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A Locus Control Region Adjacent to the Human Red and Green Visual Pigment Genes

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Summary

Deletion of sequences 5′ of the human red and green pigment gene array results in blue cone monochromacy, a disorder in which both red and green cone function are absent. To test whether these sequences are required for transcription of the adjacent visual pigment genes in cone photoreceptors, we produced transgenic mice carrying sequences upstream of the red and green pigment genes fused to a β-galactosidase reporter. The patterns of transgene expression indicate that the human sequences direct expression to both long and short wave–sensitive cones in the mouse retina and that a region between 3.1 kb and 3.7 kb 5′ of the red pigment gene transcription initiation site is essential for expression. Sequences within this region are highly conserved among humans, mice, and cattle, even though the latter two species have only a single visual pigment gene at this locus. These experiments suggest a model in which an interaction between the conserved 5′ region and either the red or the green pigment gene promoter determines which of the two genes a given cone expresses.

Introduction

The vertebrate retina contains two classes of photoreceptor cells, rods and cones. Rods mediate vision in dim light and contain the visual pigment rhodopsin. Cones mediate vision in bright light and comprise two or more classes (the number depending upon the species) that differ from one another with respect to which cone visual pigment they contain. Comparison of the relative extents of excitation of the different cone classes forms the basis of color vision. The division of photoreceptors into distinct classes implies a mechanism to produce selectively only one type of visual pigment in each photoreceptor class, presumably by selective transcription of one type of visual pigment gene.

In the human retina the three classes of cones contain visual pigments that are maximally sensitive in the blue, green, and red regions of the spectrum (Boynton, 1979). An analysis of the genes encoding these pigments reveals that the red and green pigment genes are highly homologous and reside in a head-to-tail tandem array on the X chromosome (Nathans et al., 1986a, 1986b; Vollrath et al., 1988; Feil et al., 1990). The blue pigment gene and the rhodopsin gene are located on different autosomes and show a far lower degree of homology with each other and with the red and green pigment genes (Nathans et al., 1986a, 1986b). A comparison of visual pigment genes from fish, birds, and mammals shows that the duplication events which generated the rhodopsin, blue, and red/green pigment genes occurred before the vertebrate radiation (Nathans and Hogness, 1983; Takao et al., 1988; Kuwata et al., 1990; Tokunaga et al., 1990; Yokoyama and Yokoyama, 1990). By contrast, a comparison of visual pigment genes from different primates shows that the red and green pigment genes arose via a duplication event within the Old World primate lineage (Jacobs and Neitz, 1987; Neitz et al., 1991; Ibbotson et al., 1992). The sequence similarity of red and green pigment genes is reflected in a corresponding similarity of red and green cones, as judged by their response properties and anatomic distribution (Boynton, 1979). The recent origin of distinct red and green cone pigments suggests that the regulatory mechanisms determining red versus green cone identity may be simpler than those determining the identities of other photoreceptor types.

The experiments reported here are aimed at defining cis-acting regulatory sequences that control the cell type–specific expression of human red and green cone pigment genes. The initial impetus for this work came from an analysis of human mutations responsible for the combined loss of red and green cone function, a disorder referred to as blue cone monochromacy. Blue cone monochromacy is associated with two general classes of DNA rearrangement at the red and green pigment gene locus (Nathans et al., 1989). In one class, the array contains a single visual pigment gene that is inactivated by a point mutation. In the second class, observed in approximately half of blue cone monochromats, a deletion removes sequences 5′ of the gene array. Thus far, six different deletions have been analyzed, and these range in size from 0.6 kb to 55 kb. All of the deletions encompass the region that is missing from the chromosome with the smallest deletion. The four largest deletions also remove part of the adjacent red pigment gene transcription unit, but leave intact at least one green pigment gene transcription unit. The simplest interpretation of these observations is that sequences within the small region common to all deletions are required for expression of both red and green pigment genes. This region is

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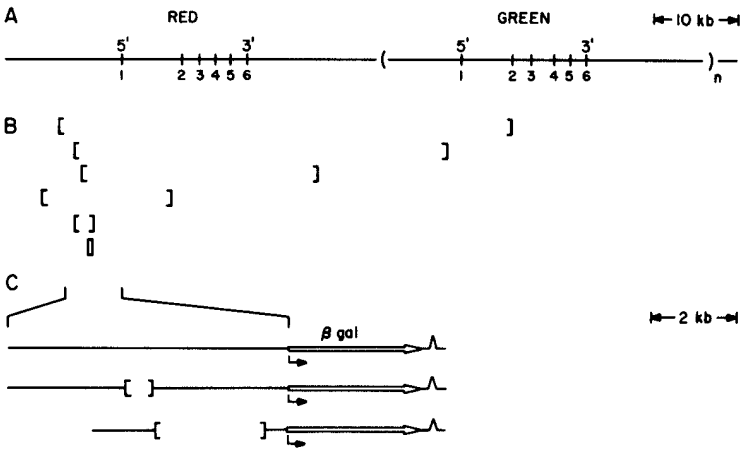


Figure 1. The Human Red and Green Pigment Gene Array

(A) The locations of red and green pigment gene exons, numbered 1–6, are indicated for the wild-type array. (B) Brackets beneath the visual pigment array show the locations of nonhomologous deletions found among blue cone monochromats (Nathans et al., 1989). (C) Transgene constructs are shown on an expanded scale. Top: the full-length construct, pR6.5lacZ, in which 6.5 kb of 5′ flanking sequences beginning at the initiator methionine codon are joined to a β-galactosidase reporter cassette. The large open arrow represents the β-galactosidase coding region (*lacZ*); the line beyond the tip of the arrows represents the mouse protamine 1 intron and polyadenylation site; the small arrow indicates the start site and direction of transcription. Middle: pR5.9lacZ, the 0.6 kb deletion construct. Bottom: pR2.1lacZ, the construct in which 1.6 kb of distal sequences are joined to the promoter-proximal 0.5 kb.

located 3 kb and 42 kb from the red and green pigment gene transcription start sites, respectively (Figure 1).

Deletions analogous to those found in the second class of blue cone monochromat genotypes have also been observed in the human β-globin locus (Curtin et al., 1985; Driscoll et al., 1989; Taramelli et al., 1986; Van der Ploeg et al., 1980). Like the red and green pigment genes, the human embryonic (ε), fetal (γ), and adult (δ and β) globin genes are arranged in a head-to-tail tandem array. Deletions 5′ of the globin gene array result in a β-thalassemia in which the remaining globin genes on that chromosome are not expressed. A segment of DNA from the deleted region, encompassing 20 kb 5′ of the structural genes, has been shown to confer high level, erythroid-specific expression on a linked reporter in transgenic mice (Grosveld et al., 1987; Ryan et al., 1989). By contrast, constructs containing only promoter-proximal sequences from the globin genes are expressed variably and at low levels in erythroid cells (Magram et al., 1985; Townes et al., 1985; Kollias et al., 1986). The 5′ flanking segment, referred to as the locus control region (LCR), coincides with a region previously shown to be accessible to DNAase I digestion in erythroid cells (Tuan et al., 1985).

