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Pineal Opsin: A Nonvisual Opsin Expressed in Chick Pineal

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Pineal opsin (P-opsin), an opsin from chick that is highly expressed in pineal but is not detectable in retina, was cloned by the polymerase chain reaction. It is likely that the P-opsin lineage diverged from the retinal opsins early in opsin evolution. The amino acid sequence of P-opsin is 42 to 46 percent identical to that of the retinal opsins. P-opsin is a seven-membrane spanning, G protein–linked receptor with a Schiff-base lysine in the seventh membrane span and a Schiff-base counterion in the third membrane span. The primary sequence of P-opsin suggests that it will be maximally sensitive to ~500-nanometer light and produce a slow and prolonged phototransduction response consistent with the nonvisual function of pineal photoreception.

All identified vertebrate opsins fit into four major groups based on conservation at the protein level, epitomized by chick green, blue, violet, and red opsins (*1*, *2*). The order of divergence of these groups is not well resolved. The green opsin cluster contains as a subgroup the rod opsins (rhodopsin) used for scotopic vision (*2*). Within the red cluster multiple duplication events gave rise to closely related "green" opsins in Old World primates and some fish (*3*). Opsins within a given class share ~65 to 95% amino acid identity, whereas identity among classes is only ~40 to 50%.

The chick pineal is thought to contain a rhodopsin-like photoreceptor with a maximal sensitivity at 500 nm (*4*), based on the action spectrum for light-induced suppression of *N*-acetyltransferase activity, which reduces melatonin synthesis. Two other pieces of evidence also suggest that the pineal gland contains an opsin-like protein: (i) Vitamin A depletion from cultured pineal cells reduces light-mediated suppression of melatonin synthesis, suggesting the action of a vitamin A–based pigment (*5*) (presumably an opsin-bound chromophore) and (ii) immunocytochemistry indicates the presence of one or more opsin-like proteins in avian pinealocytes (*6*).

To identify the opsin or opsins expressed in pineal, we used degenerate oligonucleotide primers and reverse transcriptase–polymerase chain reaction (RT-PCR) (*7*) to amplify and clone opsin-like complementary DNAs (cDNAs) from chick pineal. Using primers to conserved regions flanking the Schiff-base lysine in the seventh transmembrane domain, we recovered three distinct opsin-like cDNAs: chick red opsin, chick green opsin, and a previously unidentified opsin [pineal opsin (P-opsin)]. Ribonuclease (RNase) protection (*8*) detected abundant P-opsin RNA but no retinal opsin RNAs in pineal (Fig. 1A) (*9*). RNase protection detected no expression of P-opsin transcript in chick retinal RNA although, as expected, red, green, blue, violet, and rhodopsin transcripts were detected. Neither P-opsin nor retinal opsin RNAs were detected in chick brain by RNase protection. In situ hybridization (*10*) demonstrated expression of P-opsin in most pinealocytes (Fig. 1, B and C), but not in chick retina. Thus, P-opsin is an abundant pineal transcript absent from retina.

