BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **237,** 84–89 (1997)
ARTICLE NO. RC977081

A Novel Zebrafish Gene Expressed Specifically in the Photoreceptor Cells of the Retina[1]

Han Chang*,[2] and Walter Gilbert†
**Biophysics Program, †Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138*

Received June 23, 1997

We have identified and characterized a novel protein from adult zebrafish retina, which we named ES1. Database search revealed that the ES1 gene has significant similarity to two genes with unknown functions: the *Escherichia coli* sigma cross-reacting protein 27a (scrp27a) and the human KNP-I/GT335. *In situ* hybridization and immunohistochemistry experiments showed that both ES1 mRNA and protein are expressed specifically in adult photoreceptor cells. ES1 seems to be a cytoplasmic protein. An ES1-like antigen was also detected in photoreceptor cells of goldfish with anti-ES1 antibodies. The retina specific expression and the evolutionary conservation suggest that ES1 protein may be important for maintaining normal retina structure and function.

Because of its accessibility, simple layered structure, and well defined cell types and patterns of connection, the vertebrate retina has been subjected to extensive studies, both because of interest in understanding the retina itself, and because of interest in using the retina as a model system for the central nervous system(1). The identification and cloning of genes expressed in the retina has made it possible to dissect the molecular components of retinal development and of phototransduction pathways. Genes encoding the visual pigments, the opsin proteins, were first cloned from bovine and human(2, 3) and subsequently from many other species. Peripherin, a protein initially identified in rod outer segments(4, 5), is the product of the mouse *rds* gene(6, 7). Mice homozygous for the *rds* mutation fail to develop outer segments of photoreceptor cells. A number of other genes, all abundantly and specifically expressed in photoreceptor cells, were also found to cause degenerative retinal diseases when mutated. They include the beta subunit of rod cGMP phosophodiesterase in *retinal degeneration* (*rd*) mice(8) and rhodopsin in one type of autosomal dominant retintis pigmentosa(9). We are interested in identifying molecules expressed specifically in the retina, since these molecules often play important roles in retinal development and function. Here we describe the identification of a novel protein which is abundant in the adult zebrafish retina. We cloned the gene encoding this protein and analyzed its expression pattern. Both ES1 mRNA and protein seems to be specific for the adult retina.

MATERIALS AND METHODS

Molecular cloning and sequence analysis of ES1 gene. Poly(A)+ RNA was purified from adult eyes pooled from a hundred zebrafish using the FastTrack kit (Invitrogen). About 7 μg of poly(A)+ RNA was primed with a oligo(dT) primer, cDNA was inserted into Lambda ZAPII/EcoRI-XhoI unidirectional expression vector (Strategene), according to supplier's instruction. There are approximately 2×10^6 independent inserts in the library. The ES1 protein was separated by SDS-PAGE and transferred to nitrocellulose membrane. A trypsin digested fragment was separated by HPLC, and sequenced by Harvard Microchemistry Facility. From the 33 amino acid sequence FSHGQGMMQMNDLSKLDANSFDAVIFPGGHGIV, a degenerate oligonucleotide (GG(GCTA)ATGATGCA(GA)ATGAA(CT)GA) was designed. Using this degenerate primer and the T7 primer (inside the vector sequence), a digoxigenin-labeled DNA probe was generated by polymerase chain reaction from the zebrafish eye cDNA library. We used this probe to screen the same library, and identified 10 positive clones. One clone (5p) with the longest insert was isolated and analyzed. Nucleotide sequence was determined with the Sequenase system version 2.0 (United States Biochemical).

Northern blot analysis. Poly(A)+ RNA was purified as described above. 1 μg poly(A)+ RNA from eyes were fractionated on 1.2% agarose gel by standard procedure(10), and capillary transferred to GeneScreen nylon membrane (Du Pont). [^{32}P]-labeled DNA probe were generated from 5p plasmid by random priming using a random priming kit (BRL). Hybridization was carried out in a solution containing 50% formamide, 0.125 M sodium phosphate buffer pH7.2, 0.25 M sodium chloride, 7% SDS, and the labeled probe.

