Developmental Biology **216,** 41–56 (1999)
Article ID dbio.1999.9467, available online at http://www.idealibrary.com on IDEAL®

Extrinsic Modulation of Retinal Ganglion Cell Projections: Analysis of the Albino Mutation in Pigmentation Mosaic Mice

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Tyrosinase is a key enzyme involved in the synthesis of melanin in the retinal pigment epithelium (RPE). Mice that are homozygous for the albino allele at the *tyrosinase* locus have fewer retinal ganglion cells with uncrossed projections at the optic chiasm. To determine the site of the albino gene action we studied the projections of retinal ganglion cells in two types of pigmentation mosaic mice. First, we generated mosaic mice that contain a translocated allele of the wild-type *tyrosinase* on one X chromosome but that also have the *lacZ* reporter transgene on the opposite X chromosome. In these *lacZ/tyrosinase* mice, which are homozygous for the albino allele on chromosome 7, X-inactivation ensures that *tyrosinase* cannot be functional within 50% of the retinal ganglion cells and that these individual cells can be identified by their expression of the *lacZ* reporter gene product, β-galactosidase. The proportion of uncrossed retinal ganglion cells expressing β-galactosidase was found to be identical to the proportion that did not express it, indicating that the albino mutation associated with axonal behavior at the optic chiasm must affect ganglion cells in a cell-extrinsic manner. Second, to determine whether the RPE is the source of the extrinsic signal, we generated aggregation chimeras between pigmented and albino mice. In these mosaic mice, the extent of the uncrossed projection corresponded with the amount of pigmented cells within the RPE, but did not correspond with the genotypes of neural retinal cells. These studies demonstrate that the albino mutation acts indirectly upon retinal ganglion cells, which in turn respond by making axonal guidance errors at the optic chiasm.

Key Words: **decussation; X-inactivation; transgene; chimera; mosaicism; tyrosinase.**

INTRODUCTION

The albino phenotype is characterized by a total absence of pigmentation due to a mutation in the gene encoding the tyrosinase protein. This enzyme catalyzes the first step in the biochemical pathway controlling the synthesis of melanin. A curious feature of albino mammals is that they also exhibit abnormalities in their central visual pathways at sites removed from the normal distribution of melanin. For example, many ganglion cells in the temporal retina that normally give rise to uncrossed projections at the optic chiasm misproject to the contralateral side of the brain (Lund, 1965; Guillery, 1971). The growth patterns of ganglion cell axons in the region of the optic chiasm appear normal and the cellular organization of this structure is intact in albinos (Marcus *et al.*, 1996), suggesting that the defects associated with axonal guidance at the optic chiasm occur at a point upstream of the optic chiasm, either within the optic nerve or within the retina itself.

The melanin-producing cells are located in several positions where they could affect the developing visual pathway. These sites include the choroid, the optic stalk, and the retinal pigment epithelium. Cells that produce melanin

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0012-1606/99 $30.00

in the choroid are not likely to be involved in the development of axonal connections between the retina and the brain because these cells are derived from neural crest and they develop after the abnormality at the chiasm becomes apparent in albinos (Chan *et al.*, 1993; Chan and Guillery, 1993). There is also no correlation between pigment levels in the choroid and axon decussation patterns at the optic chiasm (Sanderson *et al.*, 1974; LaVail *et al.*, 1978; Witkop *et al.*, 1982). Alternatively, pigment-bearing cells in the optic stalk could impart a directional signal to ganglion cell axons as they exit the eye (Silver and Sapiro, 1981; Strongin and Guillery, 1981; Webster *et al.*, 1988). Normally pigmented rats, however, lack pigmented cells in the optic stalk but exhibit normal decussation patterns at the optic chiasm (Horsburgh and Sefton, 1986). Furthermore, there is no relationship between the position of an axon in the optic stalk relative to these pigmented cells and the growth of axons at the optic chiasm (Colello and Guillery, 1990, 1992).