A pineal-specific cDNA library was

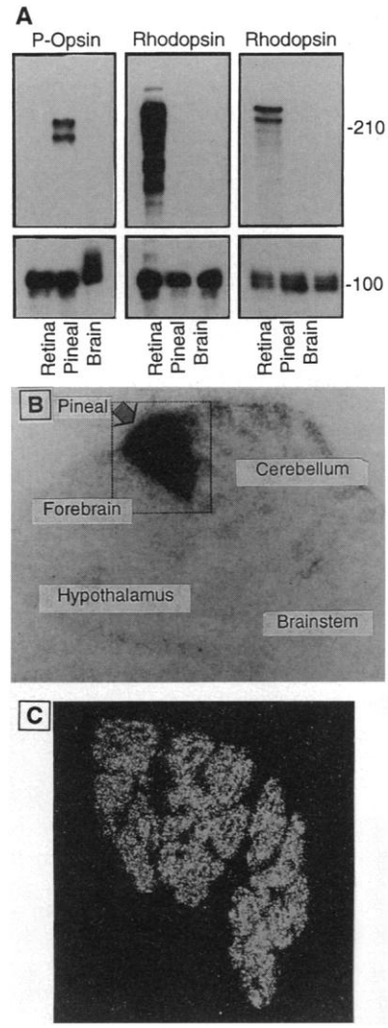

Fig. 1. Expression of P-opsin mRNA. (**A**) RNase protection assay (*8*) with RNA (~30 μg) from chick pineal, retina, or brain. (Left) Exposure for 8 hours shows abundant P-opsin expression in pineal but no expression elsewhere. (Middle) Eight-hour exposure shows that rhodopsin is not expressed in pineal; however, its protected product in retina appears to smear due to overexposure. (Right) Four-hour exposure of the rhodopsin protection shows the appropriate P-opsin bands in retina at ~210 bp. In other RNase protection assays, protected products were detected with probes made from chick green, red, blue, and violet opsins in retinal RNA but not in pineal RNA. All probes include 70 bp of vector sequence: No full-length probe could be detected after RNase digestion, indicating that only sequence-specific probe fragments of the correct size were protected. All protections included an actin standard (100-bp product) to control the amount of target RNA in each assay. (**B**) Autoradiograph of in situ hybridization (*10*) of ^{33}P-labeled P-opsin probe to a sagittal section of chick brain including attached pineal. The dotted square around the pineal gland corresponds to the view in (C). (**C**) Dark field view (40× magnification) of the boxed area of the section in (B) after the slide was dipped in emulsion (NTB 2 from Kodak), exposed for 2 weeks, and developed. The label is restricted to the pineal. Retina is not labeled by this probe and the sense probe control does not label the brain, pineal, or retina.

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screened with the cloned P-opsin PCR product (*11–13*) to obtain a full length P-opsin clone. The longest clone [1696 base pairs (bp)] with a plausible transcription start site and ATG sequence contained two stretches of open reading frame that together encode an opsin-like protein of 351 amino acid residues (Fig. 2A). We confirmed that this clone is full length by primer extension (*11*). This clone contains a 181-bp unprocessed intron (based on alignment with retinal opsin cDNAs) that was not present in the