In this paper we show that sequences 5′ of the human red and green pigment gene array are required for the expression of a linked reporter in cone photoreceptor cells in a manner reminiscent of the β-globin LCR. A comparison of the corresponding bovine, human, and murine sequences reveals significant homology within the required region and suggests a model for selective activation of red and green pigment genes in their respective cone types.

Results

Transgene Expression in Cone Photoreceptors

A previous study of human blue cone monochromacy suggested that a region between 3.1 and 3.7 kb upstream of the human red pigment gene is essential for the expression of both red and green pigment genes (Nathans et al., 1989). Guided by this work, we generated transgenic constructs containing various regions 5′ of the human red pigment gene fused to the E. coli gene encoding β-galactosidase (*lacZ*). The constructs also include a mouse protamine 1 gene intron and polyadenylation site in the 3′ untranslated region (Peschon et al., 1987).

A 6.5 kb NcoI–SphI fragment corresponding to the region immediately upstream of the human red pigment gene was obtained from genomic DNA of a male with normal color vision (J. N.) and used in the full-length construct, pR6.5lacZ (Figure 1; see Experimental Procedures). This construct was microinjected into the pronuclei of B6A F_1 × C57BL/6J embryos, and 8 independent transgenic animals were obtained, of which 7 served as founders of transgenic lines. Six of the 7 transgenic lines, as well as the infertile transgenic animal, express β-galactosidase in the retina. One transgenic line did not express β-galactosidase activity in any tissue. By morphological criteria (Carter-Dawson and LaVail, 1979a), all of the cells expressing β-galactosidase appear to be cones. As seen in Figure 2, the cell bodies and synaptic terminals are located at the outer edges of the outer nuclear and outer plexiform layers, respectively; the outer segments are shorter than those of the adjacent rods; and the cells are typically separated from one another by

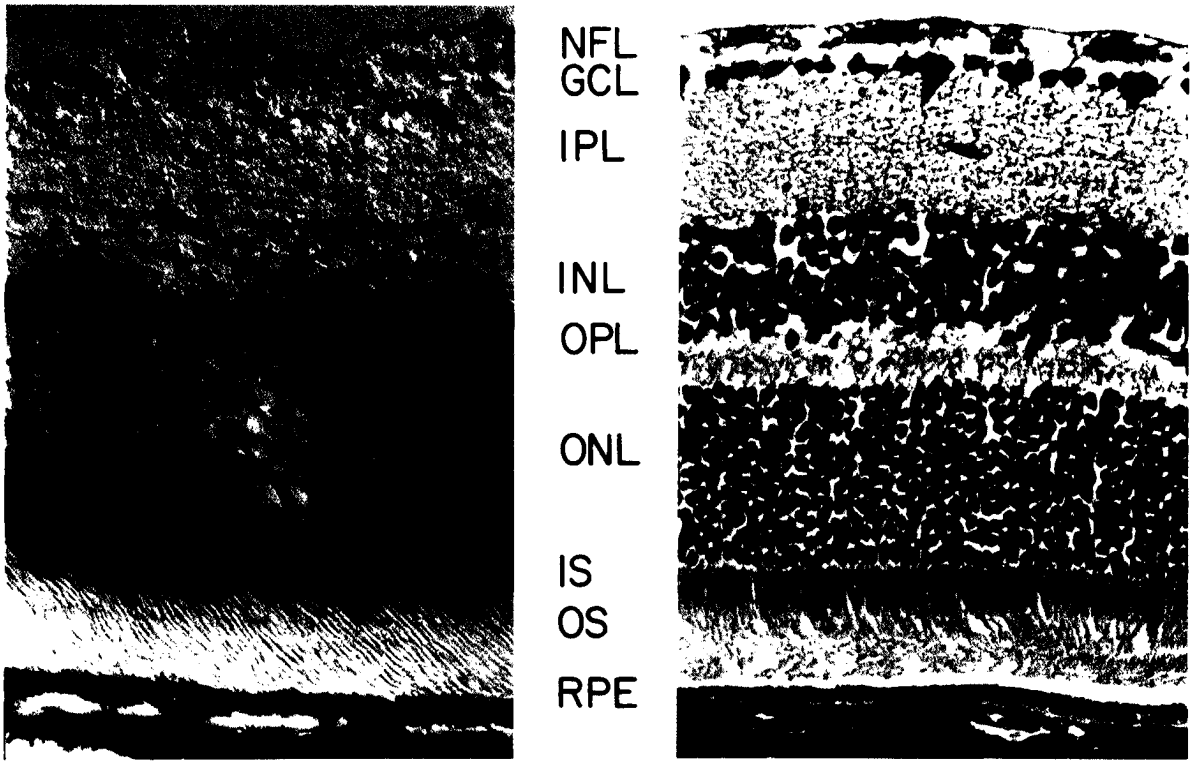


Figure 2. Transgene Expression in Cones

(Left) A 10 μm frozen section from the right eye of a 1-month-old pR6.5lacZ transgenic mouse stained for 2 hr with X-Gal. (Right) A section similar to that on the left stained with hematoxylin and eosin to reveal the retinal layers. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments; OS, outer segments; RPE, retinal pigment epithelium.

a distance of several cell diameters (compare with Figure 2 in Zack et al., 1991, for a similar section of retina in which β-galactosidase is expressed in rods).

Following backcrossing of the founders to C57BL/6J mice, retinas from 10 or more adult F_1 transgenic animals from each line were examined for β-galactosidase activity by staining whole eyes from which the sclera and choroid had been removed. For each line, the percentage of transgenic animals expressing β-galactosidase in the retina is shown in Figure 3. All of the transgenic animals in 4 lines, as well as the 1 infertile transgenic animal, show strong X-Gal staining in a subpopulation of photoreceptors distributed uniformly across the retina. These 4 lines are designated high expressing lines. In 2 lines, referred to as low expressing lines, the number of β-galactosidase-expressing cells in the retina varied markedly among transgenic animals, ranging from no expression in approximately 50% of animals to as many as several thousand β-galactosidase-expressing cells in some animals. One of the lines showed no β-galactosidase expression in the retina.

To identify different cone classes among the X-Gal-stained photoreceptors, rabbit polyclonal antibodies generated against the carboxyl terminus of the human red and green pigments and against the carboxyl terminus of the human blue cone pigment were used to stain sections and whole mounts of transgenic retinas. The antisera appear to be specific for their respective cone pigments, as determined by protein blots of recombinant pigments (Merbs and Nathans, 1992) and by the pattern and density of cones that each stains in sections of monkey retina (Y.-W. Peng, S. L. Merbs, and J. Nathans, unpublished data). In the mouse retina, each antiserum stains a subset of cone outer segments. Interestingly, the cones stained by the anti-red/green antiserum are located predominantly in the superior half of the retina, whereas the cones stained with the anti-blue antiserum are located predominantly in the inferior half of the retina (Figure 4). As the two distributions are reciprocal, the total cone density is approximately uniform across the retina (Carter-Dawson and LaVail, 1979a).

