The molecular basis of cone photoreceptor-specific gene expression is largely unknown. In this study, we define cis-acting DNA sequences that control the cell type-specific expression of the zebrafish UV cone pigment gene by transient expression of green fluorescent protein transgenes following their injection into zebrafish embryos. These experiments show that 4.8 kb of 5′-flanking sequences from the zebrafish UV pigment gene directly express specifically to UV cones and that this activity requires both distal and proximal sequences. In addition, we demonstrate that a proximal region located between −215 and −110 bp (with respect to the initiator methionine codon) can function in the context of a zebrafish rhodopsin promotor to convert its specificity from rod-only expression to rod and UV cone expression. These experiments demonstrate the power of transient transgenesis in zebrafish to efficiently define cis-acting regulatory sequences in an intact vertebrate.

The existence of separate rod and cone photoreceptors is a nearly universal feature of vertebrate retinas. Most present day vertebrates have multiple classes of cone photoreceptors, with peak sensitivities that can range from −350 to −600 nm. Comparison of excitation levels among the various cone classes provides the basis for color vision.

A fundamental step in the evolution of color vision was the generation of distinct cone cell types that differentially express the corresponding cone pigment genes. The present work addresses the mechanism of this differential gene expression in the zebrafish Danio rerio. In zebrafish, the four cone types are morphologically distinct, and each expresses a single type of visual pigment (1–3). Short single cones express UV pigment; long single cones express blue pigment; short double cones express green pigment; and long double cones express red pigment. In zebrafish, as in other teleosts, the cone and rod photoreceptors are arranged in a crystalline lattice in the plane of the retina (4–7).

Zebrafish have become widely used in genetic research largely on the basis of the advantages they afford over mammals for embryological investigations. In particular, the combination of rapid development and near transparency facilitates analyses of organogenesis and patterning (8). A second experimental advantage of zebrafish is the ease with which embryos can be genetically manipulated by injection of DNA, RNA, or morpholino oligonucleotides (9–12). In the present work, we report an in vivo analysis of cis-acting DNA sequences that control expression of the zebrafish UV pigment in the appropriate photoreceptor type. These experiments take advantage of (a) the efficiency with which transiently transgenic fish can be generated by microinjection of plasmid DNA and (b) the rapid development of the zebrafish retina, in which all terminal cell fate decisions are complete within −3 days post-fertilization (dpf); (13)). The data reveal a requirement for both proximal and distal sequences 5′ of the UV pigment gene, and they further show that a small proximal sequence can mediate UV cone-specific expression in the context of the rhodopsin promotor.

MATERIALS AND METHODS

Zebrafish Genomic Clones—A commercial zebrafish genomic lambda phage library (Clontech) and a second lambda phage library prepared by partial digestion of zebrafish genomic DNA with Sau3AI were screened using probes derived from the cloned zebrafish visual pigment cDNAs (a kind gift of Drs. Thomas Vihtelic and David Hyde; (3)). Multiple independent clones corresponding to SW51, SW52, LWS-1, and the RH2 gene cluster were isolated, restriction-mapped, and partially sequenced. One clone, encompassing −4.8 kb of 5′-flanking DNA from the UV pigment gene, was fully sequenced (GenBank™ accession number AY512505). A bacterial artificial chromosome clone encompassing the four RH2 genes was isolated and subject to shotgun sequencing (GenBank™ accession number AC153391). These analyses are in good agreement with those of Chiyan et al. (14) and Takechi et al. (15).

Production of Anti-visual Pigment Antibodies—Based on sequence comparisons and the work of Vihtelic et al. (3), the following regions from the indicated zebrafish visual pigment cDNA clones were chosen as immunogens for generating specific antibodies: amino acids 1–27 and 303–326 from the UV pigment; amino acids 1–41 and 307–347 from the blue pigment; amino acids 1–34 and 232–345 from the red (LWS-1) pigment; amino acids 310–337 from the green (RH2-1) pigment; and amino acids 310–348 from rhodopsin. Each region was amplified by PCR, expressed in Escherichia coli as a C-terminal fusion to glutathione S-transferase, purified by glutathione affinity chromatography, and used for immunization of rabbits. Antisera were tested for specificity by (a) immunoblotting at 1:10,000 dilution to a set of fusion proteins containing the same visual pigment segments joined to the E. coli b-galactosidase promotor.