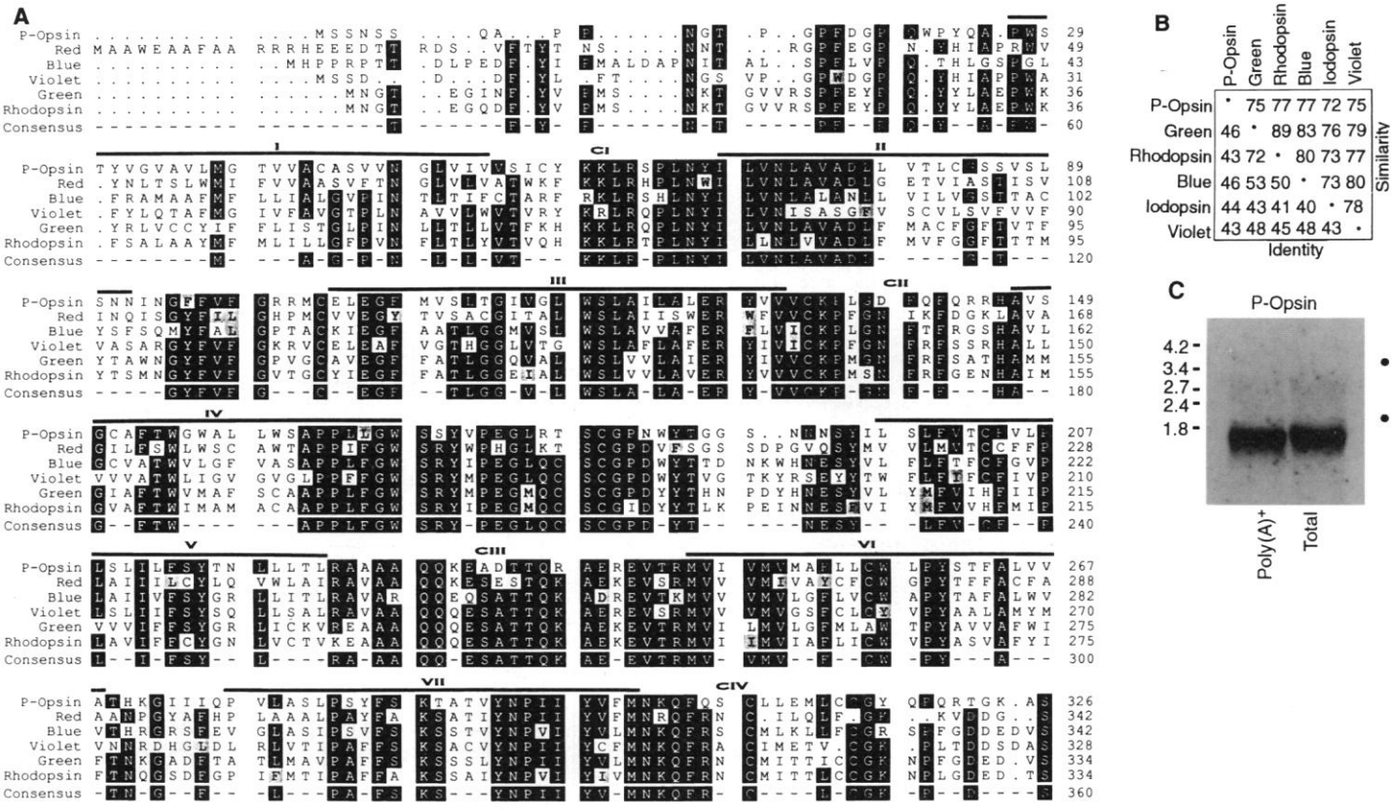
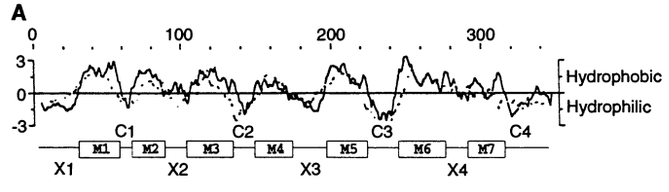


Fig. 2. Comparison of the P-opsin amino acid sequence (*12*) to that of the chick retinal opsins (*2*). (**A**) An optimized alignment of the P-opsin amino acid sequence to that of the chicken retinal opsins [single-letter code (*30*)]. Regions of amino acid identity are represented by white letters boxed in black. Conservative substitutions are shown by black letters boxed in gray. Unboxed black letters correspond to nonconsensus regions. Dots correspond to gaps introduced in the sequence to optimize alignment. A line showing consensus of four or more amino acid residues appears along the bottom (white letters boxed in black). Bars along the top of the alignment indicate transmembrane regions (I to VII) and cytoplasmic loops (CI to CIV). The alignment was determined iteratively by the Bestfit routine of the Wisconsin GCG software package (*15*) with modifications by eye for best fit of the divergent NH_2- and COOH-terminal regions. (**B**) Amino acid relatedness of chick opsins. The percent of identity between any two opsins is shown below the diagonal and the percent of similarity (identities and conservative changes) between any two opsins is shown above the diagonal. (**C**) Northern blot (*11, 14*) of pineal polyadenylated (3 μg) and total (30 μg) RNA labeled with P-opsin cDNA probe. Numbers on the left of the blot show the size of DNA markers (in kilobases) and the dots to the right of the blot show the positions of chick ribosomal RNA bands.