[1] The nucleotide sequence of ES1 gene was deposited to GenBank, Accession No. U10403.

[2] To whom correspondence should be addressed at current address: D243 Fairchild Bldg., SUMC, Stanford, CA 94305. Fax: 415-725-7598. E-mail: hanchang@leland.stanford.edu.

0006-291X/97 $25.00

After overnight hybridization at 42°C, the filter were washed at room temperature in 2×SSC/0.1% SDS for 15 min, 0.5×SSC/0.1% SDS for 15 min, and 0.1×SSC/0.1% SDS for 30 min. The final wash was for 30 min at 65°C in 0.1×SSC/0.1% SDS.

In situ hybridization. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C from 4 hr to overnight, and cut into 15 μm cryostat sections. The sections were further treated by ultraviolet light for 10 min, then Proteinase K (5 μg/ml in 2×SSC) treated for 5 min at room temperature. We used a digoxigenin-labeled cRNA probe spanning the entire ES1 cDNA sequence. The size of the probe was reduced by alkaline hydrolysis (60 mM sodium carbonate, 40 mM sodium bicarbonate, 60°C, 30 min). *In situ* hybridization was performed according the manufacturer's protocol (Boehringer Mannheim). Briefly, sections were prehybridized in hybridization buffer (50% formamide, 4×SSC, 1×Denhardt's, 0.5 mg/ml salmon testes DNA, 0.25 mg/ml yeast tRNA and 10% dextran sulfate) at 48°C for 2 hr. Hybridization was carried out overnight at the same conditions with approximately 2 μg/ml cRNA probe. After hybridization, slides were rinsed briefly in 2×SSC, and washed in 2×SSC/50% formamide at 48°C for 1 hr. Then slides were treated with RNAse A (30 μg/ml in 2×SSC) at 37°C for 30 min. Following RNAse A treatment, slides were further washed twice in 2×SSC/50% formamide at 48°C for 1 hr each time. After final rinsing in 2×SSC, slides were subjected to immunological detection.

Generating antibodies. The first 238 amino acids of ES1 gene were fused to the C terminal of glutathione S-transferase gene in pGEX-2T vector (Promega). We partially purified the inclusion bodies by a Triton X-100 washing protocol (Sambrook et al., 1989). The fusion protein was then further purified by SDS-PAGE, and electro-eluted from gel slices. Polyclonal antiserum to ES1 protein were produced in Balb/c mice by standard methods(11).

Immunohistochemistry. Cryostat tissue sections were prepared as described above. The mouse anti-ES1 antiserum was used at 1:1000 dilution. Immunostaining was visualized with FITC-conjugated secondary antibodies. For disassociation of photoreceptor cells, retinas were dissected from adult zebrafish, rinsed in L-15 media (Gibco), and cells were mechanically disassociated. Cells were then transferred to Chamber Slide culture chambers (Nunc) which has been coated with poly-L-lysine (1 mg/ml, 15 min , room temperature, Sigma), and centrifuged at 200×g for 5 min at room temperature. Cells were than fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, rinsed with PBS, and permeablized with 1% Triton X-100 in PBS. Immunostaining with anti-ES1 antibody was carried out the same as above.

Western blot. Total proteins from different tissue were obtain by boiling for 5 min in denaturing SDS sample buffer, separated by denaturing SDS-PAGE, transferred to Immobilon-P PVDF membrane (Millipore) and probed with anti-ES1 antiserum according to standard protocol. The binding of specific antibodies were detected by chemiluminescent system (TROPIX). Triton X-114 detergent phase separation experiments were carried out as described by Bordier(12).

