Dual *Bar* homeo box genes of *Drosophila* required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development

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In the *Bar* mutation of *Drosophila*, ommatidial differentiation is known to be suppressed in the anterior portion of the eye. Our structural analysis shows that the *Bar* region contains a pair of homeo box genes, *BarH1* and *BarH2*. These genes encode polypeptides similar in size and sequence and share a common homeo domain that is identical in sequence except for putative *trans*-activator-binding sites. We also show, by mosaic analysis and immunostaining with anti-*BarH1*/*BarH2* antibodies, that *BarH1* and *BarH2* are not only specifically coexpressed but also functionally required in R1/R6 prephotoreceptors and primary pigment cells in developing ommatidia. In R1/R6, the expression of *BarH1* and *BarH2* appears to be regulated by *rough* and *glass* gene products. *BarH1* and *BarH2* proteins are essential to normal lens formation, formation of three types of pigment cells, and elimination of excess cells from mature ommatidia. Taken together, our results suggest that *Bar* homeo domain proteins may play key roles in the fate-determination processes of pigment cells and cone cells.

[*Key Words*: Homeo box genes; eye development; photoreceptor; pigment cell; cell fate]

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The *Drosophila* compound eye contains ~ 800 ommatidia, each a stereotyped assembly of eight photoreceptor cells, R1–R8, and several different types of accessory cells (Ready et al. 1976). Ommatidium formation begins during the mid–third instar and occurs as a wave sweeping from the posterior to the anterior edge of the eye disc (Ready et al. 1976; Tomlinson 1985; Tomlinson and Ready 1987). At the end of larval life, recruitment of photoreceptor cells and four cone cells is complete in the most mature ommatidium (Tomlinson 1985). Cells surrounding the ommatidia at the four-cone-cell stage differentiate into three types of pigment cells and bristle group cells during the first half of pupal life (Cagan and Ready 1989).

There is no strict cell lineage relationship among ommatidial cells (Lawrence and Green 1979). Except for bristle group cells, the fates of cells that later join a developing ommatidium are thought to be determined by positional cues present on the surface of cells that have already differentiated within a unit (Tomlinson and Ready 1987; Cagan and Ready 1989). Several genes involved in determination of fate of photoreceptor cells have been cloned, and their possible roles have been clarified. The induction process of R7 is best understood at the molecular level. To date, three genes, *sevenless* (*sev*), *bride of sevenless* (*boss*), and *seven in absentia* (*sina*), have been identified whose absence causes the transformation of the R7 precursor into a non-neuronal cone cell (Banerjee et al. 1987; Hafen et al. 1987; Reinke and Zipursky 1988; Carthew and Rubin 1990). The *sev* gene, required in R7, encodes a transmembrane receptor that possesses a tyrosine kinase domain (Hafen et al. 1987). Recent results strongly suggest that the *boss* gene product is a ligand for the *sev* receptor and that these two proteins can directly interact at the R7–R8 cell boundary (Kramer et al. 1991).

The involvement of other classes of genes in photoreceptor differentiation has also been demonstrated. For instance, the *rough* (*ro*) gene, which encodes a homeo domain protein, is essential for conveying signals from the R2/R5 pair to their neighbors (Tomlinson et al. 1988). A presumptive steroid hormone receptor, coded for by the *seven up* (*svp*) gene, is required for determining the cell identity of R3/R4 and R1/R6 (Mlodzik et al. 1990). The *glass* (*gl*) gene product is a putative DNA-binding protein with zinc fingers and may be involved in photoreceptor determination (Moses et al. 1989), whereas the *scabrous* gene appears to play a central role in establishing the ommatidial periodicity at a very early

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stage of concerted ommatidium formation (Baker et al. 1990).

In contrast to studies on the fate determination of photoreceptor cells, little research has been directed toward the molecular events that begin in the pupal stage. Previously, we identified a homeo box gene in the *Bar* region as a gene possibly causing *Bar* mutation (Kojima et al. 1991). This gene (*BarH1*) and its cognate *BarH2* form a small complex at the *Bar* region and are important to pupal, ommatidial differentiation. In this paper we describe the structural features of *BarH2* and its close relationship to *BarH1*. *BarH1* and *BarH2* proteins were found to share a common homeo domain that is identical in sequence except for putative *trans*-activator-binding sites. Immunohistological and genetic evidence is also presented, showing that both *BarH1* and *BarH2* are specifically coexpressed and functionally required in R1/R6 prephotoreceptors and primary pigment cells in developing ommatidia. Analysis of Bar^- phenotypes indicated that expression of *BarH1* and *BarH2* is essential to the formation of pigment cells and cone cells. *BarH1* and *BarH2* expression was also shown to be regulated by *gl* and *ro* gene products.

Results

BarH1 *and* BarH2 *genes form a small complex in the* Bar *region*

Genomic DNA of *Drosophila melanogaster* was digested with various restriction enzymes, sized on agarose gel, and subjected to blot hybridization using the *BarH1* homeo box sequence as a probe. As shown in Figure 1b, hybridization occurred with two fragments in all digests, suggesting that the chromosome of *D. melanogaster* contains two loci that can hybridize with the *BarH1* homeo box sequence. In each lane the fragment with the stronger signal corresponded to the *BarH1* gene, whereas that with the weaker signal was found to include the sequence with a new homeo box gene, which we call *BarH2*. A genomic fragment representing *BarH2* was isolated and used as a probe for in situ hybridization to polytene chromosomes. As with *BarH1* (Kojima et al. 1991), *BarH2* mapped to 15F9/16A1 on the X chromosome (data not shown), the locus for *Bar* mutations in which ommatidium differentiation is suppressed in the anterior portion of the eye (Sturtevant 1925). Genomic DNA clones representing the *Bar* region were isolated by chromosome walking (Fig. 1a). *BarH2* was found to be located ~80 kb from *BarH1*, and all four *Bar* breakpoints examined so far (Tsubota et al. 1989) were in a narrow region terminated by *BarH1* and *BarH2*. Because these *Bar* homeo box genes are very similar in structure and are expressed in the same subset of cells in a developing ommatidium, as shown below, and the overexpression of *BarH1* causes eye malformation similar to that in *Bar* mutations (Kojima et al. 1991), it is likely that *BarH1* and *BarH2* form a small complex (*Bar* complex) closely related to eye morphogenesis.