Antibody staining and X-Gal staining were analyzed in the same retinas from transgenic lines R7 and R28, 2 representatives of the high expressing lines. Surprisingly, X-Gal staining colocalizes with staining by both anti-red/green and anti-blue pigment antisera (Figure 5). The uniform distribution of cones expressing β-galactosidase in the other high expressing lines suggests

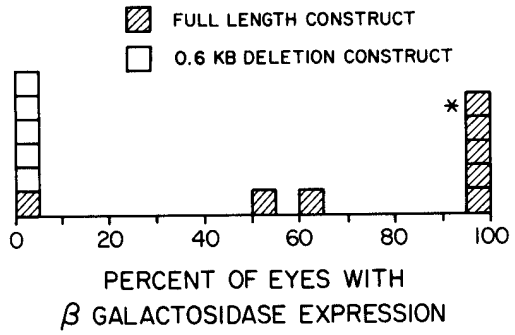


Figure 3. Percentage of Animals in Each Transgenic Line Expressing β-Galactosidase in the Retina

Whole retinas from 10 or more transgenic animals per line were tested by staining with X-Gal. Each square represents the percentage of retinas showing β-galactosidase staining. The transgenic lines on the left side of the histogram showed no stained cells in any of the retinas. The transgenic lines on the right side of the histogram showed a large number of stained cells in every retina. The asterisk indicates the result for one retina from the infertile transgenic animal. The full-length construct shows expression in 7 of 8 lines; the 0.6 kb deletion construct shows expression in 0 of 5 lines ($\chi^2 = 6.3$; $P = 0.01$)

a similar colocalization. Apparently, the 6.5 kb fragment upstream of the human red pigment gene directs expression of the β-galactosidase reporter to both classes of cones in the mouse retina.

To assess the tissue specificity of transgene expression, slices of brain, heart, lung, liver, kidney, spleen, and ovary or testes from 2 mice from each of the 7 lines were stained with X-Gal. Following incubation at room temperature for 6 hr, during which time the retinas of the high expressing lines stained intensely blue, no staining was found above the nontransgenic control level in any of the samples (data not shown).

Spatial and Temporal Regulation

β-Galactosidase activity within the eyes of transgenic animals increases with age, as determined by a solution β-galactosidase assay of individual eyes from F_1 heterozygotes from line R7 (Figure 6). β-Galactosidase activity is first detectable on postnatal days 3–4 (P3–P4), and increases roughly linearly with time until 1 month of age. By contrast, when the rhodopsin promoter directs *lacZ* expression in transgenic mice, β-galactosidase activity is first detectable at approximately P10–P15 (Zack et al., 1991). In the mouse, cones mature approximately 1 week before rods, a time differential that is consistent with the timing of β-galactosidase production seen here (Carter-Dawson and LaVail, 1979b; Young, 1985).

In addition to the gradient of cone classes reported here, several recent studies have identified gradients of various types across the retina. For example, gradients have been identified for several antigens in mouse and chicken retina (Trisler et al., 1981; Rabacchi et al., 1990; McLoon, 1991). Experiments in which the bovine and murine rhodopsin gene promoters were used to direct β-galactosidase expression revealed for some constructs a superior-temporal to inferior-nasal gradient across the retina (Lem et al., 1991; Zack et al., 1991). Over the first several months of life, the zone of transgene expression increased until it eventually encompassed most of the retina. With these precedents in mind, we examined the pattern of X-Gal staining in whole retinas from transgenic line R7 mice at various ages. As shown in Figure 7, β-galactosidase expression was observed predominantly in the inferior retina of P6 mice. The gradient of expression spreads to the superior retina by P8 and disappears by P10. One possible explanation for this pattern is that in the mouse, the class of cones enriched in the inferior retina develops earlier than that enriched in the superior retina.

Deletion Constructs

The experiments described above show that the 6.5 kb NcoI–SphI fragment is capable of directing expression of the β-galactosidase reporter to cone photoreceptors. To define more precisely the important regions within this 6.5 kb fragment, we generated two deletion constructs. The design of the first deletion construct, pR5.9lacZ, was based on the pattern of deletions observed in human blue cone monochromats. As described in Introduction, in one class of blue cone monochromats, DNA deletions of various sizes are found to encompass a region between 3.1 and 3.7 kb 5′ of the red pigment gene. To test the hypothesis that this region is required for the proper expression of the adjacent cone pigment genes, we removed this region from the 6.5 kb full-length construct by replacing a 5.8 kb HindIII fragment from construct pR6.5lacZ with the corresponding 5.2 kb fragment from a blue cone monochromat (individual HS102 in Nathans et al., 1989). Five transgenic lines were derived from this 0.6 kb deletion construct, and transgenic F_1 animals were analyzed by X-Gal staining of retina whole mounts, a format that allows single stained cones to be reliably scored. Retinas from 10 transgenic animals from each line were tested by staining overnight in X-Gal, and none was found to express the *lacZ* transgene (Figure 3).

To localize further the sequences that are required for cone-specific expression, the 0.5 kb region immediately 5′ of the red pigment gene, which we presume contains essential promoter sequences, was fused to a 1.6 kb fragment between 3.0 and 4.6 kb upstream of the red pigment gene that encompasses the essential 0.6 kb region (Figure 1). This 1.6 kb fragment includes a region of interspersed homology between murine and human sequences (see below). The resulting 2.1 kb fusion fragment was joined to the β-galactosidase reporter to generate the construct pR2.1lacZ. In 3 of 3 transgenic lines carrying pR2.1lacZ, the retinas of F_1 transgenic animals showed an X-Gal staining pattern similar to that found in the high expressing lines car-