RESULTS AND DISCUSSION

Molecular cloning and sequence analysis of ES1 gene. The ES1 protein was first noticed because it appears as a major protein band on SDS-PAGE specifically from a retinal sample, and also because it cross-reacts slightly with a retina specific antibody 7A11 (Chang and Gilbert, 1992, Soc. Neurosci., abstract). This retina specific protein was purified by SDS-PAGE, transferred to nitrocellulose membrane and digested with trypsin. The digested peptides were separated by HPLC. The sequence of 33 amino acid residues was obtained by microsequencing one of the peptides. Based on the peptide sequence, we isolated a cDNA clone for the ES1 gene from an adult zebrafish eye cDNA library. The first methionine codon at position 61 was assigned as the initiation codon(13), which is followed by an open reading frame of 810 bp and a stop codon at position 871 (Figure 1). Further downstream is the 3′ untranslated sequence including a polyadenylation signal sequence AATAAA and a poly(A)+ tail. An in frame stop codon upstream of the putative initiation codon suggests that 5p insert contains the entire coding region. Translation of the open reading frame predicts a polypeptide of 270 amino acids with a calculated mass of 30.7 kd. The 33 amino acids peptide sequence from peptide microsequencing matches perfectly the predicted protein sequence from residue 105 to 137. The predicted amino acid sequence is relatively rich in methionine (8% compared to average 1.7%).

```
   1  GGAATTCGGCACGAGGTCACTTTATCTCTCTGTGTGTTTTAAAGCTACAGTAAATCAGTC   60

  61  ATGTTGGCATCTCGGGCTCTCCTCGCCAAGCAGGCCGCAGCCATGCTGGTGCGCCAACCT  120
   1  M  L  A  S  R  A  L  L  A  K  Q  A  A  A  M  L  V  R  Q  P   20

 121  GCCTGCCTGATGCATCACGGGGGAGACTGGGGCAACTGGGGCAACACCAACATCGCTGTG  180
  21  A  C  L  M  H  H  G  G  D  W  G  N  W  G  N  T  N  I  A  V   40

 181  GTTTTCTCTGGTTGTGGTTGGTGGGACGGGACTGACATCCATGAGGCTGCATACACCATG  240
  41  V  F  S  G  C  G  W  W  D  G  T  D  I  H  E  A  A  Y  T  M   60

 241  TACCACTTGAGCCGTAATGGAGCCCGTTTTCAGATCTTTGCTCCAAACCAGCAGCAAATG  300
  61  Y  H  L  S  R  N  G  A  R  F  Q  I  F  A  P  N  Q  Q  Q  M   80

 301  CATGTCATGGATCACATGAAGATGCAGCCATCTTCCAGCGACAACAGGAACATAATGATG  360
  81  H  V  M  D  H  M  K  M  Q  P  S  S  S  D  N  R  N  I  M  M  100

 361  GAGTCTGCGCGATTCAGCCACGGTCAGGGAATGATGCAGATGAACGATCTGTCCAAACTG  420
 101  E  S  A  R  F  S  H  G  Q  G  M  M  Q  M  N  D  L  S  K  L  120

 421  GACGCTAACAGTTTTGATGCCGTCATCTTTCCTGGAGGCCACGGCATCGTCAAAAACATG  480
 121  D  A  N  S  F  D  A  V  I  F  P  G  G  H  G  I  V  K  N  M  140

 481  TCCACCTTCAGCAAAGATGGCAAAGACTGCAAGTTGAATAATGACGTGGAAAAGAGTTCTG  540