BarH2 *is a cognate homeo box gene of* BarH1

Four cDNA clones of *BarH2* were isolated (see Fig. 1a),

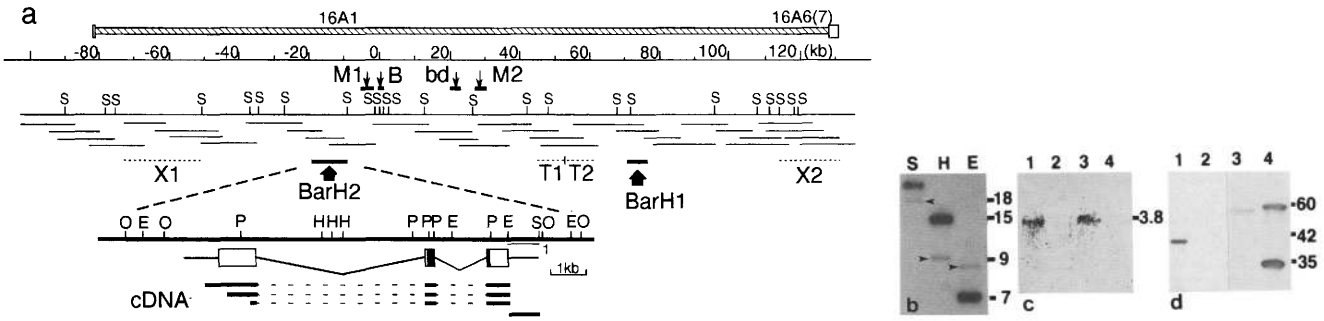

Figure 1. Chromosomal locations of the *BarH1* and *BarH2* genes, and blotting patterns of their DNA, RNA, and protein. (*a*) Chromosomal locations. The physical map was made using a series of λ clones (sizes and locations are indicated by thin lines). Thick arrows show the locations of *BarH1* and *BarH2* genes; arrows labeled M1, B, bd, and M2, respectively, indicate the positions of *Bar* breakpoints in B^{M1}, *B*, B^{bd}, and B^{M2} mutants (Tsubota et al. 1989). The hatched line shows the size and location of the deleted region in $Df(1)B^{263\text{-}20}$. The dotted line labeled *T1/T2* shows the location of a putative transposon. In the *B* mutant, *T1* was deleted. Northern blotting (S. Ishimaru, unpubl.) suggested that the cloned *Bar* region contains five transcriptional units: *X1* (a transcriptional unit related to *forked* but unrelated to eye morphogenesis); *BarH2*, *T1/T2* (a putative transposon); *BarH1*; and an uncharacterized transcriptional unit, *X2*. (*Bottom left*) An enlarged map of *BarH2*. The *BarH2*-coding regions are shown by boxes; thin horizontal lines associated with them indicate exon size and location. The solid boxes indicate a homeo domain. A thin line labeled *1* shows the location of a fragment used as a probe in Northern blot. Lines labeled cDNA show the regions cloned as cDNAs. (*B*) *Bam*HI; (*E*) *Eco*RI; (*H*) *Hin*dIII; (*O*) *Xho*I; (*P*) *Pst*I; (*S*) *Sal*I. (*b*) Blot hybridization patterns of the *Sal*I (S), *Hin*dIII (H), and *Eco*RI (E) digests of total genomic DNA. Molecular masses are shown in kb. Arrowheads indicate fragments including the *BarH2* gene. Southern hybridization was carried out using a ^{32}P-labeled *BarH1* homeo box fragment as probe (fragment 1 shown in Fig. 1a of Kojioma et al. 1991). (*c*) Northern blot analysis of *BarH2* RNA. (Lane *1*) Embryos; (lane 2) larvae; (lane 3) pupae; (lane 4) adults. *BarH2* RNA size is shown in kb. A *BarH2* fragment (fragment 1 in Fig. 1a) was used as probe. (*d*) Western blotting was carried out using antibodies against *BarH1* (lanes *1,2*) and *BarH2* (lanes *3,4*) proteins. Lysates were prepared from *E. coli* cells producing the polypeptide corresponding to the carboxy-terminal half of the *BarH1* protein (lanes *1,3*) and the *BarH2* protein (lanes *2,4*). A faint band in lane *3* represents an *E. coli* protein; the 35-kD band in lane *4* is a degradation product of the *BarH2* protein. Molecular masses (in kD) are shown at *right*.

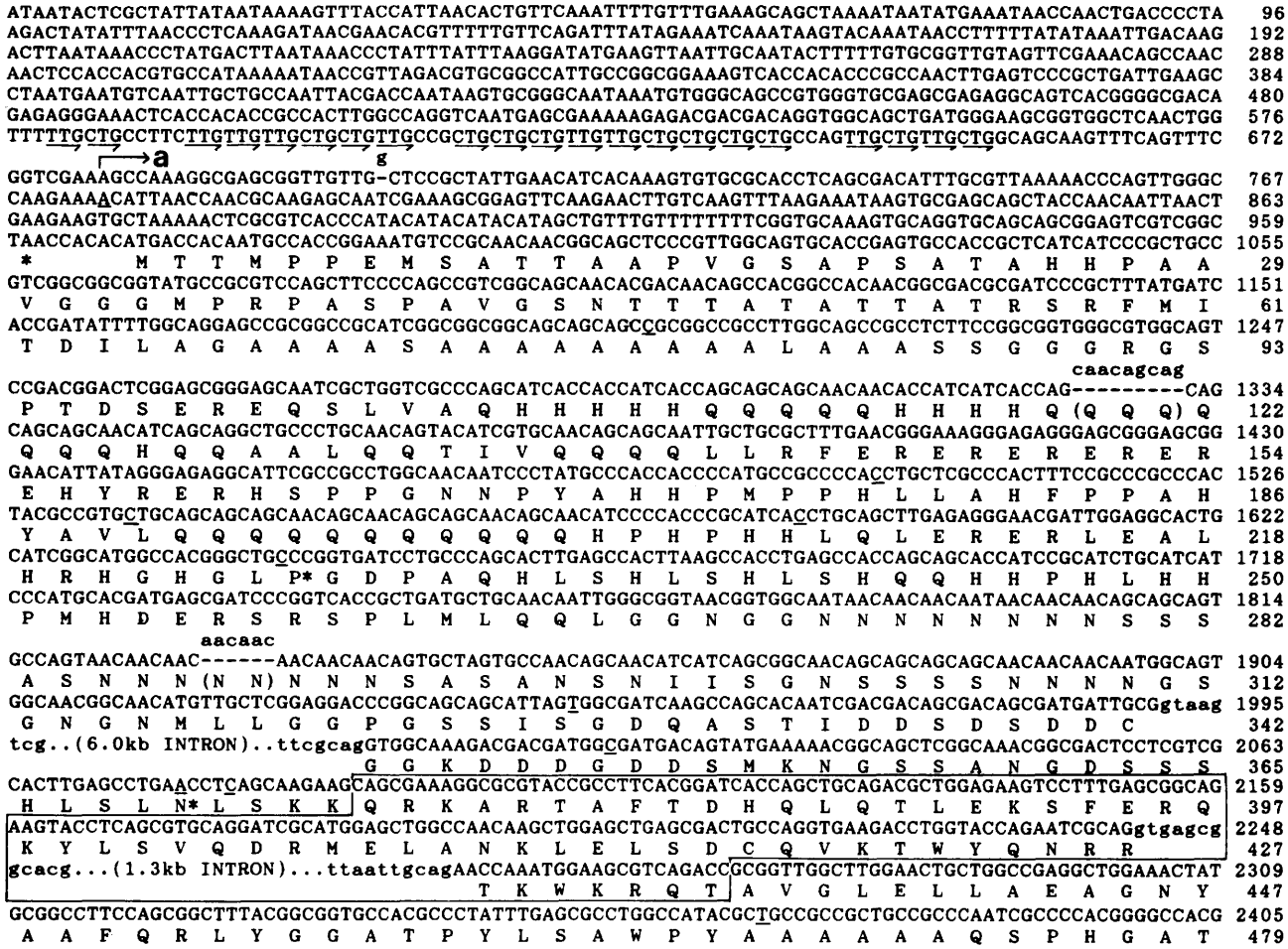

Figure 2. Nucleotide sequence of the *BarH2* gene and the amino acid sequence of its putative translation product. The horizontal L-shaped arrow (*a*) shows the 5′ end of the longest cDNA insert (see Fig. 1a). The RNA start site was located at positions 1–5 by primer extension. Horizontal arrows indicate (T/C)TG repeats found in the 5′-untranslated region. Positions where single-base substitutions occur in cDNA clones are underlined; insertions in cDNA clones are shown above the genomic nucleotide sequence. Single-base substitutions, mostly synonymous, are as follows: genomic DNA/cDNA = A/T (775), C/G, C/T, C/T, C/T, C/A, T/C, C/A, A/G, C/G, T/G, and A/T (2641) (from 5′ to 3′). (*) The positions of 2-amino-acid substitutions. Because of two short insertions, the conceptual translation product of cDNA has 5 additional amino acids, all of which are situated within repeated amino acid sequences. Presumed intron sequences are indicated by lowercase letters. Homeo box and homeo domain sequences are enclosed in the large box; the putative polyadenylation signal AATAAA is enclosed in the small box (*b*). The vertical arrow (*c*) indicates a poly(A) site.