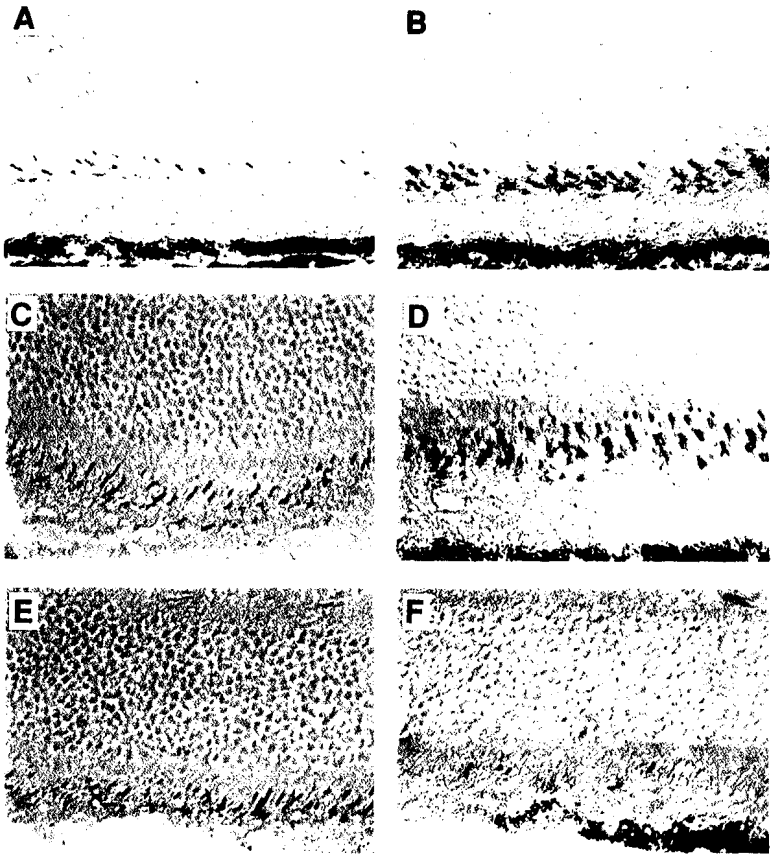


Figure 4. Reciprocal Gradients of Cone Classes within the Mouse Retina

Three regions from a single frozen section of mouse retina stained with anti-red/green pigment antiserum (A, C, and E) or with anti-blue pigment antiserum (B, D, and F). (A) and (B) are adjacent areas from one edge of the retina; (E) and (F), adjacent areas from the opposite edge of the retina; (C) and (D), adjacent areas near the posterior pole of the retina.

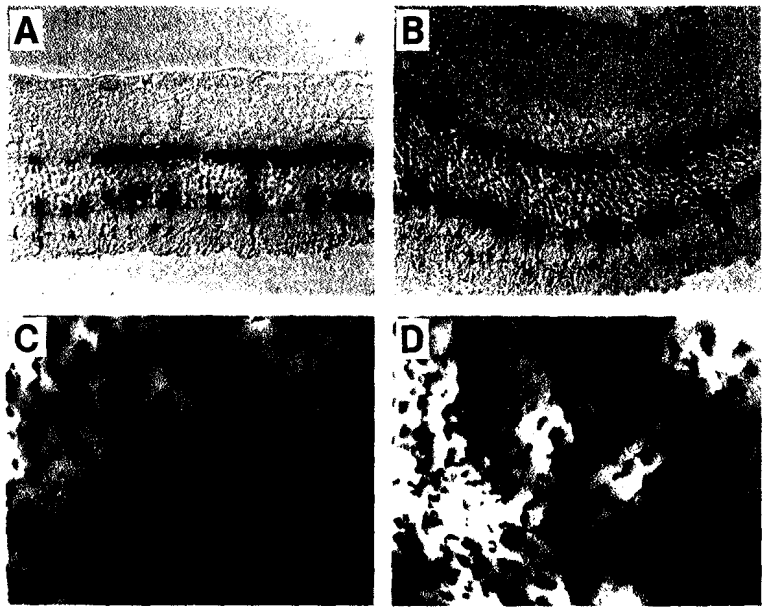


Figure 5. Localization of β-Galactosidase Activity in Identified Cone Classes in Transgenic Retinas

Frozen sections (A and B) or whole mounts (C and D) of retinas from pR6.5lacZ transgenic line R7 were double stained with X-Gal and with either anti-red/green pigment antiserum (A and C), or anti-blue pigment antiserum (B and D).

rying the full-length construct pR6.5lacZ (data not shown).

Conservation of 5′ Flanking Sequences Across Species

Because functionally important sequences tend to be conserved during evolution, such sequences can often be revealed by comparisons across species. Therefore, we determined the DNA sequence upstream of the human red pigment gene and the corresponding region of the murine and bovine homologs (Figure 8). Murine and bovine genomic DNA libraries were constructed in bacteriophage λ and screened with a

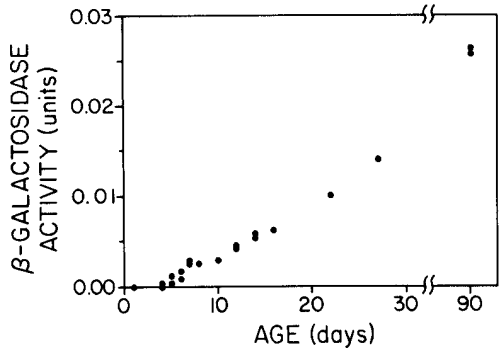


Figure 6. Developmental Time Course of β-Galactosidase Activity in Transgenic Retinas

Whole eyes from heterozygous transgenic mice (pR6.5lacZ, line R7) were homogenized and assayed for β-galactosidase activity. The enzyme activity is expressed in standard units per eye (1 unit hydrolyzes 1.0 μmol of O-nitrophenyl-β-D-galactopyranoside to O-nitrophenyl and galactose per min). Mouse eyes increase in surface area by approximately a factor of 4 between P3 and P30.

human red pigment cDNA clone. In each case, the recombinant phage isolated with this probe were derived from a single visual pigment gene. The first exon sequences from the cloned bovine and murine genes were determined and found to share 70% and 62% amino acid identity, respectively, with the first exons of the human red and green pigment genes. Nucleotide sequences were determined for 6.0 kb 5′ of the gene encoding the human red pigment, 5.5 kb 5′ of the murine red/green pigment gene homolog, and intervals 0 to 0.4 kb and 0.9 kb to 2.5 kb 5′ of the gene encoding the bovine red/green pigment gene homolog.

Sequences from each species were compared with those of the other two using a dot matrix format (Figure 9). Of the various pairwise comparisons, the human/murine and bovine/murine are the most informative, as these pairs show the lowest overall homology. The degree of homology among the three sequences is similar to that observed in a similar comparison of