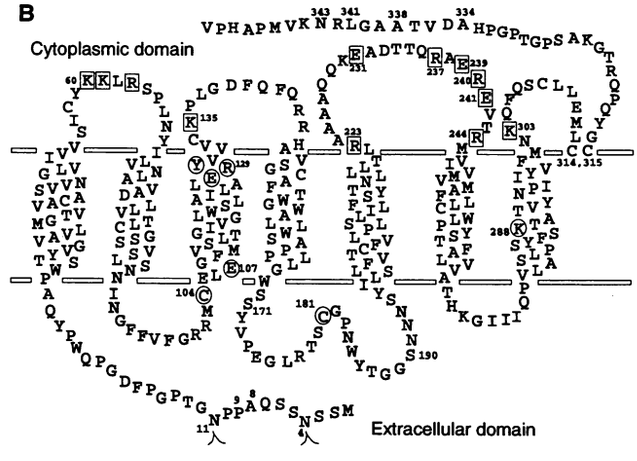


Fig. 3. Predicted secondary structure of P-opsin. (**A**) The Kyte-Doolittle (*15*) hydrophobicity profile of P-opsin with the putative transmembrane regions (M1 to M7), cytoplasmic loops (C1 to C4), and extracellular regions (X1 to X4) indicated. (**B**) The proposed secondary structure of P-opsin embedded within the membrane (represented by the two double lines). The cytoplasmic domain is shown to have four loops homologous to those of the other opsins; this face of the molecule is expected to interact with one or more G proteins (*16*). The regions below the bottom double line are presumed to be extracellular (rather than intradiscal) because pinealocytes have modified cone-like disk membranes rather than rod-like disks (*6*). Numbered amino acids are discussed in the text.

shorter clones. The predicted size of the fully processed transcript is 1515 bp, which corresponds to the size of the band detected by northern (RNA) blot (*11*, *14*) of pineal RNA (Fig. 2C). The predicted amino acid sequence is 42 to 46% identical with that of retinal opsins (Fig. 2B), suggesting that P-opsin does not fit into any of the previously identified classes of opsin genes.

P-opsin is a typical seven-membrane spanning protein like the retinal opsins and other GTP-binding protein (G protein)–linked receptors (Fig. 3A) (*15*). Regions of conservation in the retinal opsins are largely conserved in P-opsin (Fig. 2A). The following observations suggest a functional similarity between P-opsin and the retinal opsins (Fig. 3B): (i) a lysine (K288) in the appropriate position within the seventh membrane spanning region to act as the Schiff-base linkage with 11-*cis* retinal (*16*); (ii) a glutamic acid (E107) within the third membrane span to serve as the Schiff-base counterion (*16*); (iii) a pair of cysteines (C104 in the third membrane span and C181 in the third extracellular loop) which are thought to promote folding and in rhodopsin to stabilize the meta II form (*16*, *17*); (iv) predicted N-linked glycosylation sites (N4, N11) (*16*); (v) a pair of cysteines (C314,315) in the carboxyl tail that are palmitoylated in rhodopsin and anchor the tail to the membrane and thus form a fourth cytoplasmic