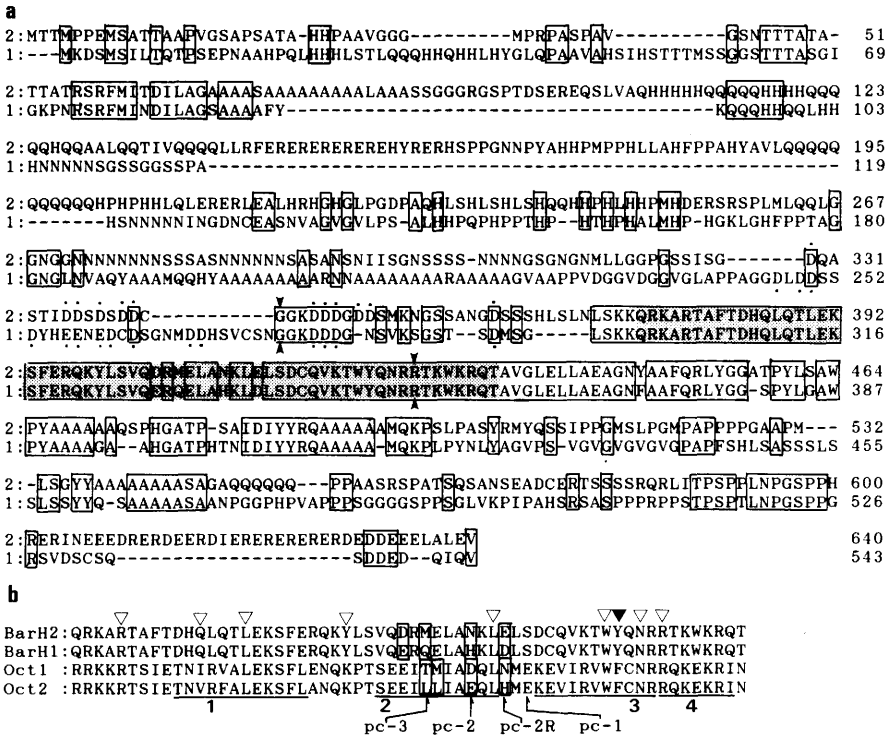

Figure 3. Amino acid sequence homology and other structural features of the *BarH2* protein. (*a*) Homology beween *BarH1* and *BarH2* proteins. Identical amino acids are enclosed in boxes; the hatched region corresponds to the homeo domain. Small dots indicate the positions of acidic amino acids found just upstream from the homeo domain. Locations of introns are indicated by arrowheads. (*b*) Sequence comparison of two *Bar* homeo domains and *Oct*-1 and *Oct*-2 homeo domains (Stern et al. 1989). (▽) The positions of 8 amino acids conserved in homeo domains of *Drosophila* (Kojima et al. 1991). (▼) The location of the helix-3 tyrosine residue characteristic of *BarH1* and *BarH2*. The amino acids differing between *BarH1* and *BarH2* are boxed. Similarly, the amino acids that are possibly protein–protein interaction sites in *Oct*-1 and their counterparts in *Oct*-2 are boxed. Helices 1–4, predicted to be present in the homeo domain (Qian et al. 1989), are indicated along with the positions of *pc* mutations and the *pc-2* revertant *pc-2R* (Hawley et al. 1983; Hochschild et al. 1983; Stern et al. 1989).

and the nucleotide sequences of cDNA inserts, along with those of their genomic counterparts, were determined (Fig. 2). The *BarH2* gene contains three exons that would encode a polypeptide of 640 (or 645)-amino-acid residues (Figs. 1a and 2). To define the transcriptional start site, primer extension experiments were carried out with a 5′-end-labeled oligonucleotide, complementary to nucleotides 361–379, as a primer (see Fig. 2). The major extension product is 355–360 nucleotides long, suggesting that the RNA start site is within positions 1–5. A putative polyadenylation signal, AATAAA, is located 4–5 bp upstream from the poly(A) site (positions 3750–3751). Thus, the predicted size of the *BarH2* transcript is ~3.75 kb, which, following polyadenylation, would agree well with the 3.8-kb RNA observed in Northern blots (see Fig. 1c).

The most striking feature of the putative *BarH2* protein is that its carboxy-terminal half contains a homeo domain identical at 56 of 60 positions to the *BarH1* homeo domain (Figs. 2 and 3a). The third DNA recognition helix of the *BarH2* homeo domain showed precisely the same sequence as that of its *BarH1* counterpart, having characteristic phenylalanine–tyrosine substitution (Fig. 3b; Kojima et al. 1991); this suggests that both *BarH1* and *BarH2* proteins recognize an identical set of DNA sequences as their targets. As shown in Figure 3a, sequence homologies can be extended beyond the homeo domains in both directions, and the genomic sequences encoding both proteins appear to be interrupted at two identical sites. Most nonhomologous regions are occupied by repeated amino acid sequences. Taken together, these results indicate that *BarH1* and *BarH2* are cognate homeo box genes.

Coexpression of BarH1 *and* BarH2 *in two prephotoreceptor cells and primary pigment cells*

Northern blot analysis indicated that *BarH2* is expressed only in embryogenesis and metamorphosis, as is the case of *BarH1* (Kojima et al. 1991) (Fig.1c). To examine spatial expression patterns, polyclonal antibodies against *BarH1* and *BarH2* proteins were produced. Virtually no cross-reaction could be found (Fig. 1d). As will be described elsewhere, both *BarH1* and *BarH2* are expressed in a limited number of identical neuronal cells in embryos and pupae. Here, expression is described only in cells of a developing ommatidium, except for bristle group cells. The spatial distribution of *BarH1* and *BarH2* proteins was virtually identical for the wild-type eye disc obtained from late third-instar larvae (Fig. 4a–c). The basal region under the morphogenetic furrow, where nearly all the cells were stained (see insets in Fig. 4a and c), contains unpatterned precursors for R1, R6, R7, and accessory cells. In a more distal region, from column 5–7 to the posterior edge of the eye disc, a particular pair of cells can be seen clearly by staining (Fig. 4a–c). The unique locations of the stained pairs, with respect to the equator (Fig. 4d) and *BarH1–svp* double-staining pattern (Fig. 4e), show almost unaminously that the stained pairs are R1/R6 prephotoreceptor pairs.