 141  S  T  F  S  K  D  G  K  D  C  K  L  N  N  D  V  E  R  V  L  160

 541  AAGGACTTCCACCGCGCTCGCAAACCCATTGGGTTGTCCAGCATGGCTCCTCTCCTGGCG  600
 161  K  D  F  H  R  A  R  K  P  I  G  L  S  S  M  A  P  L  L  A  180

 601  TGTCGAGTTCTGCCCAGTCTGGAGGTCACGATGGGTTATGAGCGGGACGAGAGCAGCCGC  660
 181  C  R  V  L  P  S  L  E  V  T  M  G  Y  E  R  D  E  S  S  R  200

 661  TGGGGCCGCTGGCCAAATACCAACATGGTGCAGGCGGTCAAGAGCATGGGGGCCCGTCAC  720
 201  W  G  R  W  P  N  T  N  M  V  Q  A  V  K  S  M  G  A  R  H  220

 721  AACACCCGTGAACCTTACGAAGCCTATGTGGACGAGAAAAACAAGGTGATCAGCACTCCG  780
 221  N  T  R  E  P  Y  E  A  Y  V  D  E  K  N  K  V  I  S  T  P  240

 781  ACCTTCATGTGGGAGACCGACTACCACTACCACTACATCTTTGACGGCATCGGGAACATG  840
 241  T  F  M  W  E  T  D  Y  H  Y  H  Y  I  F  D  G  I  G  N  M  260

 841  GTGAAGCACGTCATGCGCATGACCGCCAAATGAGTCTCACCAGACCACAAACACCTGAAG  900
 261  V  K  H  V  M  R  M  T  A  K  *                              280

 901  GCTTTCACACTTAACTTTAGGTTGACCAGAATCCACTGTGATGTGGGCGTACAGAGAGTA  960
 961  AATAAAGAAAAGCATGCAATGCAAAAAAAAAAAAAAAAAAAAACTCGAG  1008
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FIG. 1. Nucleotide and predicted amino acid sequence of ES1. The region corresponding to the sequence obtained by peptide sequencing is underlined. The polyadenylation signal is marked by asterisks.

We searched databases with ES1 amino acid and nucleotide sequence by BLASTN and BLASTP programs. Two genes with unknown function were found to have significant similarity to the ES1 gene: the *Escherichia coli* sigma cross-reacting protein 27A (scrp27a)(14) and the human KNP-I/GT335(15, 16). The ES1 protein is

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es1          1 MLASRALLA.KQAAAMLVRQPACLMHHGGDWGN......WGNTNIAVVFS
gt335        1 MAAVRALVASRLAAASAFTSLSPGGRTPSQRAALHLSVPRPAARVALVLS
scrp27a      1 ..................................MITMKKIGVILS

es1         44 GCGWWDGTDIHEAAYTMYHLSRNGARFQIFAPNQQQMHVMDHMKMQPSSS
gt335       51 GCGVYDGTEIHEASAILVHLSRGGAEVQIFAPDVPQMHVIDHTKGQPSEG
scrp27a     13 GCGVYDGSEIHEAVLTLLAISRSGAQAVCFAPDKQQVDVINHLTGEA.MT

es1         94 DNRNIMMESARFSHGQGMMQMNDLSKLDANSFDAVIFPGGHGIVKNMSTF
gt335      101 ESRNVLTESARIARG....KITDLANLSAANHDAAIFPGGFGAAKNLSTF
scrp27a     62 ETRNVLIEAARITRG....EIRPLAQADAAELDALIVPGGFGAAKNLSNF

es1        144 SKDGKDCKLNNDVERVLKDFHRARKPIGLSSMAPLLACRVLP.SLEVTMG
gt335      147 AVDGKDCKVNKEVERVLKEFHQAGKPIGLCCIAPVLAAKVLR.GVEVTVG
scrp27a    108 ASLGSECTVDRELKALAQAMHQAGKPLGFMCIAPAMLPKIFDFPLRLTIG

es1        193 YERDESSRWGRWPNTNMVQAVKSMGARHNTREPYEAYVDEKNKVISTPTF
gt335      196 HEQEEG...GKWPYAGTAEAIKALGAKHCVKEVVEAHVDQKNKVVTTPAF