Another candidate target of the albino gene is the retinal pigment epithelium (RPE), although how the cells of the RPE might impart a signal acting upon retinal ganglion cells or their axons is unclear. Gap junctional connections exist between the neuroepithelial and the pigmented epithelial cells during early development (Fujisawa *et al.*, 1976; Hayes, 1976; Townes-Anderson and Raviola, 1981), creating a possible means by which the RPE might influence these cells, but this has not yet been proved. The relationship between melanin in the RPE cells and the retinofugal pathway has been examined in female mice that exhibit pigmentation mosaicism due to an X-autosome translocation involving the *tyrosinase* locus (Cattanach, 1961). When bred onto an albino background, these mice are homozygous for the albino allele on chromosome 7, but possess a wild-type copy of *tyrosinase* on one of the two X chromosomes. Following random X-inactivation (Lyon, 1961), an average of one-half of the RPE cells retain a functional *tyrosinase* due to its presence on the active X chromosome and express pigment. Unexpectedly, these pigmentation mosaic mice exhibited more degenerating axons in the optic tract following enucleation of the ipsilateral eye compared to normally pigmented mice (Guillery *et al.*, 1973). In a follow-up study by Guillery and colleagues using a different technique to mark uncrossed ganglion cells, mosaic mice were found to contain a highly variable number of uncrossed retinal ganglion cells (Guillery *et al.*, 1987). These results suggest that the overall degree of melanin in the RPE may not bear a simple relationship with decussation pattern at the optic chiasm, if they are related at all.

Recent studies have called into question the restricted expression of *tyrosinase* to only melanin-bearing cells within the CNS (Beermann *et al.*, 1992a; Tief *et al.*, 1996), raising the possibility that another site of action for the albino gene defect might be within the retinal neurons themselves. In order to examine this hypothesis directly, we have generated mosaic mice carrying the *tyrosinase* locus translocation on one X chromosome (Cattanach, 1961) and that also have the *lacZ* reporter transgene on the other X chromosome (Tan *et al.*, 1995). The presence of the transgene product, β-galactosidase, is an unambiguous marker for ganglion cells with an inactivated *tyrosinase* and vice versa. We can therefore assess directly the relationship of an active or inactive copy of *tyrosinase* to laterality at the optic chiasm by comparing the proportions of uncrossed retinal ganglion cells with a silenced or potentially active *tyrosinase*.

A second approach, aimed at assessing the relative contributions of tyrosinase activity in the neural retina versus RPE, is to examine chimeric mice made by aggregating mouse embryos derived from an albino strain and a pigmented strain, the latter of which also carries the *lacZ* reporter transgene (Goldowitz *et al.*, 1996). If the albino gene acts in an intrinsic manner within ganglion cells to modify axonal behavior at the optic chiasm, then the size of the population of uncrossed retinal ganglion cells should be related to the proportion of the temporal retina that descends from each of the two genotypes. Alternatively, if the primary determinant of axonal behavior at the optic chiasm is related to pigmentation levels, then the size of the population of uncrossed retinal ganglion cells should be related to the extent of pigmentation in the RPE.

MATERIALS AND METHODS

Animals: lacZ/tyrosinase Mosaic Mice

Female mice harboring the Tyr^{c} locus translocation from autosome 7 to the X chromosome [Is(In7;X)Ct] were obtained from Dr. Bruce Cattanach (MRC Radiobiology Unit, Chilton, Didcot, UK). These mice were bred with male albino BALB/c mice to produce female offspring with a mosaic coat color. Those offspring were then mated with albino male mice carrying the *lacZ* reporter transgene on the X chromosome to produce 30 females of the same variegated coat color status (*lacZ/tyrosinase*), possessing the *tyrosinase* locus translocation on one X chromosome and the *lacZ* transgene on the opposite X chromosome (Fig. 1A). Sections cut through the RPE of these double-mosaic mice used in the present study show the complementarity of the two markers (Fig. 1B): cells of the RPE that lack melanin express the blue, β-galactosidase reaction product, and vice versa.