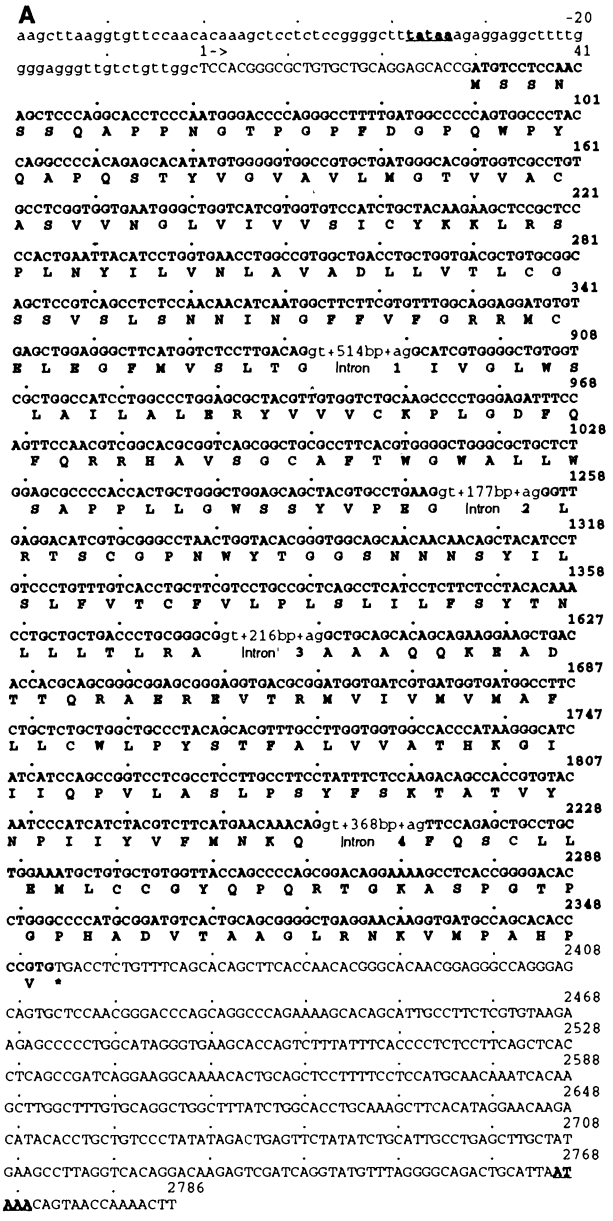


Fig. 4. The genomic structure of P-opsin and the evolutionary relationship of P-opsin to the retinal opsins. (**A**) Coding sequence from P-opsin genomic clone GP3 (*22*) as well as some 5′ flanking DNA −79 bp 5′ of the transcription start site and extending to the end of the coding sequence. DNA encoding protein is shown in uppercase bold typeface and the amino acid translation appears below. The positions of the introns are shown in lowercase nonbold typeface. Included are the intron-exon splice donor sites and the number of bases in between. The 5′ and 3′ untranslated regions are shown in uppercase nonbold typeface. The putative TATAA box is shown in bold and underlined in the 5′ flanking region as is the polyadenylation signal sequence in the 3′ untranslated region. (**B**) The reconstructed phylogenetic tree of vertebrate opsins including P-opsin. The tree was reconstructed with the PHYLIP package of programs (*1*, *23*). The tree is unrooted. Initial trees constructed with a selection of invertebrate opsins indicated that invertebrate opsins are strongly resolved from vertebrate opsins; thus, *Drosophila* opsin was chosen as the outgroup to draw the tree. Numbers in parentheses at each node show the number of trees with this pairing (n = 100 sample sets). Values of <60% pairing indicate that the appropriate pairing is unresolved and pairing of <70% is weak. All sequences are available through GenBank. Species designations are as follows: CAV, cavefish (*Astyamax fasciatus*); ZEF, zebrafish (*Brachydanio rerio*); GOF, goldfish (*Carassius auratus*); SGB, sandgoby (*Pomatoschistus mintus*); FRG, frog (*Rana pipiens*); XEN, (*Xenopus laevis*); XLTAD, tadpole (*X. laevis*); CHK, chick (*Gallus gallus*); BOV, cow (*Bos taurus*); OVI, sheep (*Ovis aries*); MUR, mouse (*Mus musculus*); CG, hamster (*Cricetulus griseus*); HUM, human (*Homo sapiens*); Can, dog (*Canis familieris*); LNP, lamprey (*Lampetra japonica*); GEC, lizard (*Gekko gekko*); ANC, lizard (*Anolis carolinensis*); CJAC, marmoset (*Calithrix jaccus*); and DRMGH1, fruit fly Rh1-6 (*Drosophila melanogaster*). The number after the P in the gene designation indicates absorption maxima. Other abbreviations are as follows: RHO, rhodopsin; GR, green. Major groupings agree with previous opsin phylogenetic trees (*1*, *2*), consistent with the suggestion that P-opsin is a separate lineage (that is, a fifth major group) within the vertebrate opsin family.