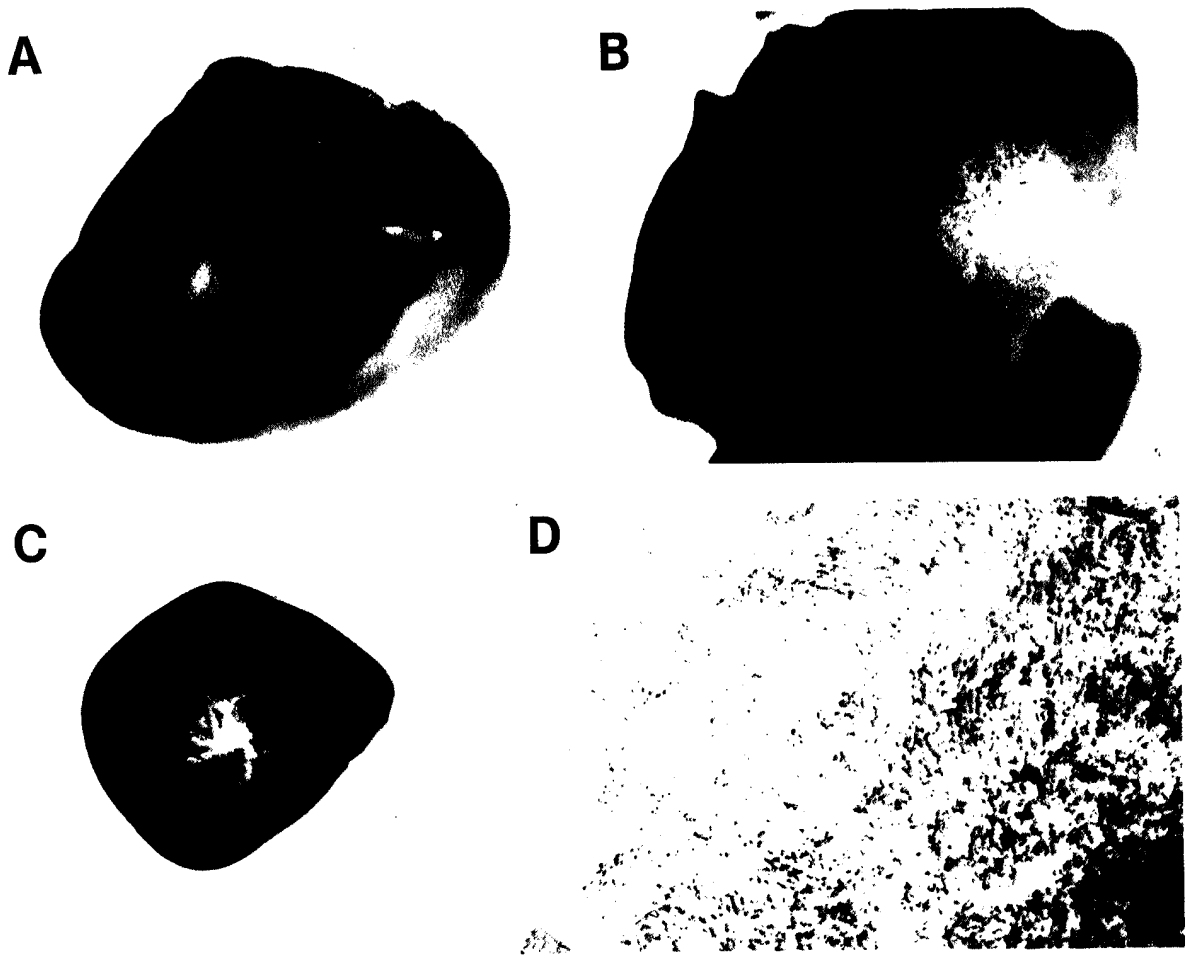


Figure 7. A Gradient of Transgene Expression in Early Postnatal Retinas

X-Gal-stained whole retinas from pR6.5lacZ transgenic line R7 are shown at the following postnatal ages: 6 days (A), 8 days (B), and 1 month (C). (D) A piece of the retina from (B) shown at higher magnification. The gradient of decreasing transgene expression is apparent proceeding from lower right to upper left. Each small stained object is a single photoreceptor cell.

sequences 5′ of the rhodopsin gene (Zack et al., 1991). In each pairwise comparison, the same two regions of homology were observed. A proximal region of homology begins near the start site of transcription and extends approximately 150–200 bp 5′ of that point; a distal region of homology begins in the human sequence at approximately 3.2 kb 5′ of the transcription start site and extends, with several interruptions, to 4.5 kb. Interestingly, the essential 0.6 kb encompasses the most conserved part of the distal homology region, including a core of 37 bp that is identical in all three species. The 37 bp sequence has no significant homology with any sequence in the GenBank DNA data base.

Discussion

Identification of the LCR

The experiments reported here demonstrate that sequences between 3.1 and 3.7 kb 5′ of the human red and green pigment gene locus are required to obtain cone photoreceptor-specific expression of a β-galactosidase reporter gene. 6.5 kb of wild-type DNA immediately 5′ of the human red pigment gene conferred cone-specific expression in 7 of 8 transgenic lines. A second construct, which differed from the wild type by deletion of sequences between −3.1 and −3.7 kb, showed no expression in the retina or other tissues in 5 of 5 transgenic lines. A third construct, in which the promoter-proximal 0.5 kb from the human red pigment gene was fused to sequences between −3.0 and −4.6 kb, produced cone-specific expression in 3 of 3 of three transgenic lines in a manner similar to the wild-type construct.

The 0.6 kb deletion in the second transgene construct corresponds precisely to a deletion found in one blue cone monochromat family. All of the blue cone monochromats who have nonhomologous DNA rearrangements have deletions that encompass this region. The lack of expression seen with the 0.6 kb deletion construct argues for a model in which the primary lesion in this class of blue cone monochromats is a lack of transcription of the red and green pigment gene array. Because the 0.6 kb deletion construct was generated by substituting a 5.2 kb fragment from the DNA of a blue cone monochromat for the corresponding wild-type fragment, we cannot exclude the possibility that there may be small sequence differences outside of the deleted region that contribute to the lack of activity of this fragment.

Consistent with an essential role for the 0.6 kb region, we observe within this segment a high degree of sequence conservation in bovine, human, and murine DNA. The conserved region includes a core of 37 bp of perfect identity, embedded within a 200 bp region of 70% identity. The only other region within the 5′ flanking DNA with a comparable degree of sequence identity is the 150–200 bp adjacent to the start site of transcription. In all three species, the distal homology region is located between 2 and 4 kb from the nearest visual pigment gene transcription start site. In the mouse, it is inverted relative to the human and bovine sequences.

These experiments show that the tandem array of human red and green pigment genes, like the tandem array of embryonic, fetal, and adult β-globin genes, requires 5′ flanking regulatory sequences—an LCR—for their appropriate expression. The β-globin LCR has been shown to confer erythroid-specific expression on a heterologous promoter and to function in transgenic mice regardless of integration site (Grosveld et al., 1987; Ryan et al., 1989). It is not yet known whether the red and green pigment gene LCR can confer cone-specific expression on a heterologous promoter. The extent to which LCR activity is affected by the integration site also remains to be determined.