Aggregation Chimeras

Aggregation chimeras were generated using standard techniques (Le Douarin and McLarin, 1984). Briefly, females were superovulated and mated with like-genotype males overnight. The following morning, successful matings were determined by the presence of vaginal plugs. The 8-cell embryos were harvested at 2.5 days postcoitus and fused overnight in an incubator following enzymatic removal of the zona pellucida. The resulting aggregation blastocysts were then implanted into the uterus of a pseudopregnant host. Two strains of albino mice, BALB/c and ICR, and the pigmented *lacZ*-ROSA26 transgenic strain (Friedrich and Soriano, 1991) were used to generate chimeras. The genotype of transgenic retinal cells was distinguished from albino cells by the presence of

a blue staining in their cytoplasm after detection for β-galactosidase. Therefore, any cell exhibiting reactivity for β-galactosidase is genetically normal at the *tyrosinase* locus and an unstained cell is genetically albino.

Analysis of Uncrossed Ganglion Cells in the lacZ/tyrosinase Transgenic Mosaic Mice

Adult transgenic mice were anesthetized with Avertin and mounted in a stereotaxic headholder, and a craniotomy was performed. Animals were given two to four 0.1-μl injections of a 50% solution of horseradish peroxidase (HRP; Sigma Type VI) dissolved in 2% dimethyl sulfoxide at stereotaxic coordinates previously determined to target the optic tract and visual nuclei. Mice were given a lethal injection of sodium pentobarbital 24 h later, and the dorsal margin of the limbus of each eye was marked on the cornea with a heated pin. Mice were perfused intracardially with 10 ml of 0.9% saline followed by 50 ml of 2% paraformaldehyde in 0.1 M sodium phosphate buffer (PB; pH 7.4 at 20°C), and retinas were dissected immediately after the dorsal mark was transferred to a radial cut running from the dorsal margin of the retina to the optic nerve head. The RPE was confirmed to display the pigmentation mosaicism characteristic of these animals at the time of dissection (Guillery *et al.*, 1987). Retinas were further fixed in the flattened state in 2% paraformaldehyde + 0.2% glutaraldehyde for a maximum of 15 min before being rinsed in phosphate buffer and then processed free-floating for β-galactosidase histochemistry. The reaction solution contained the following reagents: 50 ml of 0.1 M PBS–Triton (0.1%), 8 mg potassium ferricyanide, 105 mg potassium ferrocyanide, 2 mg $MgCl_2$, 10 μl NP-40, 5 mg sodium deoxycholate, and 8 mg of X-gal dissolved in 200 μl dimethylsulfoxide. Detection was carried out for 16 h at 37°C in a shaker oven. Following the reaction, retinas were washed in 0.1 M PB and processed for HRP histochemistry using *p*-phenylenediamine + catechol as the chromogen (Perry and Linden, 1982). Retinas were then prepared as whole-mounts. Brains of these same animals were sectioned at 50 μm and every other section was collected and processed for HRP histochemistry, as above, to confirm the injection sites. Ten normal C57BL/6 *lacZ* transgenic mice were prepared identically, for comparison. A total of eight *lacZ/tyrosinase* mosaic mice and five control, *lacZ* transgenic mice were determined to have excellent retrograde HRP labeling, with no evidence of spread of the HRP across the midline, as well as strong β-galactosidase labeling. These mice were used for further analysis.

Each of the retinas ipsilateral to the injection site was drawn at a magnification of ×40, including the region of the temporal retina containing uncrossed retinal ganglion cells (the temporal crescent). From this region, an area near the widest extent of the temporal crescent was sampled using a X100 oil immersion objective. A sequential series of 90 μm × 90 μm fields was sampled until a minimum of 100 ipsilaterally projecting ganglion cells were encountered. This sample of ganglion cells is approximately one-tenth of the total number of cells making up the uncrossed projection in mice (Rice *et al.*, 1995). Each cell was scored for the absence or presence of β-galactosidase activity. A two-tailed Student's *t* test was performed to determine whether the two samples were drawn from the same population, while a separate test for variance was performed to determine whether one of the two sampled populations showed greater variability.