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1 The abbreviations used are: dpf, days post-fertilization; hpf, hours postfertilization; GFP, green fluorescent protein; PFA, paraformaldehyde; PBS, phosphate buffered saline; MBP, maltose-binding protein.
UVR Cone Pigment Expression

The full complement of zebrafish cone pigment sequences has been defined by analysis of retinal cDNA clones and their corresponding genes (Refs. 3 and 14 and see also this study). As an initial step in identifying and analyzing zebrafish cone pigment promoters, we isolated multiple overlapping lambda and bacterial artificial chromosome genomic clones encompassing the zebrafish UV, blue, green, and red pigment genes. In agreement with the report of Chinen et al. (14), we identified a single copy each of the UV pigment gene (SWS1) and the blue pigment gene (SWS2), one of the two highly homologous copies of the red pigment gene (LWS1-1), and four highly homologous copies of the green pigment gene (RH2-1, RH2-2, RH2-3, and RH2-4). To visualize different classes of retinal photoreceptors, rabbit polyclonal antibodies were raised against bacterial fusion proteins derived from the N terminus and/or from the C-terminal region of each of the major classes of zebrafish visual pigments. Among the resulting antisera, only those directed against the N termini of the UV and blue pigments appear to specifically recognize the pigment used for immunization, as determined by immunoblotting against visual pigment fusion proteins and by immunostaining of adult zebrafish retinas (Fig. 1A).

In an initial survey to identify cis-acting regulatory sequences of the UV pigment gene, transgenic fish were generated by injection with GFP reporter plasmids under the control of 10.5 or 4.8 kb 5′-flanking sequences of the UV pigment gene (SWS1), 5.2 kb 5′ of the blue pigment gene (SWS2), 10.5 kb 5′ of one of the green pigment genes (RH2-1), 8 kb 5′ of a second green pigment gene (RH2-3), or 4.5 kb 5′ of the red pigment gene (LWS1). Among this group, only the two UV pigment promoter-GFP plasmids produced detectable GFP expression in the retina (see below). Expression of the UV pigment promotor-GFP transgenes was first observed at 3 dpf and reached a maximal level at 5 dpf. Expression was not observed outside the retina, and within the retina, expression was confined to photoreceptor cells. The analyses described in this study have therefore focused on the UV pigment gene 5′-flanking region. Fig. 1B shows the structure of the UV pigment gene as deduced from the analysis of four overlapping lambda phage clones, and Fig. 1C shows a partial sequence of the 5′-flanking DNA.

Requirement for Distal Sequences of the UV Pigment Gene—The experiments described in this study involve the analysis of GFP expression between 5 and 7 dpf following injection of covalently closed circular DNA at the one or two cell stage. We will refer to this procedure as “transient transgenesis” by analogy with transient transfection of cultured cells and to distinguish it from the classic transgenesis approach, which requires screening for germline transmission of a stably integrated transgene. When live fish were examined under a dissecting microscope equipped with UV excitation and a GFP filter set, individual GFP-expressing photoreceptors were readily observed at 5 dpf. A simple rating system was devised to assess the number and intensity of the expressing cells (Fig. 2A). Eyes with greater than ~50 fluorescent cells were rated ++ +, eyes with ~5–50 fluorescent cells were rated +++, eyes with one or a few fluorescent cells were rated +, and eyes with no fluorescence were rated −. In general, those fish with larger numbers of fluorescent cells per eye also showed greater fluorescence intensity per cell. In the analyses that follow, expression efficiency for each construct has been quantitated by scoring multiple injected embryos and plotting the resulting data as a histogram. In interpreting these data, we have confined our attention to differences of ~2-fold or greater, a value in excess of the random experimental variation observed for the sample sizes used here.