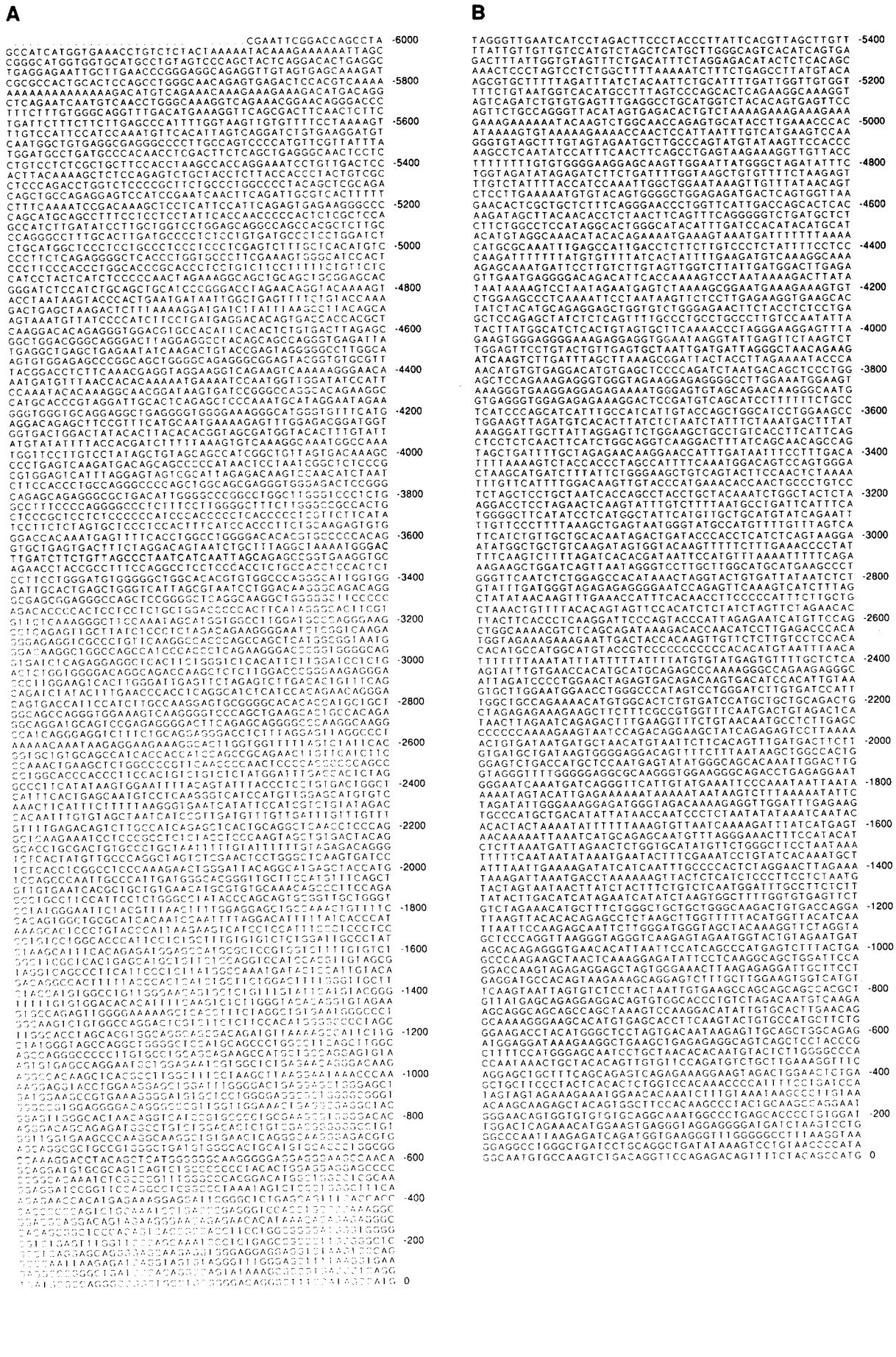
Transgene Expression in Different Classes of Cone Photoreceptors

Two immunologically distinguishable classes of cones are present in the mouse retina. One class is recognized by antibodies directed against the carboxyl terminus of the human blue pigment, and a second class is recognized by antibodies directed against the carboxyl terminus of the human red and green pigments. The 6.5 kb transgene construct is expressed in both classes of cones, an unexpected finding given the great evolutionary distance between blue cones and red/green cones and the lack of obvious sequence homology between bovine, human, and murine blue and red/green pigment gene 5′ flanking regions (I. Chiu and J. Nathans, unpublished data). This pattern of transgene expression suggests both similarities among cones and differences between cones and rods with respect to a subset of transcription factors. We surmise that regulatory sequences responsible for repression of human red/green pigment gene expression in blue cones may be missing from the 6.5 kb construct or may have diverged to such an extent that the human sequences are no longer recognized in mice.

In the course of this work we made the incidental observation that the two immunologically distinguishable classes of cone photoreceptors are not uniformly distributed across the mouse retina. Antibodies directed against the human red and green pigments bind to a class of cones that is enriched in the superior half of the retina, whereas antibodies directed against the human blue pigment bind to a class of cones that is enriched in the inferior half of the retina. The density of each cone class changes by approximately 10-fold across of the retina. The reciprocal nature of the two distributions produces a total cone density that is approximately uniform.

Models for Selective Expression of Red and Green Pigment Genes

By analogy with models in which embryonic and adult chicken globin genes compete for an enhancer (Choi and Engel, 1988), the presence of an LCR adjacent to


A
B

C

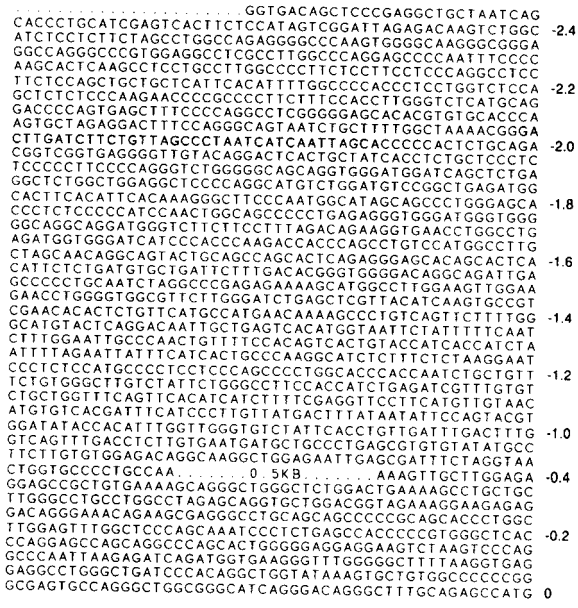


Figure 8. DNA Sequences 5′ of the Human Red Pigment Gene, and the Bovine and Murine Red/Green Pigment Gene Homologs

Nucleotide sequences are numbered with negative numbers starting from the G in the initiator methionine ATG (number 0) and proceeding away from the gene. In the human sequence (A), the start site of transcription corresponds to nucleotide −43. The conserved 37 bp region is highlighted (note that in the mouse [B] it is inverted). The bovine sequence (C) is interrupted by a region of approximately 0.5 kb, for which the sequence was not determined. A partial sequence of this region from human DNA reported earlier (Nathans et al., 1989) was missing one of the 4 G's between bases −3618 and −3621.

the red and green pigment gene array suggests a mechanism by which each red or green cone activates only one type of visual pigment gene (Figure 10). The high degree of homology among bovine, human, and murine sequences in this region suggests that the LCR predated the divergence of these species. Since the murine and bovine genomes contain a single red/green ancestor gene, the LCR apparently predated the duplication event that generated the red and green pigment genes. This line of reasoning implies that the LCR evolved to activate a single promoter, and it seems reasonable to suppose that this activation occurs via direct contact between the two DNA segments and their associated proteins. If we further suppose that the LCR can accommodate only one such interaction and that the interaction is stable over an extended period of time, then, as observed, each cell in which the LCR-promoter complex is active would stably express only a single gene from the array.