Analysis of Uncrossed Ganglion Cells in the Aggregation Chimeras

Aggregation chimeras and controls were anesthetized, injected with HRP, perfused, and histochemically reacted as described above, with the exception that the retinas were processed for HRP histochemistry and the uncrossed ganglion cells were quantified prior to the detection of β-galactosidase. Differences in the protocols for preparing these retinas from those described above were as follows: The eyes were enucleated and the muscle was removed from each eye to allow for photographic documentation of the level of pigmentation within that eye when viewed from the ventrotemporal aspect (e.g., Fig. 5B), before retinal dissections were prepared. Separate dissections were performed to produce whole-mounts of both neural retina and the pigment epithelium, which remained attached to the sclera. Following the Hanker-Yates histochemical reaction for detecting HRP (Perry and Linden, 1982), retinas were temporarily mounted in an equal mixture of phosphate buffer and glycerol. The entire population of normal and displaced ipsilaterally projecting ganglion cells was then plotted and quantified with the aid of a drawing tube attached to a Zeiss microscope using a 40× objective and differential interference contrast microscopy. Next, retinas were washed free of the slide in PB and reacted to demonstrate the distribution of the *lacZ* transgenic retinal cell populations using the β-galactosidase reaction as described above. Therefore, the quantification of ipsilaterally projecting ganglion cells was done prior to any knowledge of the genotypic composition of the neural retina. Coverslips were applied with a polyvinyl alcohol and glycerol solution (Gelvatol).

Lineage analysis of the retina has shown that retinal clones in chimeras are uniform in structure. Each clone contains the same ratio of the major cell types as the retina itself (Williams and Goldowitz, 1992). Therefore, to obtain an estimate of the relative proportions of ganglion cells in the temporal crescent derived from the albino versus the pigmented strain, we determined the proportions of neural retinal cells in this region that are derived from the pigmented strain in whole-mounts. Images of the whole-mounts were acquired in Adobe Photoshop using a digital camera attached to a dissecting microscope. The overall retinal area and the area of the temporal crescent were determined from camera lucida drawings using a digitizing tablet (Summagraphics) and the program MacMeasure. Whole-mounts were analyzed using the program NIH Image. The percentage of β-galactosidase-positive cells, which are derived from the pigmented strain, in the total retina and in particular areas such as the temporal crescent was determined by converting the color images to grayscale and adjusting the threshold so that transgene-positive clones were covered by black pixels. The percentage of β-galactosidase-positive cells was determined by dividing the area occupied by the β-galactosidase-positive cells by the total area under study. The placement of the threshold is subjective and measurements obtained with NIH Image were repeated several times with different threshold values. In all cases, the threshold was set so as to minimize inclusion of areas occupied by albino cells, which are β-galactosidase negative.

Quantification of Ganglion Cell Axons in the Optic Nerve of Aggregation Chimeras

Following perfusion, the optic nerves from aggregation chimeras were dissected and postfixed in 1.25% paraformaldehyde and 1.25% glutaraldehyde, rinsed in 0.1 M PB with 6% sucrose, and transferred to cold 2% osmium tetroxide in a 6% sucrose