loop (*16*); (vi) a ERY or DRY (E128-Y130) motif in the third membrane span that is thought to participate in receptor–G-protein interactions (*16*); and (vii) several charged amino acids in the cytoplasmic loops (K60, K61, R63, K135, R223, E231, K237, E239, R240, E241, R244, K303) conserved in the chick retinal opsins and thought to be important for G-protein activation (*18*).

P-opsin also has several unique features when compared to the retinal opsins. The multiple serines and threonines in the carboxyl tail of the retinal opsins are not present in P-opsin (amino acids 334, 338, 341, and 343). These residues are phosphorylated in metarhodopsin by rhodopsin kinase, which increases the affinity of rhodopsin for S-antigen (arrestin) and decreases its affinity for transducin (thereby shutting off the transduction cascade) (*19*). P-opsin may be inactivated by a different mechanism or it may produce a prolonged light response as observed in arrestin mutants of *Drosophila* (*20*). In addition, P-opsin has an alanine at amino acid 232 rather than the conserved serine present in the retinal opsins. In rhodopsin this mutation reduces its ability to activate rod transducin by half (*21*), suggesting that the interaction of P-opsin with its G-protein partner may be similarly altered.

A chicken genomic library was screened with the P-opsin cDNA as a probe (*11*, *13*, *22*). We obtained 12 positive clones, one of which (GP3) contained the entire protein-coding region (Fig. 4A). The genomic organization of the P-opsin gene is similar to that of the short-wavelength opsin genes (it has five exons rather than the six exons of the red and red-like green opsins) except that the position of the second intron is displaced 15 nucleotides in the 3′ direction.

A preliminary evaluation of the evolutionary relationship of P-opsin to the retinal opsins was assessed by the neighbor-joining method with the PHYLIP package of programs (*1*, *23*). Individual runs with the full fixed alignment of sequences often pair P-opsin with the red and red-like green branch of the opsin phylogenic tree (putative node A′, Fig. 4B), but when bootstrap resampling is applied, P-opsin pairs more often with the blue, violet, green, and rhodopsin branch of the tree (node A), consistent with its genomic organization (Fig. 4A). However, this pairing occurs only 56 out of 100 times, which is inadequate to resolve the node. The phylogenic tree analysis suggests that P-opsin branches early from the other opsin groups but does not resolve its pairing. Several other pairings in this tree are also not adequately resolved, particularly the blue and violet clusters and the nodes marked B to F. Some of these nodes (including the node giving rise to P-opsin) may be better resolved as more opsin sequences become available.

Although no attempt was made in this tree to equate node position with evolutionary time, several lines of evidence suggest that the divergence of each of the major opsin groups occurred before the vertebrate radiation. (i) Homologous genes for each group are found across multiple vertebrate classes. (ii) Identity between groups is greater at the nucleotide level than at the amino acid level, suggesting selection for functional divergence by amino acid substitution in critical regions. (iii) The genomic structure is highly conserved between groups. Based on the homology of P-opsin with the retinal opsins (and assuming that evolutionary rates are equivalent among vertebrate opsin genes), it appears that the P-opsin gene is at least as old as the vertebrate lineage.

The function of P-opsin may differ significantly from that of the retinal opsins because photoreception in pineal cells seems to differ in important ways from that of retinal photoreceptors. Pineal cells retain the ability to respond to light after many days in dispersed cell culture and with repeated exposure to bright light (*24*), despite the apparent absence of any retinal isomerase or retinal pigment epithelium (RPE) equivalent cell type [RPE cells appear to be the sole site of re-isomerization of all-*trans* retinal to 11-*cis* in vertebrate retina and thus are required for regeneration of active retinal chromophore after photobleaching (*25*)]. Perhaps pineal opsin or opsins can bind all-*trans* retinal and photoconvert it to 11-*cis* as do the invertebrate pigments (*26*).