The expression of *BarH1* and *BarH2* in R1 and R6 cells not only persisted at the larval stage but also at the early

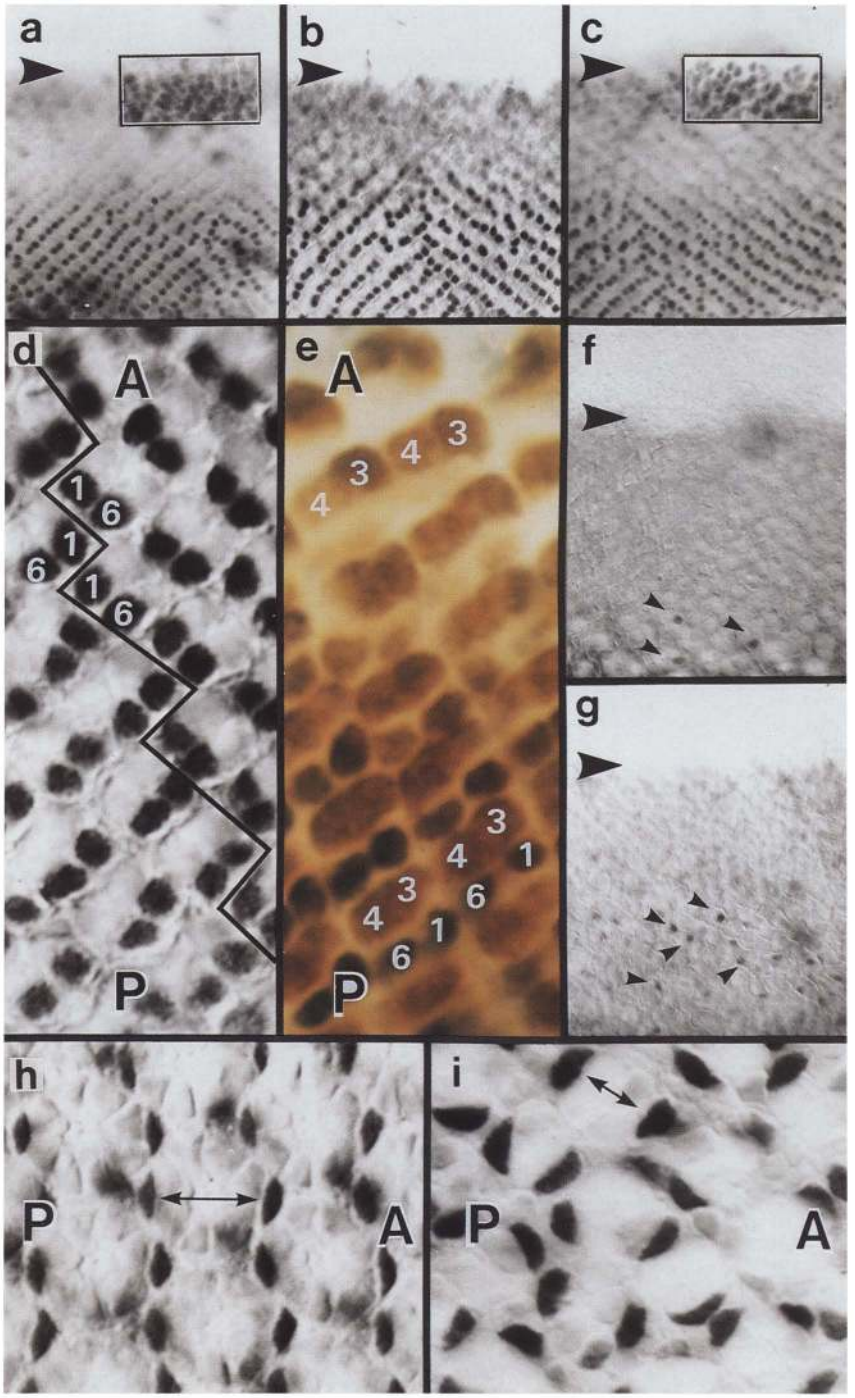

Figure 4. Cell type-specific expression of *Bar* homeo box genes. (A) Anterior; (P) posterior. (*a–c*) Staining patterns of wild-type eye discs by anti-*BarH1* (*a*), anti-*BarH2* (*c*), and their mixture (*b*) (456×). (*d*) A high-magnification (1520×) picture of *b*. The morphogenetic furrow is indicated by the arrowhead. Insets in *a* and *c* show the basal region under the morphogenetic furrow. The solid zigzag line in *d* indicates the equator. Cells labeled *1* and *6*, respectively, indicate R1 and R6 prephotoreceptors. (*e*) *BarH1–svp* double staining (1520×). (Black) *BarH1*-positive nuclei; (brown) *svp*-positive cells. Note that the *BarH1*-positive nuclei are surrounded by *svp*-positive cytoplasm, showing *BarH1*-positive cells to be simultaneously *svp*-positive. The fact that *svp* expression occurs in R3/R4 in the five-cell precluster stage (Mlodzik et al. 1990) permits us to easily distinguish R3/R4 pairs (cells labeled *3*, *4*) from R1/R6 pairs (cells labeled *1*, *6*). (*f*, *g*) Staining patterns of larval discs derived from gl^1 (*f*) and ro^1 (*g*) mutants (480×). Mixed antibodies were used. (Small arrowheads) *BarH1/BarH2*-positive cells; (large arowheads) morphogenetic furrow. *h* and *i*, respectively, show staining patterns of the nuclei of the wild-type and *ro* mutant primary pigment cells by the mixed antibody (1520×). Pupal eye discs (~70 hr APF) were examined. Arrows indicate nuclei of the paired primaries. The aberration in orientation of nuclei in the *ro* ommatidia is probably the result of a reduction in the number of cone cells per ommatidium (T. Kojima, unpubl.).

pupal stage. The level of expression decreased considerably at ~30 hr after puparium formation (APF) at 20°C, at which time *BarH1* and *BarH2* were expressed in anterior and posterior primary pigment cells (Figs. 4h and 5). This expression could still be detected up to 80 hr APF (Fig. 5).

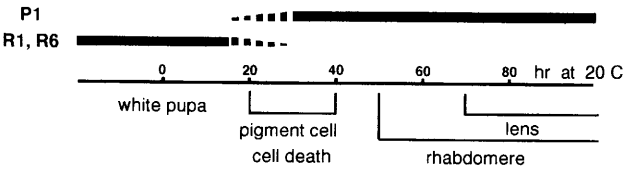

Figure 5. Time course of the expression of *BarH/BarH2* genes in R1/R6 and primary pigment cells during pupal development at 20°C. Periods for pigment cell formation, lens formation, rahbdomere formation, and cell death (Cagan and Ready 1989) are shown at *bottom*. (P1) primary pigment cells.

