus may be analogous to the putative silencer protein that binds to factor which "silences" expression in cones.

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