The pineal shows two responses to light (both of which are maximally sensitive near 500 nm) (*4*, *27*): (i) Melatonin synthesis is acutely suppressed and (ii) the phase of the circadian rhythm of melatonin production is shifted (entrainment). Several lines of evidence suggest that two separate transduction pathways mediate these responses. The acute transduction pathway is sensitive to pertussis toxin and to vitamin A depletion, whereas the entrainment pathway is not (*5*, *24*, *28*). One would expect that P-opsin could mediate one or both of these transduction pathways. The identification and cloning of P-opsin should provide the means to study the molecular mechanisms underlying nonvisual phototransduction.

Note added in proof: Subsequent to submission of our manuscript, Okano *et al.* (*29*) reported the cloning of an opsin cDNA (named pinopsin) that is expressed in chick pineal. The inferred amino acid sequences of P-opsin and pinopsin differ at only 2 positions out of 351, suggesting that they are the same gene products.

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9. Although RNase protection assays failed to detect the retinal pigments in pineal that were amplified by PCR (red and green), there appear to be a small number of rhodopsin and iodopsin immunoreactive cells in the pineal (*6*). This suggests that the pineal may express some retinal opsins in a small subset of cells detectable by PCR but not by RNase protection assay.
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screened with a ^{32}P-labeled probe generated by PCR, with the 210-bp P-opsin cDNA obtained by RT-PCR as template. Filters were hybridized with the probe overnight at 65°C in Church buffer (*13*) and washed at high stringency in 0.2× saline sodium citrate (SSC), 0.5% SDS at 65°C. More than 30 individual partial P-opsin cDNA clones and one full-length clone were obtained (ranging in size from 800 to 1696 bp). Some of the partial clones (~1/3) had the sequence CAG (glutamine) for amino acid 28 rather than TGG (tryptophan). This may be due to a polymorphism in the population or to RNA editing.

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22. Genomic clone GP3 and others were obtained by screening of a chicken genomic library contained in Lambda Fix II (Stratagene) with a random-primed probe made from the full-length P-opsin cDNA clone. After overnight hybridization at 65°C in Church buffer, filters were washed at high stringency (0.2× SSC, 0.5% SDS, 65°C). Lambda DNA from purified positive plaques was subcloned into pBluescript II KS+, mapped by restriction endonuclease digestion, and sequenced. Both of the genomic clones that were sequenced through this region contain the sequence CAG (glutamine) at amino acid 28 rather than TGG (tryptophan) as found in the majority of cDNAs (*12*).
23. Tree reconstruction was performed as described in figure 3 of (*1*) with PHYLIP version 3.5c, assembled by J. Felsenstein [*Cladistics* **5**, 164 (1989)]. An alignment of sequences was generated by McCAW [M. O. Dayhoff, R. M. Schwartz, B. C. Orcutt, *Atlas of Protein Sequencing and Structure*, M. O. Dayhoff, Ed. (National Bioscience Research Foundation, Washington, DC, 1978), vol. 5, p. 345; G. D. Schuler, S. F. Altschul, D. J. Lipman, *Protein Struct. Funct. Genet.* **9**, 180 (1991)], with minor adjustments made by hand. Distance matrices were calculated with PRODIST, which uses the Dayhoff PAM 001 matrix. NEIGHBOR was used to generate pairwise groupings with the neighbor-joining method described by N. Saitou and M. Nei [*Mol. Biol. Evol.* **4**, 406 (1987)]. Randomization of sequence entry order had no effect on tree reconstruction. Bootstrapping (SEQBOOT) generated multiple (n = 100) randomly resampled data sets from the alignment [J. Felsenstein, *Evolution* **39**, 783 (1985); *Annu. Rev. Genet.* **22**, 521 (1988)]. CONSENSE produced a consensus tree from the data set. No attempt was made to associate branch length with amino acid substitution rate. Similar results were obtained using the accepted-mutation parsimony (AMP) method of Kolakowski and Rice, available by e-mail at lfk@receptor.MGH.Harvard.Edu.
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29. T. Okano, T. Yoshizawa, Y. Fukada, *Nature* **372**, 94 (1994).
30. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Massive Cell Death of Immature Hematopoietic Cells and Neurons in Bcl-x–Deficient Mice