As an initial step in defining sequence elements required for UV pigment expression, we constructed and analyzed a nested deletion series within the 5′-flanking region of the UV pigment gene. Fig. 2B shows the GFP expression level associated with each of nine constructs that form a deletion series from the 5′ end of the UV pigment gene 5′-flanking DNA. Constructs with 10.5, 4.796, or 4.620 kb of 5′-flanking sequence (numbered with respect to the initiator methionine codon) conferred high levels...
of GFP expression with ~70% of surviving fish showing detectable photoreceptor expression. Further deletion of 5’ sequences between ~4.620 and ~3.835 kb dramatically reduced the number of GFP-expressing cells, and even further deletion to ~2.692 kb led to expression in only a few photoreceptors in a small minority of the injected fish. 5’ sequences of 2.285 kb or less produced no detectable GFP expression. We will refer hereafter to the essential sequences located ~4.5 kb upstream of the UV pigment coding region as the “distal region.” As noted above, these experiments involved injecting covalently closed circular DNA, and therefore, the decreased expression seen with progressively smaller 5’-flanking regions cannot be due to exonucleolytic degradation of sequences adjacent to a free DNA end.

For each construct that conferred detectable photoreceptor expression, retinas from multiple fish were double-immunostained for GFP and the UV pigment. These analyses were generally conducted on whole mount retinas and viewed by confocal microscopy, a format that permits an assessment of colocalization across the entire retina. For each of the constructs described above, we observed GFP expression only in cells that were also stained with the anti-UV pigment antibody, as shown at low magnification for two retinas in Fig. 2, C and E. Higher magnification views of whole mount retinas and conventional frozen sections confirmed this conclusion and showed that GFP is predominantly in the cell body and the UV pigment is exclusively in the outer segment (Fig. 2D and data not shown). The same UV cone specificity and subcellular localization were also observed in two lines of zebrafish carrying a stably integrated transgene in which GFP is controlled by a 5.5-kb 5’-flanking region of the UV pigment gene (Fig. 2F). Takechi et al. (15) have recently reported similar observations for a stably integrated transgene with a 5.5-kb 5’-segment from the zebrafish UV pigment gene.

In transiently transgenic fish, GFP is observed in only a subset of UV cones, typically ~55% of UV cones for those constructs with the highest expression levels. As compared with transiently transgenic fish, the stable fish lines exhibited a larger fraction of GFP-expressing UV cones: ~100% of UV cones in the line shown in Fig. 2, F–H, and ~70% of UV cones in a second stably transgenic line (data not shown). This variation in transient transgene expression most likely reflects a combination of mosaicism for the plasmid DNA and epige-
FIG. 2. Role of distal 5' sequences in expression of zebrafish UV pigment transgenes. A, semiquantitative scoring system for transgenic GFP expression in living fish at 5 dpf. Examples are shown of eyes with scores of ++++, ++, +, and −. In the examples with +++ and ++ scores, numerous fluorescent photoreceptors are seen. In the +++ panel, the back of the contralateral eye is seen at the top of the image. The central green object in the +++ image is the lens (arrow), from which the fluorescent light is strongly refracted at the angle of this micrograph. Only a single fluorescent photoreceptor (arrow) is seen in the eye scored +, and no fluorescent cells are seen in the eye scored −. B, structure of transgene constructs with progressively deleted 5' regions (left) and the corresponding GFP expression levels in the retina (right). The number of transgenic fish scored for each construct is indicated to the right of the histogram. The distal (DR) and proximal (PR) regions are indicated above the construct maps; the pBS vector is not shown. The rightward green arrow represents GFP coding sequences. All constructs were injected as covalently closed circular DNA. Expression was limited to UV cones. ZUV, zebrafish UV pigment gene. C–E, whole mounts of transgenic retinas double-labeled for GFP and UV pigment showing examples of high (C and D) and low (E) densities of transgene-expressing cells directed by the constructs beginning at bp −4796 or bp −4216, respectively. In both cases, the transgene is expressed specifically in UV cones. A higher magnification image of a whole mount retina viewed at an angle to the axis of the photoreceptor cells (D) shows the accumulation of UV pigment in the outer segment and GFP in the cell body. All analyses were performed on fish at 1 week of age. F–H, UV cone-specific expression in a stable line of zebrafish carrying GFP under the control of the 10.5-kb 5' region. A frozen section through the head immunostained for GFP (green) and the UV pigment (red) is shown at low magnification (F) and at high magnification (H). In F, the layer of UV cones within each of the two retinas is strongly stained, whereas the rest of the retina, the brain, and other head structures are unstained. G, whole mount retina stained as in C and E. In some whole mount images, such as the one in G, the intensity of the immunostaining signals vary in a graded fashion across the image because the high curvature of the zebrafish retina causes the layer of fluorescent cells to progressively deviate from the relatively narrow plane of the optical section. In the overlaid image (rightmost panel in G), this effect, together with the different subcellular locations of the GFP and visual pigment proteins (panels D and H), leads to a continuous gradient across the image in the relative intensities of the two colors in GFP-expressing cells.
...metic effects that repress expression in a subset of cells. Variegated expression is also commonly observed in mice carrying visual pigment transgenes (17–19).