Functional requirement of the Bar *complex in R1/R6 prephotoreceptors and primary pigment cells*

Although no point mutatation has been detected in *Bar*

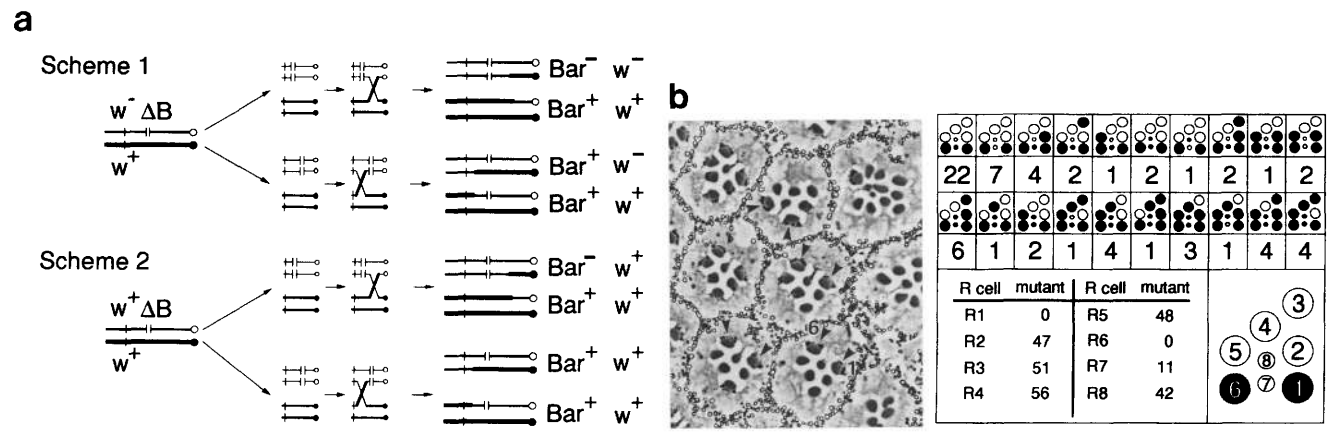

Figure 6. Mosaic analysis of *Bar*$^-$ clones. (*a*) Two schemes of somatic recombinantion. (ΔB) *Bar* deficiency. Scheme 1 shows the procedures for the first series of mosaic analysis. Virgin females heterozygous for *Df(1)B*$^{263\text{-}20}$ *w/FM7* were crossed with Canton-S males. After X-ray irradiation (1200 rads) of first/second-instar larvae, the mosaic eyes, with fused, bulging ommatidia (*Bar*$^-$ phenotype; Kojima et al. 1991), were isolated and used for subsequent mosaic analysis. Scheme 2 shows the procedures for the second series of mosaic analysis. This scheme is essentially identical to scheme 1, except for the use of virgin females heterozygous for *Df(1)B*$^{263\text{-}20}$ *w*$^+$/+. Note that all flies used in the second series of mosaic analysis are wild type for the *white* (*w*) gene. (*b*) (*Left*) Thin section of mosaic eye tissue obtained from the first series of mosaic analysis. Phase-contrast microscopy was used. Wild-type photoreceptors have pigment granules in the cytoplasm directly adjacent to the base of the rhabdomeres; *Bar*$^-$ cells lack pigment granules entirely. Arrowheads labeled *1* and *6*, respectively, show the pigment granules found in R1 and R6 photoreceptors. (*Right*) Diagrams of 71 normally constructed ommatidia containing both wild-type and *Bar*$^-$ photoreceptors are shown at *top*; the distribution of photoreceptors with the mutant phenotype *Bar*$^-$ is shown at *bottom*. (○) *Bar*$^-$ photoreceptors; (●) Wild-type photoreceptors. The number of ommatidia are shown *below* each diagram.

homeo box genes at present, most of the *Bar* region (16A1–A7) is known to be deleted in *Df(1)B*$^{263\text{-}20}$ (Sutton 1943). As summarized in Figure 1a, our structural analysis revealed that the deleted region in *Df(1)B*$^{263\text{-}20}$ is ~210 kb long and contains five transcriptional units: *BarH1*, *BarH2*, two units (*X1* and *T1/T2*) not relevant to eye morphogenesis, and one uncharacterized unit (*X2*). Using this deletion, mosaic analysis was carried out according to scheme 1 in Figure 6a, to examine whether the *Bar* complex, comprised of *BarH1* and *BarH2*, is required in ommatidial cells expressing *BarH1* and *BarH2* during eye development.

As shown in Figure 6b, only R1 and R6 cells were found to be genotypically wild-type photoreceptors in the 71 mosaic ommatidia that were normal in rhabdomere appearance and orientation, suggesting that the *Bar* region genes are required in the R1/R6 pair, coexpressing *BarH1* and *BarH2*, for the formation of normal ommatidia. The expression of the genes deleted in *Df(1)B*$^{263\text{-}20}$ also appears to be required in anterior and posterior primary pigment cells. We examined the distribution of pigment cell type granules in 45 of the 71 mosaic ommatidia with normal appearance. All of them were judged to contain paired primary pigment cells that were genotypically wild type, as determined by the presence of a typical circular distribution of dense pigment granules associated with nuclei, located just beneath the apical surface (see Fig. 8a, below). Twenty-five percent of these mosaic ommatidia were found to be partially surrounded by the unpigmented "white" area at the level of the secondary (and/or tertiary) pigment cells. Thus, the agreement of the results of mosaic analysis and of immunostaining by anti-*BarH1/BarH2* antibodies was striking, supporting the notion that *BarH1* and/or *BarH2* genes are functionally required in R1/R6 and primary pigment cells for normal eye development, although the possible involvement of *X2* in eye morphogenesis cannot be excluded.

Requirement of the Bar *complex for pigment cell formation*

In the region devoid of *Bar* gene function, many fused, bulging ommatidia were present (Kojima et al. 1991). A longitudial view of the *Bar*-deficient region indicated striking morphological changes in the internal region of the mutant retina (Fig. 7a). The apical surface of the retina protruded outwardly more than in the wild type, whereas no appreciable change in the basal surface could be observed. A tangential section (Fig. 7b) showed both the interommatidial distance and area occupied by nonphotoreceptor cells to increase in the *Bar*-deficient region. *Bar* deficiency thus may induce an increase in retinal cell mass per unit area. However, no pupal eye cell counts could be carried out because of technical difficulties due to a low frequency of the appearance of the mosaic eye and the absence of a proper cell marker.

The pigment cells appear to be necessary for the elimination of excess cells from the ommatidia (Cagan and Ready 1989), and consequently, a second series of somatic recombination analyses were conducted with a fly strain heterozygous for *Df(1)B*$^{263\text{-}20}$ *w*$^+$/+, according to