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bcl-x is a member of the *bcl-2* gene family, which may regulate programmed cell death. Mice were generated that lacked Bcl-x. The Bcl-x–deficient mice died around embryonic day 13. Extensive apoptotic cell death was evident in postmitotic immature neurons of the developing brain, spinal cord, and dorsal root ganglia. Hematopoietic cells in the liver were also apoptotic. Analyses of *bcl-x* double-knockout chimeric mice showed that the maturation of Bcl-x–deficient lymphocytes was diminished. The life-span of immature lymphocytes, but not mature lymphocytes, was shortened. Thus, Bcl-x functions to support the viability of immature cells during the development of the nervous and hematopoietic systems.

Apoptosis (programmed cell death) is a poorly understood process that occurs in many tissues during early development and throughout adult life in many organisms. The protooncogene *bcl-2*, whose gene product inhibits certain forms of apoptosis (*1*), is widely expressed during mouse development and in long-lived cells such as neurons and stem cells of many tissues in an adult mouse (*2*). Although early embryonic lethality was expected on the basis of its expression pattern, *bcl-2*–ablated mice were shown to remain viable, and major abnormalities were limited to the hair color, polycystic kidney development, and decreased lymphoid cell life-span (*3*, *4*). In particular, the nervous system developed normally. These findings may be attributed to redundancy, because *bcl-2* is only one of a larger family of related genes (*5*, *6*). As expected, other members of the *bcl-2* family can inhibit apoptosis in in vitro assays (*5*, *6*).

bcl-x, a member of the *bcl-2* gene family, can be alternatively spliced to produce two protein isoforms (Bcl-x_L and Bcl-x_S), one of which (Bcl-x_L) inhibits apoptosis (*6*). Bcl-x in mice is expressed highly during development and in the brain, thymus, and kidney in adult, predominantly in the Bcl-x_L form (*7*, *8*). To elucidate the functional and developmental role of Bcl-x, we used homologous recombination in embryonic stem (ES) cells to generate mice lacking both Bcl-x_L and Bcl-x_S (*9*) (Fig. 1). Heterozygous

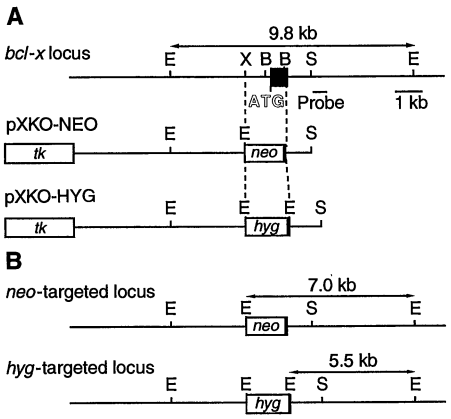

Fig. 1. Target disruption of the *bcl-x* gene (*9*). (**A**) Genomic structure surrounding coding exon of mouse *bcl-x*, and structure of the pXKO-NEO and pXKO-HYG targeting vectors. The position of translation initiation site is shown (ATG). (**B**) Predicted structure of the targeted *bcl-x* locus. The location of the hybridization probe, a 0.4-kb Kpn I–Pst I fragment, and expected sizes of the Eco RV fragments that hybridize with the probe are indicated. B, Bam HI; E, Eco RV; S, Spe I; X, Xho I. The restriction map of Bam HI is not complete.

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