scrp27a    158 TDID.............TAEVLEEMGAEHVPCPVDDIVVDEDNKIVTTPAY

es1        243 MWETDYHYHYIFDGIGNMVKHVMRMTAK
gt335      243 MCETA..LHYIHDGIGAMVRKVLELTGK
scrp27a    196 M..LAQNIAEAASGIDKLVSRVLVLAE.
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FIG. 2. Amino acid sequence alignment of ES1, KNP-I/GT335, and scrp27a proteins. Identical amino acid residuals are highlighted with black background; similar amino acid residuals are shaded in gray.

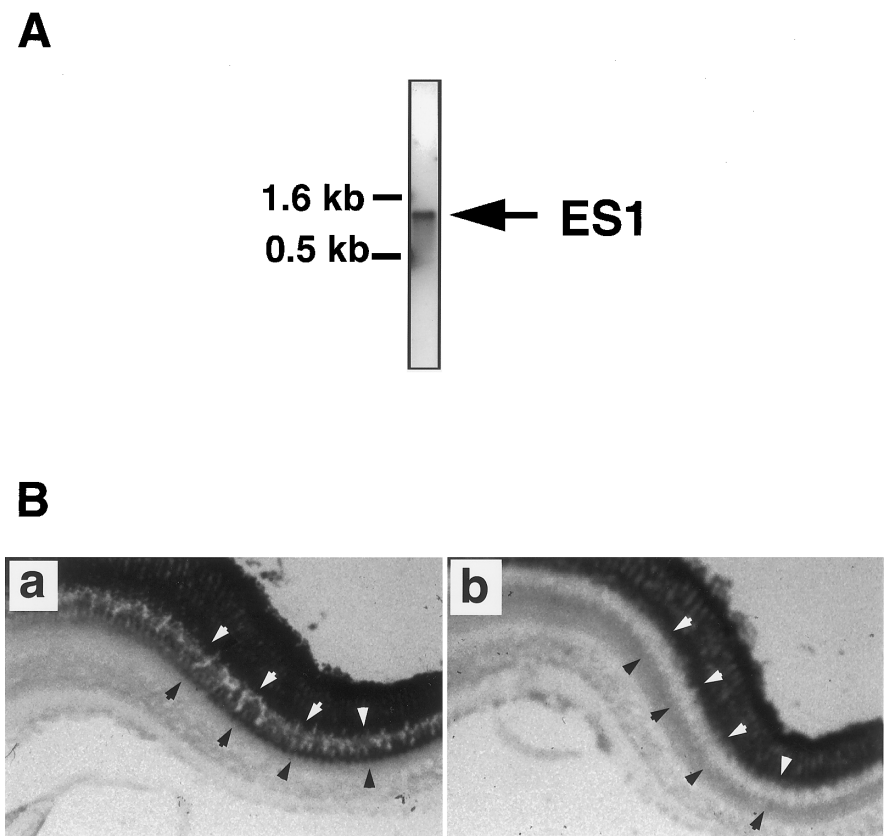

FIG. 3. Expression of ES1 mRNA. (A) Northern blot analysis of mRNA from adult zebrafish eyes with a probe spanning the whole ES1 cDNA inserts. About 1 μg of poly A(+) RNA was loaded. Arrow points to the single ES1 band detected, which is about 1.2 kb. (B) Localization of ES1 transcripts in zebrafish photoreceptor cells. (a) Adult zebrafish eye horizontal sections hybridized with digoxigenin labeled antisense cRNA probe. Between arrows is the photoreceptor cell layer, which shows a strong signal. The broad dark layer is the pigment epithelium layer (pe). (b) Adjacent section hybridized with sense cRNA probe as control. The photoreceptor cell layer between arrows shows no staining.

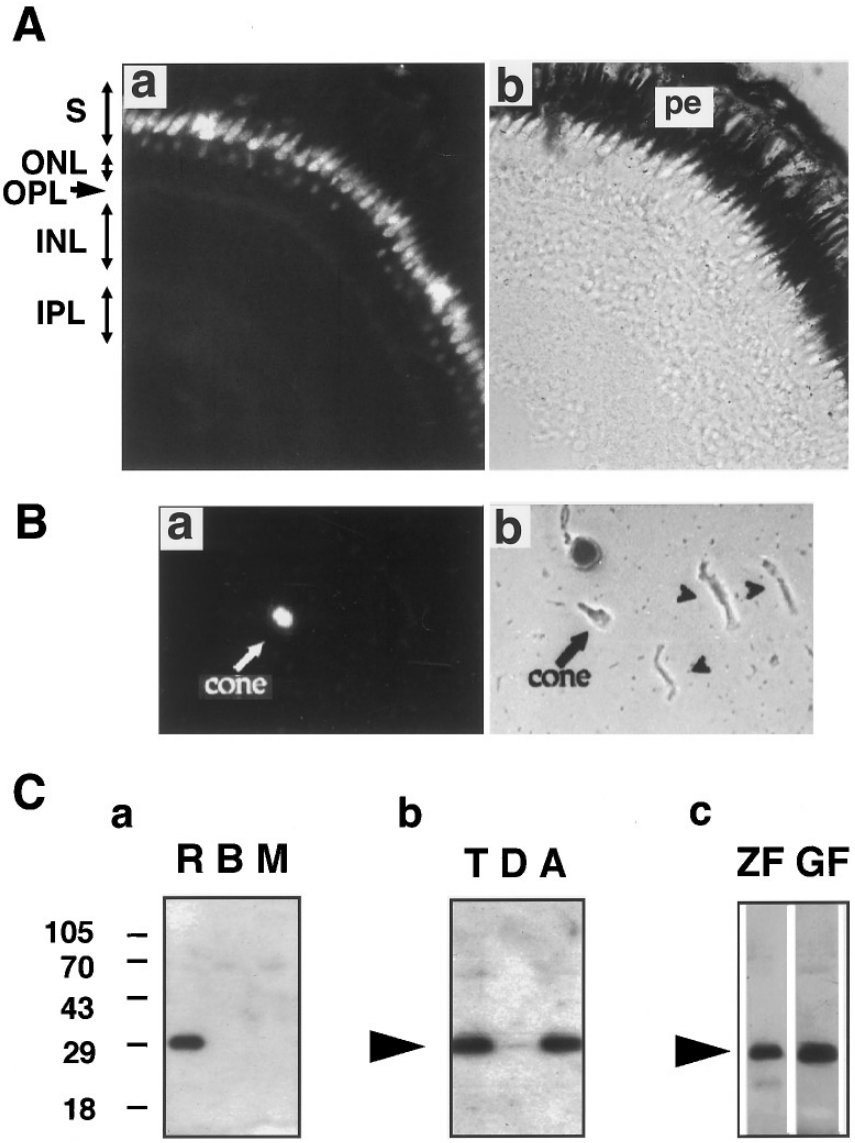

FIG. 4. Expression of ES1 protein. (A) Immunofluorescent labeling photoreceptor cells with anti-ES1 antiserum. (a) 15 μm horizontal cryostat sections of adult zebrafish eyes are stained with anti-ES1 antibodies. Cone photoreceptor cells are clearly labeled. S, outer and inner segments of photoreceptor cells; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer. (b) Phase contrast view of the same area. Dark area on the upper-right is the pigment epithelium layer (pe). (B) Immunofluorescent staining of disassociated photoreceptor cells. Photoreceptor cells were disassociated as described in Experimental Procedures. (a) Immunofluorescent staining with anti-ES1 antiserum. (b) Phase contrast image. The cone photoreceptor cell is clearly stained (arrow), while rod photoreceptor cells are not stained (arrowheads). (C) Western blot analysis. (a) Total proteins from various tissues of adult zebrafish were separated by SDS-PAGE (12% polyacrylamide gel), transferred to PVDF filter, and probed with anti-ES1 antiserum. ES1 protein was only detected in the retina sample. R, retina; B, brain; M, muscle, skin, and connecting tissues. (b) Western blot of Triton X-114 fractionated retina protein. Arrowhead points to bands labeled by the anti-ES1 antiserum. Clearly the ES1 protein largely partitioned into the aqueous phase. T, unfractionated total protein; D, detergent phase; A, aqueous phase. (c) Expression of an ES1-like protein in adult goldfish retina. Total proteins were separated by SDS-PAGE (12% polyacrylamide gel), transferred to PVDF filter, and probed with the anti-ES1 antiserum. Arrowhead points to bands labeled by the anti-ES1 antiserum. ZF, zebrafish retina; GF, goldfish retina.