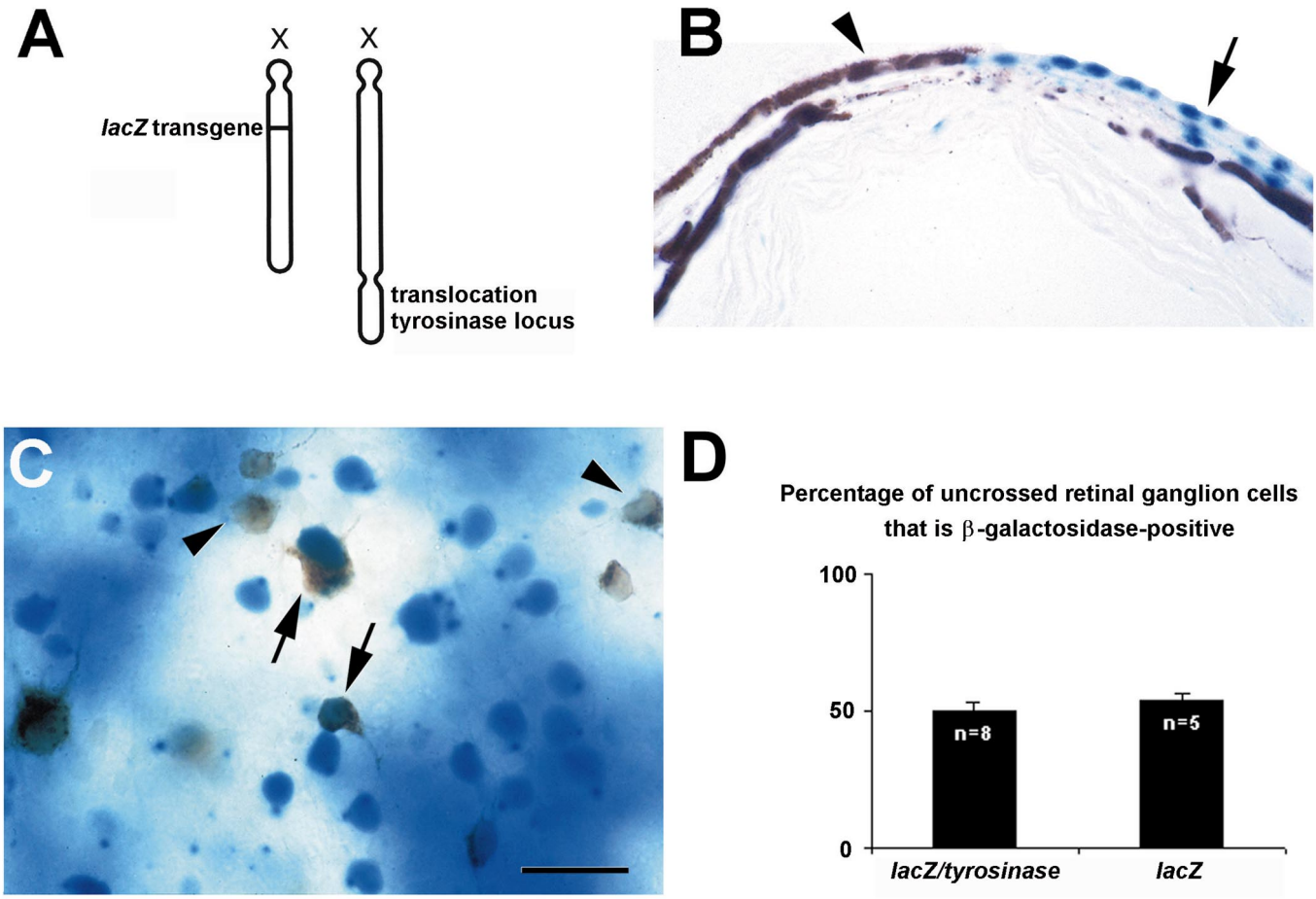


FIG. 1. The presence of the *lacZ* transgene and the translocated region of chromosome 7 containing the *tyrosinase* gene are shown schematically on the two X chromosomes in A. Random X-inactivation produces the complementary double-mosaicism of these mice, illustrated in the cross section of the RPE shown in B. RPE cells either express the *lacZ* transgene and produce β-galactosidase, consequently appearing blue (arrow), or express *tyrosinase* and synthesize melanin, appearing brown (arrowhead; 5-μm-thick transverse resin-embedded section). The curvature of the RPE became inverted at the time of embedding, so that the choroid is down in this field of view). (C) In the temporal retina viewed in whole-mount preparation, the uncrossed retinal ganglion cells are identifiable by their brown HRP label (arrowheads). Some of these retrogradely labeled cells express the *lacZ* transgene (arrows), which appears blue, indicating that *tyrosinase* must be silenced in these cells due to the inactivation of the opposite X chromosome. (D) The proportion of uncrossed cells expressing *lacZ* is 53.7 $\pm$ 2.9% (SEM) in pigmented control mice (right), in which the X-active status should not be related to axonal behavior at the optic chiasm. This proportion is unchanged (49.6 $\pm$ 3.7%) in the *lacZ/tyrosinase* pigmentation mosaic mice (left), in which one of the two X chromosomes now carries *tyrosinase* on an albino background. The scale bar in C is approximately 35 μm for both B and C.