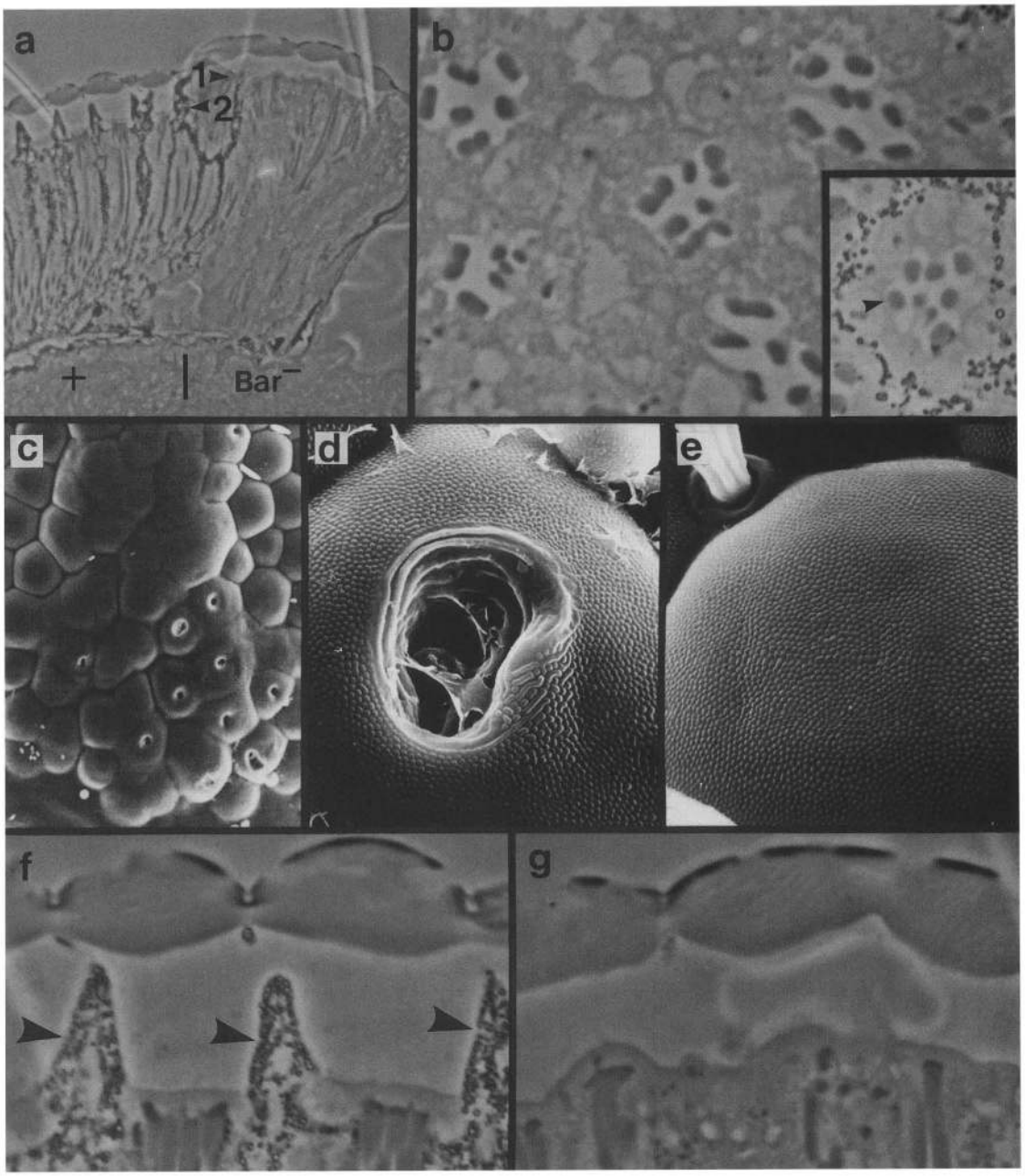

Figure 7. Morphological features of the *Bar*-deficient region of the scheme 1 mosaic eye. (*a*) A radial section of the mosaic ommatidia (320×). Arrowheads *1* and *2* show the apical surface levels of the mutant and wild-type regions, respectively. (*b*) Tangential section of the Bar^- region (1600×). (*Inset*) An example of ommatidia with normal pigment cells and, presumably the Bar^- R6 cell (arrowhead). (*c–e*) Ommatidial surface structures observed by a scanning electron microscope. (*c,d*) Bar^- mutant (500× and 5000×, respectively). (*e*) Wild type (5000×). Longitudial view of the wild-type (*f*) and Bar^- (*g*) lenses (1600×) are shown. Arrowheads indicate the extensions of the secondary pigment cells.

scheme 2 in Figure 6a. Surprisingly, pigmentation in the *Bar*-deficient region was remarkably low and mutant eye color was almost white despite the fact that all fly strains used were w^+/w^+ in genotype. A close examination of tangential sections revealed extensive changes in pigment cell morphology. At the level of primary pigment cells, typical semicircular primaries with dense pigment granules appeared to be considerably decreased in number, possibly without being compensated by their unpigmented counterparts (Fig. 8a). This finding was confirmed by examination of serial sections. Most, if not all, nonphotoreceptor cells, situated at the level of secondary (or tertiary) pigment cells, were found to be without any pigment granules (Fig. 8b). In contrast, almost all

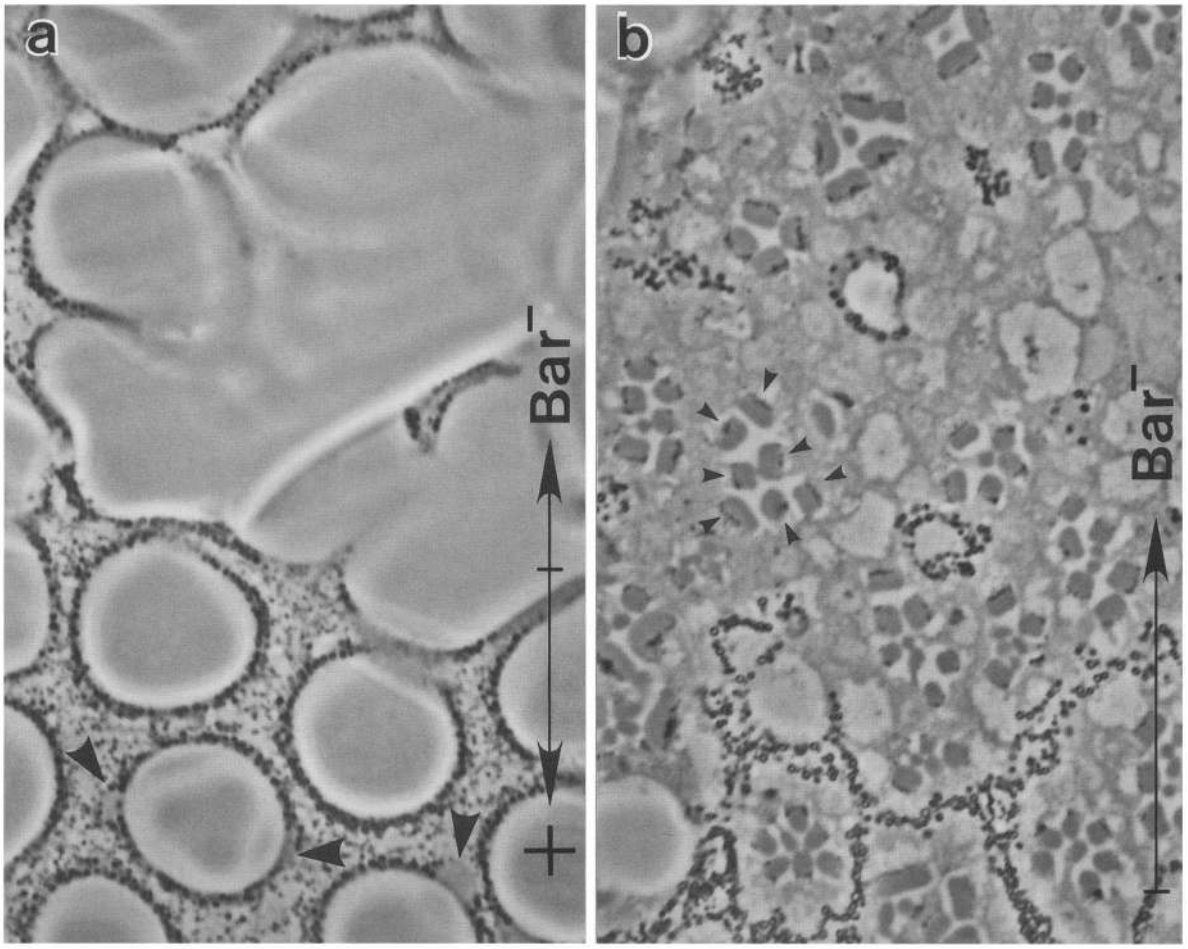

Figure 8. Tangential section of the Bar^- region of the scheme 2 mosaic eye. (*a*) Primary pigment cell level (1600×). Arrowheads indicate nuclei of primary pigment cells in the ommatidium that are normal in appearance. Note that the paired primary pigment cells are usually observed as a circle with dense pigment granules, which are surrounded by fine pigment granules of the secondary (or tertiary) pigment cells. (*b*) Secondary and tertiary pigment cell level (1600×). In contrast to pigment cells, almost all photoreceptors in the Bar^- region appear to have normal pigment granules (arrowheads).