27% identical to the scrp27a and 51% identical to the KNP-I/GT335 protein (Figure 2). With the exception of about 20 amino acids near the N-terminus, the similarity of three gene extends through the entire sequences. PROSITE and BLOCK searches with sequences of ES1, scrp27a, and KNP-I/GT335 proteins gave no clues of their functions. Interestingly, the homology between ES1 and scrp27a (72 identical amino acids) is lower than that between KNP-I/GT335 and scrp27a (101 identical amino acids).

Expression of ES1 mRNA. Northern blot analysis of mRNA from adult zebrafish eyes (Figure 3A), using the entire 5p cDNA insert as a probe, shows that the ES1 gene produces a single transcript of 1.2 kb. We further examined ES1 expression in the adult zebrafish retina by in situ hybridization experiments using a digoxigenin (DIG) labeled antisense cRNA probe transcribed from the 5p plasmid. As in Figure 3B, an intense signal was detected in the photoreceptor cell layer in adult zebrafish retina, but not in other retinal layers. While no signal was detected with a sense control probe. Using the same DIG-labeled probe, we did not detected any signal in adult zebrafish brain sections, indicating none or very little ES1 is expressed in the adult brain (data not shown).

Expression of ES1 protein. The retinal distribution of ES1 protein was examined by indirect immunofluorescent staining of cryostat sections (Figure 4A). In adult zebrafish, ES1 protein is located exclusively in the photoreceptor cell layer of the retina. This pattern is similar to that we observed in in situ hybridization experiments described above. There are four types of photoreceptors in zebrafish retina: short single cones, long single cones, double cones, and rods; each type of cones can be identified by its position in the photoreceptor layer(17, 18). Close examination of the adult retina sections reveals that the inner segments of all cone cells were strongly stained with anti-ES1 antiserum. No staining appears in the nucleus of photoreceptor cells (the outer nuclear layer, Figure 4A), suggesting that ES1 is not a nuclear protein. Due to the partial obstruction by pigments of the retina, it is difficult to examine the staining of rod photoreceptor cells. To address these issues, we disassociated adult zebrafish photoreceptor cells and stained individual photoreceptor cells with anti-ES1 antibody. As in Figure 4B, rod photoreceptor cells are not stained. Furthermore, we do not detect any staining of the outer segment of cones. In summary, ES1 protein is only detected in the inner segments of cone photoreceptor cells.