solution for 2 h. The nerves were then washed overnight in 0.1 M PBS, stained for 1 h in 0.5% uranyl acetate, dehydrated, and embedded in Spurr's resin. Transverse ultrathin sections were mounted on single-slot formvar grids and photographed with a Jeol 2000 electron microscope. Estimates of retinal ganglion cell number were obtained by sampling the retinal ganglion cell axons in the optic nerve as previously described (Rice *et al.*, 1995; Williams *et al.*, 1996). The total number of uncrossed cells estimated by light microscopy was divided by the total number of axons in the optic nerve to determine the percentage of retinal ganglion cells with an ipsilateral projection, otherwise known as the decussation ratio.

RESULTS

Within the temporal region of the retina ipsilateral to the HRP injections, individual retrogradely labeled cells could be readily discriminated as being either β-galactosidase positive or negative (Fig. 1C). The brown, HRP label was granular and cytoplasmic, being easily discriminable from the blue, β-galactosidase nuclear label. In control mice (the X-inactivation, *lacZ* transgenic mosaic mice), each ganglion cell has a functional tyrosinase allele on each chromosome 7; therefore, the expression of the *lacZ* transgene should

bear no relationship to the decision of its axon to decussate or project ipsilaterally. One would predict, therefore, that the population of uncrossed (or for that matter, the crossed) retinal ganglion cells should show an equal probability of having either an active or an inactive X-linked, *lacZ* transgene. This was in fact the case, evidenced by the percentage of uncrossed retinal ganglion cells that were transgene-active, or blue, which was about 50% (53.7 ± 2.9% SEM; Fig. 1D).

The *lacZ/tyrosinase* mice that are homozygous for the albino allele on chromosome 7, by contrast, have a potentially functional *tyrosinase* on one X chromosome, but due to random X-inactivation, approximately 50% of all cells will have inactivated this gene. If *tyrosinase* contributes in a cell-intrinsic fashion within individual ganglion cells to influence axonal growth at the optic chiasm, then one would expect a correlation between *tyrosinase* inactivation with crossed or uncrossed projection status in the temporal crescent. Put another way, the temporal retina will contain an equal number of ganglion cells that are blue and white, but now blue or white status is a direct indicator of potential *tyrosinase* status and should correlate with decussation behavior. If the population of temporal retinal ganglion cells suffers decussation errors due to the absence of a functional *tyrosinase,* then we should detect an imbalance of uncrossed cells with an active copy of *tyrosinase.*