photoreceptor cells in the Bar^- region were found to have photoreceptor-type pigment granules adjacent to the base of rhabdomeres (Fig. 8b). Thus, *Bar* gene functions are required for the formation of three types of pigment cells; without them, unpatterned, excess cells are not likely to be eliminated from the interommatidial space.

Figure 7b also shows that the shape and arrangement of rhabdomeres is considerably affected by *Bar* gene deficiency. Most or all extensive distortions in rhabdomere structure might be the result of the secondary effects from failure of excess cell elimination described above, as only small structural changes such as the appearance of one to two additional rabdomeres (see inset in Fig. 7b) and abnormal orientation of the ommatidium could be detected in most ommatidia possessing mutated, presumably R1 and/or R6 cells, but being surrounded by normal pigment cells.

Involvement of the Bar *complex in lens formation*

High-magnification electron micrographs of lens surfaces (Fig. 7c–e) showed an appreciable fraction of mutant lens units with a cavity at the center, in which at least several upper layers of lens cuticle were damaged extensively. This cavity might have resulted from defects in cone cells, considering the fact that lens formation initially occurs just over the apical surface of cone cells from which a large portion of lens material is secreted (Cagan and Ready 1989). Defects in lens structure that are similar or much more severe and are attributable to cone cell defects have been reported in transgenic flies containing a transcriptional fusion between the *sev* promoter and a *ro* minigene (Basler et al. 1990; Kimmel et al. 1990).

Longitudial views of wild-type and mutant lenses are shown in Figure 7, f and g. Most of the ommatidia in the mutant region showed no protrusions or extensions of secondary pigment cells. In the wild-type region, the features were evident just beneath lens-unit junctions. The second lens material, probably required for proper connection of the lens units, is secreted from the microvilli on the protruded area of secondary pigment cells (Cagan

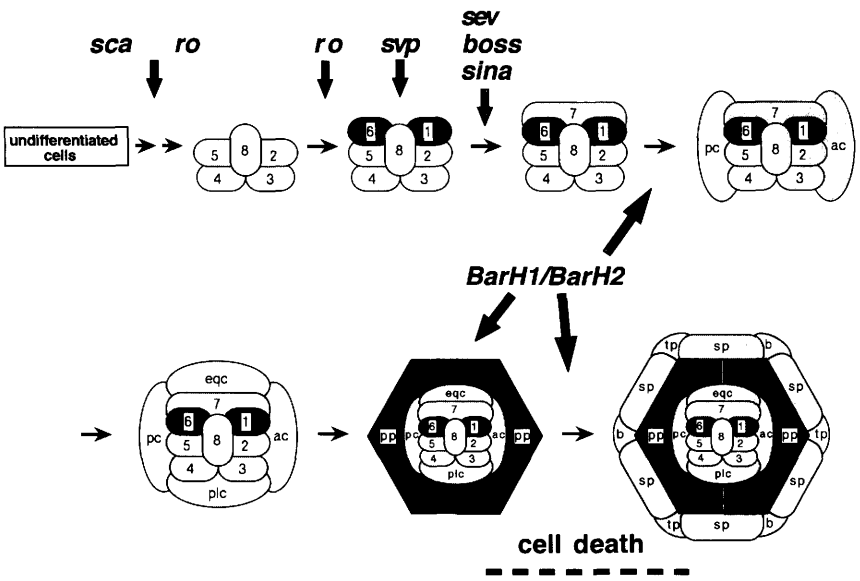

Figure 9. Possible inductive pathways in late ommatidial development. (*sca*) *scabrous*; (*ro*) *rough*; (*svp*) *seven up* (*sev*) *sevenless*; (*boss*) *bride of sevenless*; (*sina*) *seven in absentia*. The sequence of ommatidial differentiation is indicated by horizontal arrows; stippled vertical arrows indicate the steps that require the products of various regulatory genes, including *BarH1* and *BarH2*. Whether *BarH1/BarH2* genes are needed for differentation of R1/R6 and/or R7 has not yet been determined. The *gl* gene is expressed in all developing photoreceptors including R1/R6 (Moses and Rubin 1991). Cells expressing *BarH1* and *BarH2* genes are shown by solid areas. (*1*) Prephotoreceptor R1; (*2*) R2; (*3*) R3; (*4*) R4; (*5*) R5; (*6*) R6; (*7*) R7; (*8*) R8. (*ac*) Anterior cone cell; (*pc*) posterior cone cell; (*eqc*) equatorial cone cell; (*plc*) polar cone cell; (*pp*) primary pigment cell; (sp) secondary pigment cell; (*tp*) tertiary pigment cell; (*b*) bristle group cells. The broken line shows the period for the elimination of cells not incorporated into ommatidia (Cagan and Read 1989).

and Ready 1989); thus, the absence of cellular extensions of secondary pigment cells might result in the aberrant lens-unit fusion in the *Bar*$^-$ region.

Requirement of ro *and* gl *gene products for the expression of* BarH1 *and* BarH2 *in R1/R6*

The *gl* gene encodes a transcription factor with zinc fingers, which is required when prephotoreceptor cells with acquired neural characteristics progress to a photoreceptor cell-type identity (Moses et al. 1989). As shown in Figure 4f, the expression of *BarH1* and *BarH2* in R1 and R6 required the *gl* gene product. Thus, *BarH1* and *BarH2* proteins in R1 and R6 cells might function as a pair of subordinate transcription factors of *gl*. As shown in Figure 4g, *ro* was also required for the expression of *BarH1* and *BarH2* in R1/R6. Therefore, as with R3/R4 (Tomlinson et al. 1988), *ro*-dependent signals from R2/R5 might also be required for the normal development of R1/R6. In contrast, the effects of *ro* mutation on the expression of *BarH1* and *BarH2* were slight, if present at all, in the case of primary pigment (Fig. 4i) and unpatterned cells under the morphogenetic furrow (data not shown).

Discussion

Here, we have shown that the *Bar* region contains two cognate homeo box genes, *BarH1* and *BarH2*. Homeo domains encoded by these two genes are not only similar in amino acid sequence but also share a unique feature; that is, the phenylalanine residue in helix 3, conserved in all metazoan homeo domains examined so far (Qian et al. 1989), is replaced by a tyrosine residue.