The model described above accounts for the mutually exclusive expression of red and green pigment genes, but does not address the mechanism by which a single cone chooses between the two genes. We can envision two types of mechanisms for this choice. In one general and now familiar model, a set of regulatory molecules, including transcription factors, differs between red and green cones and orchestrates the choice of red versus green pigment gene interaction with the LCR, as well as the production of any additional proteins that differ between the two cell types. A second and less general model is suggested by the X linkage of the red and green pigment genes and by the extreme similarity of the red and green cones. In this model, the interaction of a visual pigment promoter with the LCR determines red versus green cone identity. Male hemizygosity and female X inactivation allow this decision to be made at only one red and green pigment gene locus per cell, obviating any requirement for coordinating or suppressing promoter-LCR interactions at a second locus. If the choice were made randomly, the result would be a fine-grained mosaic of red and green cones across the retina. This model of stochastic promoter choice suggests the possibility that red and green cones are distinguished only by the pigment contained within them. If this were the case, it would imply that red versus green cone specificity in cell-cell contact and information transfer is not predetermined during development.

Experimental Procedures

Cloning and Sequencing of Genomic DNA

The isolation of the human red pigment gene and its 5′ flanking DNA has been previously described (Nathans et al., 1986a, 1989). Genomic clone gJHN60 was used as a template for DNA sequencing. To isolate the murine and bovine homologs of the human red and green pigment genes, Sau3A partial digest genomic DNA libraries were constructed in bacteriophage λ EMBL3 as described (Frischauf et al., 1983; Sambrook et al., 1989). The libraries were screened with a human red pigment cDNA clone, hs7 (Nathans et al., 1986a). A set of overlapping recombinants were characterized from each species and identified as containing red and green pigment gene homologs by sequencing the regions homologous to the first exon, which is specific for this class of visual pigments. For each 5′ flanking region reported here, both strands were sequenced using a combination of overlapping deletions and appropriately spaced synthetic oligonucleotide primers.

Generation of Transgenic Mice

For the construct pR6.5lacZ, a 6.8 kb NcoI fragment from clone gJHN60 was inserted into placF (Peschon et al., 1987) at the NcoI site, and subclones with the correct orientation were partially sequenced to verify a perfect fusion at the translation start site. A 9.8 kb SphI partial digest fragment containing the 6.5 kb human red pigment gene 5′ region and the *lacZ* reporter cassette was gel purified for embryo injection as described (Zack et al., 1991). To construct the deletion plasmid pR5.9lacZ, a 5.8 kb HindIII fragment from the 5′ flanking DNA in pR6.5lacZ was replaced by the corresponding 5.2 kb HindIII fragment derived from blue cone monochromat HS102 (Nathans et al., 1989), in which DNA between bases −3124 and −3715 5′ of the human red pigment gene are deleted (Figure 8). A 9.2 kb SphI partial digest fragment, corresponding to the 9.8 kb SphI partial digest fragment described above, was used for embryo injection. To construct the deletion plasmid pR2.1lacZ, a 1.6 kb BamHI-StuI fragment, spanning bases −3009 to −4564 5′ of the human red pigment gene, was ligated at the BamHI site to a BamHI-NcoI fragment spanning bases 0 to −496 5′ of the human red pigment gene. The resulting 2.1 kb StuI-NcoI fragment was inserted into a placF

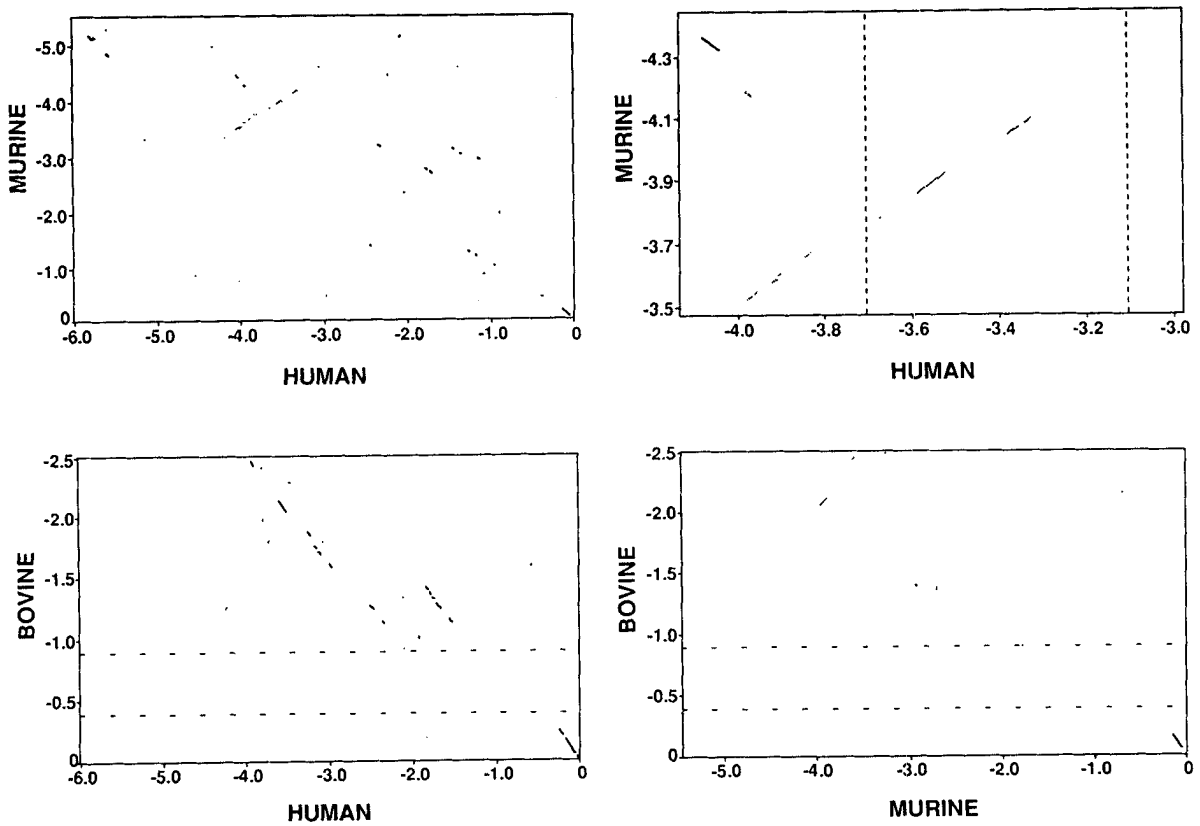


Figure 9. Dot Matrix Comparison of DNA Sequences 5′ of the Human Red Pigment Gene and the Bovine and Murine Red/Green Pigment Gene Homologs

Each sequence shown in Figure 8 is compared with the other two sequences on both strands. Homology along the same strand relative to the direction of transcription appears as a diagonal oriented from upper left to lower right, whereas homology between this strand and the opposite strand appears as a diagonal from upper right to lower left. The comparison was performed with MacVector software (IBI) with a window size of 30 bases and a criterion of 65% identity. Sequences are numbered as in Figure 8. Upper right, the region surrounding the essential 0.6 kb (between bases −3124 and −3715; indicated by dashed vertical lines) in the human/murine comparison is shown enlarged. Note that in the human/murine and bovine/murine comparisons, the homologous sequences are on opposite strands. The pair of horizontal dashed lines that intersect the bovine axes demarcate the region that has not been sequenced.