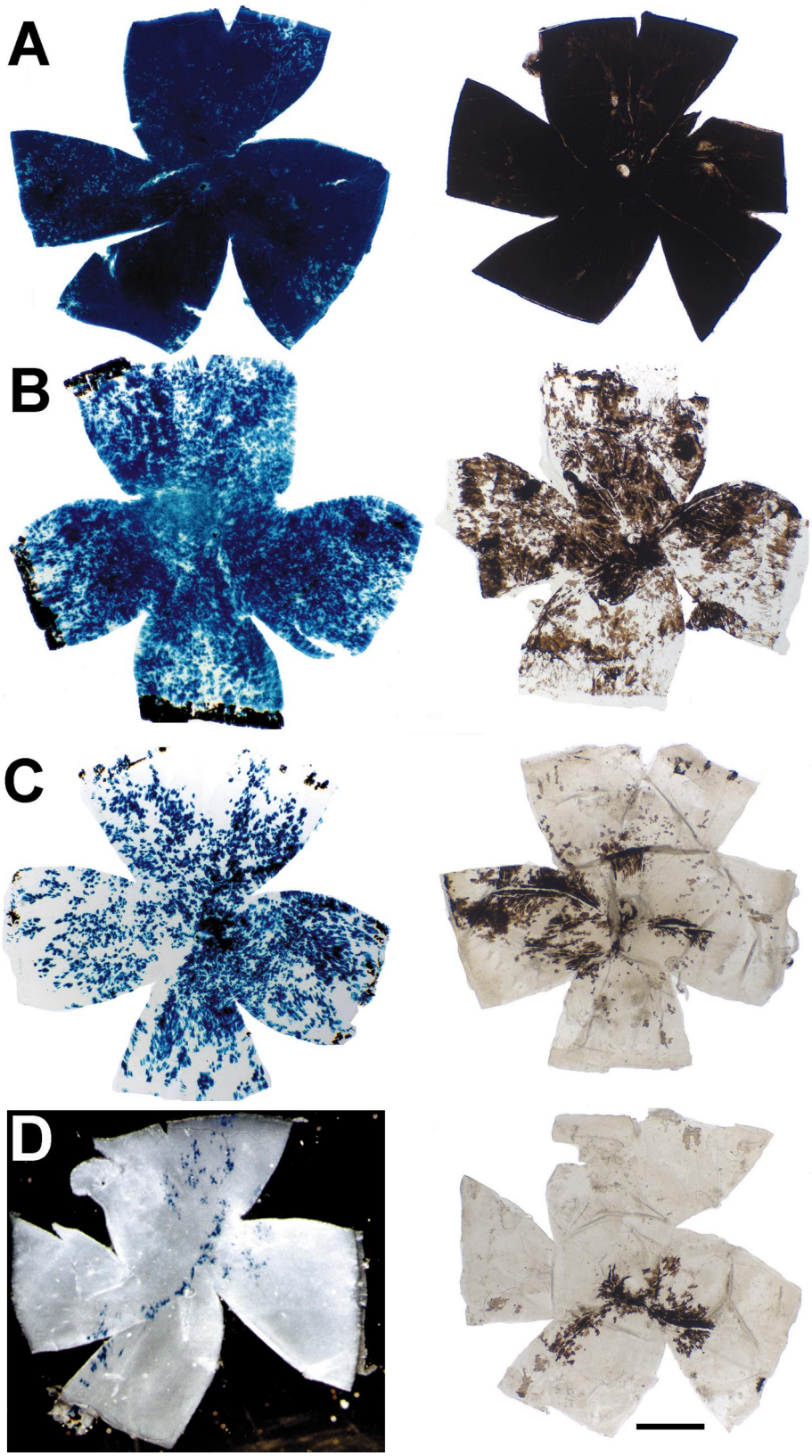
In fact, the percentage of uncrossed retinal ganglion cells containing an active *lacZ* transgene, and therefore possessing a silenced *tyrosinase,* was similar to that observed in normal animals, being about 50% (49.6 ± 3.7%; Fig. 1D; one-way ANOVA, $F(1,11) = 0.67$, n.s.). The frequency of blue to white uncrossed retinal ganglion cells did not appear to change. Neither did the variances for these percentages differ between the two populations (Fig. 1D; Levene statistic for variance, $F(1,11) = 3.9$, n.s.), despite the conspicuous variance reported for the total size of the uncrossed projection in these *tyrosinase* locus translocation mosaic mice (Guillery *et al.*, 1987). These results demonstrate that the presence or absence of a functional *tyrosinase* in ganglion cells within the temporal crescent does not contribute to axonal behavior at the optic chiasm.

The data on the retinofugal projections in the *lacZ/tyrosinase* pigmentation mosaic mice support the hypothesis that *tyrosinase* acts in a non-cell-autonomous manner to affect ganglion cell trajectories at the optic chiasm. In a second set of experiments, we used aggregation chimeras to test the relationship between pigmentation and retinal projections at the optic chiasm. Chimeras were generated between a pigmented strain, which harbors a *lacZ* transgene expressed in all cells (Friedrich and Soriano, 1991), and an albino strain. A total of 18 chimeras were generated and these exhibited varying degrees of coat color mosaicism. Similarly, the eyes of chimeric animals ranged from heavily pigmented to sparsely pigmented.