A finding of particular interest resulting from sequence analysis shows that all 4 amino acids in the homeo domain were located within or near the second helix, possibly containing protein–protein interaction sites (see Fig. 3b; Stern et al. 1989). The locations of 3 of the 4 amino acids were the same as those of 3 amino acids of the *Oct*-1 homeo domain, shown previously to be responsible for interaction with the herpes simplex virus *trans*-activator *VP16* (Batterson and Roizman 1983; Stern et al. 1989). The coincidence of these positions with those of positive control (*pc*) mutations of the λ repressor, which hinder the interaction of the repressor with *Escherichia coli* RNA polymerase without affecting DNA binding, has been reported previously (see also Fig. 3b, bottom; Hawley and McClure 1983; Hochschild et al. 1983; Stern et al. 1989). Thus, although DNA sequences recognized by *BarH1* and *BarH2* proteins appear identical, their activities might be modulated by different *trans*-activators, by direct homeo domain–*trans*-activator interactions.

Figure 9 summarizes the possible induction/determination pathways of late ommatidial differentiation, in which *BarH1* and *BarH2* gene products might function as cooperative transcriptional factors in R1/R6 and primary pigment cells. The expression of the *BarH1/BarH2* genes in R1/R6 is at least partially under the control of *ro* and *gl* proteins (Fig. 4f,g). Because functional *ro* expression occurs only in R2/R5 (Tomlinson et al. 1988; Kimmel et al. 1990) and there is physical contact between R2/R5 and R1/R6 (Tomlinson 1985), *ro*-dependent signals from R2/R5 might be required for the normal development of R1/R6 as in the case of R3/R4 (see Fig. 9; Tomlinson et al. 1988). In contrast, *gl* is expressed in all photoreceptor cells, including R1/R6, in developing ommatidia (Moses and Rubin 1991), *BarH1* and *BarH2*

genes in R1/R6 might thus be directly regulated by *gl* protein, and their gene products might serve as subordinate transcription factors of this protein, which is essential in acquiring photoreceptor identity (Moses et al. 1989).

Some defects in lens structure appeared as a result of abnormal secretion of lens material from cone cells (see Fig. 7a–c). Because two of the four cone cells (anterior and posterior cone cells) come in contact with R1/R6 at the initial stage of differentiation (Tomlinson and Ready 1987) and there is no expression of *BarH1* and *BarH2* in cone cells (see Fig. 4), it is possible that cone cell development requires at least partially *BarH1/BarH2*-dependent signals from R1/R6 (see Fig. 9), without which aberrant lens formation results.

Bar gene deficiency may lead to very serious defects in pigment cells. Most of the major defects in the Bar^- region (see Figs. 7 and 8) appear attributable to some functional or developmental defects in pigment cells. *Bar* homeo box genes are not only expressed but also seem to be functionally required in primary pigment cells, which appear to decrease in number in the Bar^- region of the mosaic eye (see Fig. 8a). Thus, it is feasible that the *Bar* homeo box genes expressed in the primary pigment cells determine, at least partially, primary pigment cell fate (see Fig. 9).

Secondary and tertiary pigment cells must come in contact with primary pigment cells from different ommatidia to acquire cell identity (Cagan and Ready 1989). Mosaic analysis and immunohistochemistry in this study failed to show either functional or nonfunctional expression of *Bar* homeo box genes in secondary or tertiary pigment cells. Furthermore, the morphology of cells situated at the level of secondary pigment cells in the Bar^- region appears to differ from that of the normal secondary pigment cell (Fig. 7g). The fates of secondary and tertiary pigment cells might thus be regulated by *BarH1/BarH2*-dependent signals from primary pigment cells (see Fig. 9). The abnormal differentiation of pigment cells in the Bar^- region would appear to result in the failure of excess unpatterned cells to be adequately eliminated from mature ommatidia, with a consequent increase in retinal cell mass.

Materials and methods

Molecular analyses

Genomic clones were isolated from our genomic DNA library of *D. melanogaster*, and cDNA clones were isolated from our pupal cDNA library (Kojima et al. 1991). Nucleotide sequence was determined as described previously (Emori et al. 1985). RNA was prepared essentially as described previously, sized on agarose gel, and subjected to Northern blotting (Kojima et al. 1991).

The *BarH2* RNA start was determined by primer extension. An oligonucleotide complementary to nuclotides 361–379 was end-labeled with [^{32}P]ATP (3000 Ci/mmole) and T4 polynucleotide kinase, and used as a primer for extension. About 2 x 10^4 cpm of labeled primer was annealed to 100 μg of total pupal RNA for 12 hr at 45°C. After extension was carried out at 37°C for 2 hr, reaction products were analyzed by electrophoresis on a standard sequencing gel. All other procedures were essentially as described by Sambrook et al. (1989).

Antibodies and immunocytochemistry

Anti-*BarH1* and anti-*BarH2* antibodies, along with lysates of *E. coli* cells producing *BarH1* or *BarH2* proteins, were prepared, as will be detailed elsewhere. Eye discs were dissected from third-instar larvae and pupae, and immunostained essentially according to Tomlinson and Ready (1987). However, 10% horse serum was replaced by 5% goat serum, and all reactions were carried out at room temperature. For detection of the primary antibody, biotinylated anti-rabbit antibody (Vector) and Vectastain ABC kit (Vector) were used. Nickel chloride was added upon color development. Osmium intensification was omitted. In the case of double staining, a mixture of anti-*lacZ* monoclonal antibody (Promega, 1 : 200 dilution) and anti-*BarH1* was used as the primary antibody, whereas HRP-conjugated anti-mouse IgG (Cappel, 1 : 400 dilution) and biotinylated goat anti-rabbit were used as the secondary antibody (Vector, 1 : 400 dilution). Diaminobenzidine staining solution (0.5 mg/ml DAB, 0.003% H_2O_2 in PBS) was used in the first cycle of staining, and the second staining was done using Vistastain ABC kit and DAB solution with 0.01% cobalt chloride. An enhancer-trap line, svp^{H162}, was kindly supplied by G. Rubin and used for examination of *svp* expression. Flies with ro^1 and gl^1 mutations were obtained from Mid-America *Drosophila* Stock Center (Bowling Green State University, Bowling Green, OH).

Mosaic analysis

Mosaic analysis was conducted using a *Bar* deficiency of $Df(1)B^{263\text{-}20}$, in which both *BarH1* and *BarH2* genes are deleted (see Fig. 1a). Virgin females heterozygous for $Df(1)B^{263\text{-}20}$ *w/FM7* and $Df(1)B^{263\text{-}20}$ w^+*/FM7*, respectively, were crossed with Canton-S males in the first and second series of mosaic experiments (see Fig. 6a). X-ray irradiation (1200 rads) of first/second-instar larvae was used to induce somatic recombination. In the first series of experiments, about half of the mosaic eyes found in female progeny heterozygous for $Df(1)B^{263\text{-}20}$ *w/+* contained the ommatidium cluster showing characteristic Bar^- phenotypes (see Fig. 7c). The mosaic eyes showing Bar^- phenotypes were isolated and used for further analysis. Fixation and sectioning were performed as described by Tomlinson and Ready (1987). The presence or absence of pigment granules in each ommatidial cell was determined by examining its entire depth.

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Note added in proof

The sequences reported in this paper have been submitted to GenBank (accession no. M82885-7 for the genomic *BarH2* sequences and accession no. M82884 for the *BarH2* cDNA sequence).

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Dual Bar homeo box genes of Drosophila required in two photoreceptor cells, R1 and R6, and primary pigment cells for normal eye development.

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