To further define the role of the distal region, we tested additional 5′ deletions as well as a set of internal deletions within the −4.615- to −4.216-kb region (Fig. 3). The most interesting result of this analysis is the apparent redundancy of the distal element with sequences between −4.769 and −10.5 kb. Deleting 5′ sequences between −4.615 and −4.216 kb in the context of the 10.5-kb 5′ region had little or no effect on GFP expression, whereas the same deletion in the context of the −4.769-kb 5′ region dramatically decreased GFP expression. Although deletion of distal region sequences resulted in a marked decrease in the number of transgene-expressing cells per retina, UV cone specificity was unaffected (Fig. 3B). The experiments in Fig. 3A further indicate that the full activity of the distal region requires the presence of sequences distributed throughout the −4.615- to −4.216-kb interval since deletion of either half of this interval markedly impairs function.

**Requirement for Proximal Sequences 5′ of the UV Pigment Gene**—The sequence of the promoter proximal region of the UV pigment gene reveals a presumptive TATA box 92 bp 5′ of the initiator methionine codon (Fig. 1C). To define transcriptional regulatory elements within this region, we tested deletions of sequences between −450 and −224, −201, −179, −155, or −101 bp (Fig. 4A). Deletions up to and including position −201 had little or no effect on GFP expression, but further deletion led to a progressive decrease in the efficiency GFP expression with a complete loss of GFP expression upon deletion of sequences between −450 and −101 bp. Consistent with this result, deletion of sequences between −201 and −109 bp completely eliminated GFP expression. Interestingly, deletion of sequences between −450 and either −179 or −155 produced a relaxation of photoreceptor specificity, with expression in both UV cones and at least one other photoreceptor type (Fig. 4C). By contrast, deletions that extend only to −224 or −201 retained UV cone specificity (Fig. 4B).

To further explore the sequences relevant to UV cone-specific expression, the region from −100 to −1, which includes the TATA box, was replaced with the corresponding region from the blue pigment gene (Fig. 4A, fourth construct from bottom). This construct directed UV cone-specific expression, arguing against a cone subtype-specific function for sequences within this region. In examining the UV pigment 5′ proximal region between bp −201 and −101 for potential transcription factor binding sites, we were struck by the presence of two copies of the sequence CCGTTCG between bp −135 and −110. Insertion of unique restriction sites at positions −135 and −110, flank ing the two CCGTTCG sequences, did not alter the efficiency or UV cone specificity of transgene expression (Fig. 4A, bottom construct). However, replacing the −135 to −110 region with a sequence of identical length but composed of the inserted restriction sites and a central region of irrelevant DNA significantly decreased the average number of GFP-expressing cells per retina, although it did not alter UV cone specificity (Fig. 4A, second from bottom construct). Taken together, the analyses of the proximal region suggest that sequences in-

![Figure 3](http://www.jbc.org/Downloadedfrom/article-2018-07-25.png)
involved in both expression efficiency and UV cone specificity are located within the interval −201 to −109.