We further tested the presence of ES1 protein in various adult zebrafish tissues by western blot. As in Figure 4C, the ES1 antigen is expressed specifically in the retina, with an apparent molecular weight of about 29 kd. No detectable ES1 protein was present in either the brain or in the sample of non-neuronal tissues (including muscle, skin, and connecting tissue).

We tested whether ES1 protein is associated with membrane with Triton X-114 detergent phase separation experiment(12). Integral membrane protein will partition into the detergent-rich pellet phase, whereas soluble proteins will remain in the upper aqueous phase. As shown in Figure 4C, ES1 protein largely partitioned into the aqueous phase, suggesting that ES1 is not a integral membrane protein.

ES1 protein was not detected in 4 day and 12 day old zebrafish embryos by immunostaining tissue sections and by western blots (Data not shown). Immunostaining of retina sections does not show any staining until about 20 days postfertilization (Data not shown). It has been reported that although all photoreceptor cells can be identified at 12 day postfertilization, they do not complete major differentiation processes and start to be adult-like until about 20 day postfertilization(17, 19). Therefore it seems that ES1 protein is only expressed in mature photoreceptors.

The sequence homology of the zebrafish ES1, the *Escherichia coli* scrp27a, and the human KNP-I/GT335 genes suggests that they share a common ancestor. However, since a much wider expression pattern was observed for the KNP-I/GT335 gene(15, 16), the KNP-I/GT335 does not seem to be the human ortholog of the zebrafish ES1 gene. On the other hand, we detected an ES1-like protein band in goldfish retinal samples with the anti-ES1 antiserum (Figure 4C). Indirect immunofluorescent staining of goldfish retina sections shows that only the photoreceptor cells are stained with the anti-ES1 antiserum (Data not shown). These experiments suggest that goldfish has a ES1-like gene.

ACKNOWLEDGMENTS

We thank members of Dr. Gilbert's group, Dr. J. E. Dowling, and members of Dr. Dowling's group for advice and valuable discussion. This work was supported by grants from NIH (to W.G.).

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