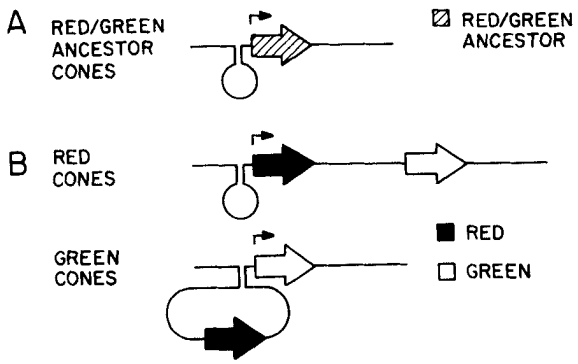


Figure 10. Model for Selective Activation of Cone Pigment Genes by Pairing with the LCR

(A) In mammals carrying a single visual pigment gene that is the ancestor of the human red and green pigment genes, the LCR is hypothesized to interact stably with the promoter of that gene in the appropriate class of cones. (B) In humans and other Old World primates, the LCR is hypothesized to interact stably with the promoter of the red pigment gene in red cones and with the promoter of the green pigment gene in green cones.

plasmid that had been digested with SmaI and NcoI. A 5.6 kb KpnI–HindIII fragment containing the fused human red pigment gene 5′ flanking sequences and the *lacZ* reporter cassette was used for embryo injection. Prior to injection, each fragment was diluted to 300 copies per 5 pl in 2.5 mM Tris–HCl (pH 7.5), 0.25 mM EDTA. Transgenic mice were generated by pronuclear injection of $B6AF_1$ (female) × C57BL/6J (male) embryos, using established techniques (Hogan et al., 1986).

Mice were screened either by Southern blotting or polymerase chain reaction. The latter was performed as described (Zack et al., 1991) with the following changes: 1% SDS was used in the tail digestion buffer instead of 2% NP-40, the digested tail pieces were extracted with phenol–chloroform, and the DNA was precipitated with ethanol and dissolved in 200 µl of Tris–EDTA. One microliter was used per reaction.

Histochemistry and Immunocytochemistry

Following cervical dislocation and prior to enucleation, each eye was lightly cauterized at the superior pole of the cornea. For X-Gal histochemistry, enucleated eyes were fixed in 0.5% gluteraldehyde in phosphate-buffered saline (PBS) with 2 mM $MgCl_2$ for 30–60 min at room temperature, followed by an 18 hr incubation at 4°C in 30% sucrose in PBS. Eyes were then frozen in 2-methylbutane prechilled in liquid nitrogen and embedded in OCT compound. Sections (5–10 µm thick) were cut in a cryostat at

−22°C and mounted on gelatin-coated slides. Mounted sections were rinsed in PBS with 2 mM $MgCl_2$ containing 0.5% gluteraldehyde for 3–5 min at room temperature as described (Zack et al., 1991). Following X-Gal staining, sections were postfixed with 0.5% gluteraldehyde for 20 min at room temperature and mounted in 50% glycerol in PBS.

For double staining with X-Gal and anti-cone pigment antibodies, the sclera and choroid were peeled away from the retina starting at the optic nerve, after which the cornea and lens were removed. The free retina was fixed in 4% paraformaldehyde in PBS with 2 mM $MgCl_2$ for 30–60 min at room temperature, stained in X-Gal for 1–24 hr as described (Zack et al., 1991), postfixed with 4% paraformaldehyde in PBS with 2 mM $MgCl_2$ for 2 hr at room temperature, and cryoprotected in 30% sucrose in PBS for 18 hr at 4°C. The retina was frozen, mounted, and sectioned as described above. Immunocytochemistry was performed as previously described (Zack et al., 1991) using affinity-purified anti-human red/green or anti-blue pigment antibodies at a dilution of 1:1000 (see below).

For flat mounts of doubly stained retinas, the choroid and sclera were removed as described above. The exposed retina was stained first with X-Gal and then with affinity-purified antibodies as described above. The doubly stained retina was peeled away from the lens and cornea and mounted in 50% glycerol under a glass coverslip with the photoreceptor side facing up.

Production and Purification of Antibodies against the Human Cone Pigments

DNA segments encoding the first 57 or last 38 aa of the human red pigment (all of which are shared by the human green pigment) and the first 33 or last 42 aa of the human blue pigment were separately inserted into the polylinker of the T7 gene 10 expression vector pGEMEX (Promega). Each cone pigment-derived peptide was produced as a carboxy-terminal extension of the T7 gene 10 protein (Studier et al., 1990). The four fusion proteins were purified by preparative SDS-polycrylamide gel electrophoresis and were used to immunize rabbits. Antisera were initially tested by immunoflourescent staining of transiently transfected tissue culture cells expressing recombinant human cone pigments (Merbs and Nathans, 1992). Each was observed to stain cells transfected with the corresponding cDNA clone but not untransfected cells. Protein blots of each of the three recombinant human cone pigments and human rhodopsin showed binding of the anti-blue carboxyl terminus antisera specifically to the blue pigment, but no significant binding of the anti-red/green carboxyl terminus antisera to any of the pigments. The antisera were also analyzed by staining frozen sections of cynomolgus monkey (Macaca fascicularis; Y.-W. Peng, S. L. Merbs, and J. Nathans, unpublished data). The distribution and number of cones stained with the four types of antisera were consistent with the identities of the cone types corresponding to each immmunogen. Antisera directed against the cone pigment carboxyl termini were affinity purified and used for immunocytochemistry. For affinity purification, 250 μg of the T7 gene 10 fusion protein used to prepare the antiserum was electrophoretically blotted onto nitrocellulose following SDS-polyacrylamide gel electrophoresis and lightly stained with 0.2% Ponceau S in 3% trichloroacetic acid. A strip of nitrocellulose containing the immobilized fusion protein was excised, incubated in 10% bovine serum albumin in 50 mM Tris (pH 8.0), 150 mM NaCl (TBS) for 1 hr at room temperature, washed 3 times in water, and incubated in 1 ml of serum diluted 1:100 in TBS with 0.5% Tween-20 for 18 hr at room temperature with gentle rocking. Following several washes in TBS with 0.5% Tween-20, the bound antibody was eluted from the nitrocellulose filter with 0.3 ml of 50 mM glycine-HCl (pH 2.3), 500 mM NaCl, 0.5% Tween-20, 100 μg/ml bovine serum albumin followed by neutralization with 30 μl of 1 M Tris (pH 8.5). The elution procedure was repeated 3 times, and the eluates were combined and adjusted to a final concentration of 10 mM Tris (pH 8.0), 150 mM NaCl, 100 μg/ml bovine serum albumin, 0.05% Tween-20.

Solution β-Galactosidase Assay

This procedure was performed as previously described (Zack et al., 1991).

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