UV Pigment Proximal Promotor Sequences Can Redirect the Specificity of the Rhodopsin Promotor—The experiments described above largely involve inferring function based on the effect of DNA deletions. However, the possibility that sequences responsible for high expression efficiency and for UV cone specificity might be intermingled, redundant, or overlapping complicates the interpretation of deletion analyses. To partially circumvent this problem and to specifically look for sequences that confer cell type specificity of expression, we asked whether sequences from the proximal region of the UV pigment gene could alter the specificity of a heterologous promotor. For this experiment, we have used a 1.8-kb segment from the zebrafish rhodopsin 5’ region as the heterologous promotor (Fig. 5, A and B). This segment directs rod-specific expression of a GFP reporter (Fig. 5, B and C) (see also Refs. 20 and 21). As seen in Fig. 5, C–E, and as described by Fadool (7), at 5 dpf, the development of the rod mosaic is incomplete and still spatially heterogeneous.

Fig. 5, B and D, shows that insertion in either orientation of the segment −215 to −110 of the UV pigment gene proximal region at position −139 in the zebrafish rhodopsin promotor alters the specificity of expression. Quantitation of photoreceptor specificity by double staining with antibodies directed against GFP and either rhodopsin or the UV pigment showed that expression is found at roughly equal frequency in rods and UV cones, although with wide variation among different retinas (Fig. 5B, right). Double staining with anti-blue pigment antibodies showed that transgene expression in blue cones is extremely
Fig. 5. Altered cell type-specific expression following insertion of 106 bp from the proximal region of the zebrafish UV pigment gene into the rhodopsin promoter. A, DNA sequence of the proximal region from the zebrafish rhodopsin promoter. The initiator methionine codon and conserved transcription factor binding sites are indicated. The site of insertion of the NdeI-Nhel linker at position 139 is indicated by a red arrow. B, structure and expression of rhodopsin promotor transgene constructs with different insertions at position 139. Left, each construct contains the region −1781 to −50 from the rhodopsin promoter. From top to bottom, the insertions are: NdeI and Nhel restriction sites only (red rectangles); insertion of sequences from −215 to −110 from the UV pigment promoter (purple line) in either the forward or the reverse orientation; or insertion of sequences from −277 to −167 from the zebrafish blue pigment promoter (which has the same relative location with respect to the TATA box as the −215 to −110 region of the UV promoter; blue line). All constructs were injected as covalently closed circular DNA. Center, semiquantitative scoring of transgene expression as in Figs. 2–4. Right, photoreceptor cell type specificity of transgene expression by immunostaining for GFP and rods (tan; monoclonal antibody 1D1), UV cones (purple), or blue cones (blue) or double staining for rods and UV cones while monitoring intrinsic GFP fluorescence (red). Each symbol represents the percent of GFP-expressing cells that stain with the indicated antibodies within a single transgenic retina. The number of retinas analyzed is indicated by the first number to the right of the data points (identically color-coded); the total number of photoreceptor cells scored is listed in parentheses. Thus, for the second construct, the 21 purple circles represent 21 retinas that were double-stained for UV cones and GFP and from which 1832 GFP-stained photoreceptors were scored. ZUV, zebrafish UV pigment gene. C, whole mount of a retina carrying the rhodopsin promotor transgene (with the NdeI-Nhel linker at position 139) double-labeled for GFP and either rods or UV cones. The transgene is expressed in rods but not in UV cones; the arrowhead at the far right indicates one of many transgene-expressing rods among non-expressing UV cones. D and E, whole mounts of retinas carrying the rhodopsin promotor transgene with the −215 to −105-bp fragment of the UV pigment inserted at position 139 in the forward orientation double-labeled for GFP and either rods or UV cones (D) or in the reverse orientation and double-stained for rods and UV cones with simultaneous analysis of intrinsic GFP fluorescence (E). The transgene is expressed only in rods and UV cones. At this optical plane, GFP-expressing rods are large and uniformly labeled; GFP-expressing UV cones are smaller and often show a ring of green fluorescence. In D, the arrowhead in the fourth panel indicates one GFP-expressing UV cone among both GFP-expressing and non-expressing rods, and the arrowhead in the eighth panel indicates one GFP-expressing rod among both GFP-expressing and non-expressing UV cones. In E, GFP-expressing UV cones are pale blue, and GFP-expressing rods are yellow in the overlaid images. The regions within the white squares are shown at higher magnification on the far right. Within a single cell, the imperfect overlap in GFP fluorescence and visual pigment immunostaining arises from the different subcellular localizations of GFP and visual pigment (Fig. 2).
UV Cone Pigment Expression

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rare. To determine the extent to which rods and UV cones together account for the GFP-expressing cells, retinas transgenic for the rhodopsin promoter carrying the −215- to −110-bp segment from the UV pigment promoter in reverse orientation were subject to double immunostaining for rods and UV cones while simultaneously monitoring intrinsic GFP fluorescence (Fig. 5, B and E). This analysis demonstrated that nearly 100% of transgene-expressing cells are either rods or UV cones. To control for the possibility that disruption of the rhodopsin promoter at position −139 might be the cause of transgene expression in UV cones, a 110-bp segment of the blue pigment gene promoter was inserted at the same location (Fig. 5A, bottom construct). Transgenic fish carrying the blue pigment promoter insertion expressed the transgene efficiently but showed little or no expression in UV cones, implying that insertion per se in the rhodopsin promoter at position −139 is not responsible for UV cone expression.

DISCUSSION

The experiments reported here represent a first step in dissecting cone-specific transcriptional controlling elements in zebrafish. Using transient transgenesis, we have shown that 5′-flanking sequences from the zebrafish UV pigment gene direct expression specifically to UV cones, that this activity requires both distal and proximal sequences, and that at least one proximal element located within the interval −215 to −110 bp can function in the context of a rhodopsin promoter to convert its specificity from rod-only expression to rod and UV cone expression.

The distal sequences characterized here are located within a several hundred bp region −4.5 kb 5′ of the UV pigment coding region, and they appear to be largely redundant with as yet uncharacterized sequences between −10.5 and −4.8 kb. The function of the distal sequences could be limited to the production of a promoter configuration that is generally permissive for transcription in a manner that is not cell type-specific since progressive deletion of the distal sequences leads to a progressive decrease in the efficiency of UV cone expression without a concomitant change in cell type specificity. However, we cannot rule out more complex models in which the distal sequences also carry cell type-specific information. By contrast, the partial loss of UV cone specificity attendant with deletion of sequences between −450 and −155 bp, together with the chimeric UV pigment/rhodopsin promoter experiments, clearly establish a small proximal region as a carrier of UV cone-specific information. In the chimeric UV/rhodopsin promoter experiments, the absence of expression in other cone types argues that the proximal region from the UV pigment gene carries information that specifically directs UV cone expression. Whether other sequences in the UV pigment promoter function to direct expression generally to cones remains an open question.

The failure to observe transgene expression with large 5′-flanking segments derived from the zebrafish red, green, and blue pigment genes suggests that additional enhancer or enhancer-like sequences reside within introns or at a greater distance 5′ or 3′ of the coding regions of these genes. These observations are in contrast to those obtained with transgenes derived from the human red and blue pigment genes and the mouse UV pigment gene, in which cases the sequences required for cone-specific expression reside within a region several kb 5′ of the start site of transcription (18, 19, 22). Like the zebrafish UV pigment gene, the human red pigment gene requires both proximal and distal elements.

At present, the most extensively studied vertebrate visual pigment promoter is that for rhodopsin. Multiple transcription factors (and their binding sites) have been described, including the transcription factors Crx and NRL, both of which have been shown to be essential for normal photoreceptor development in mammals (23–30). In Drosophila, multiple transcription factors and cis-acting controlling sequences have been defined for several visual pigment genes, and these have been shown in vivo to control the differential activation of visual pigment genes in the appropriate photoreceptor types (31, 32).

Transient transgenesis in zebrafish has the potential to enormously accelerate the experimental analysis of vertebrate transcriptional regulatory sequences, as it has recently been demonstrated for a piz-size-specific transcriptional control element (21). The ease and efficiency of plasmid injection into zebrafish embryos and the rapidity of zebrafish development allow expression analyses to be conducted with dozens of independent transgenic fish on a time scale of 1–2 weeks. Similarly rapid methods exist for creating both stably and transiently transgenic Xenopus tadpoles (33). The imminent completion of the zebrafish genome sequence together with this transgenesis methodology will greatly facilitate the definition of both cis-acting sequences and trans-acting factors for cone pigment gene expression in this organism.

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