In every chimeric retina analyzed, there was extensive intermingling of clones derived from the pigmented and albino genotypes (Fig. 2). Clusters of cells derived from the pigmented parental genotype were often found to be completely surrounded by cells derived from the albino strain in both retina and RPE. As a group, the left and right eyes of chimeric mice exhibited a similar balance of the two parental genotypes, although there was some variability within single cases. The percentage of *lacZ*-positive neural retinal cells (derived from the pigmented strain) ranged from 98 to 2%, which is similar to that obtained in a previous study in which clones of cells were identified using *in situ* hybridization with strain-specific probes (Williams and Goldowitz, 1992). The pigmented genotype was the more predominant in the majority of chimeric retinas (12/18).

Qualitative examination of the genotypic composition within the neural retina and RPE revealed a good correlation among the chimeras analyzed. The whole-mounts shown in Fig. 2 illustrate this relationship between the blue component in the neural retina, derived from the pigmented strain, and the brown component in the corresponding pigment epithelium in four different chimeras. (The pigmentation in the choroid, which results from neural crest-derived melanocytes, was distinguishable from that in the RPE by the shape of the cells; e.g., arrows in Fig. 3F). In all cases in which the neural retinas contained large clusters of blue, β-galactosidase-positive cells, the corresponding RPE contained large patches of melanin-bearing cells (Figs. 2A and 2B). In a few cases, the albino genotype was predominant and only a few blue cells were found in the neural retina (Fig. 2D, left). The corresponding RPE in these cases contained only scattered populations of pigmented cells (Fig. 2D, right).

Although there was a good correlation in the proportions of genotypic composition in the neural retina and the RPE among chimeric retinas, there were often gross disparities in the location of cells with similar genotypes in these two layers. For example, large polyclones (aggregates of cells derived from the same parental strain) of neural retinal cells derived from the albino genotype (Fig. 3C) were frequently found over large patches of cells that contained melanin in the RPE (asterisks in Fig. 3D). These same regions showed smaller patches of blue cells in the neural retina directly above nonpigmented regions of the RPE (compare arrows in Figs. 3C and 3D). The converse was also evident in that large cohorts of blue cells (e.g., asterisk in Fig. 3E) were found directly above cells in the RPE that lacked melanin (Fig. 3F). The discordance in position of like-genotype cells in neural retina and RPE occurred in both peripheral (Figs. 3C and 3D) and central (Figs. 3E and 3F) retinal regions. These results show that while the overall proportion of parental genotypes in neural retina and RPE is similar, the cells derived from the same parental genotype are often found in different locations within these structures. This discordance is expected given the derivation of these two tissues from distinct compartments in the developing optic vesicle (Mann, 1964; Graw, 1996). This spatial dissociation permits us to examine the relationship between pigmentation in the RPE and decussation patterns of the overlying



population of ganglion cells in the temporal retina of chimeric mice.

Four of the chimeric retinas ipsilateral to the injection site exhibited patterns of retrograde labeling that were deemed complete in that the labeled cells covered a surface area of approximately 20% of the retina and they were confined to the temporal crescent. We quantified two populations of ganglion cells in the retina ipsilateral to the injection site. The first population of ganglion cells is located in the ganglion cell layer and is referred to as the normal population of uncrossed cells. The second population is located in the inner nuclear layer and is referred to as the displaced population of uncrossed ganglion cells. The displaced ganglion cells are more severely affected by the albino mutation than the normal population, in that they contain proportionally fewer uncrossed ganglion cells (Dräger and Olsen, 1980; Balkema and Dräger, 1990; Rice *et al.,* 1995).

The BALB/c albino mice have approximately 1200 ipsilaterally projecting ganglion cells. Of the uncrossed retinal ganglion cells, around 8% (104) of these are displaced ganglion cells (Rice *et al.,* 1995). In the *lacZ,* pigmented mouse, there are 1541 uncrossed cells and about 13% (199) of these cells are displaced from the ganglion cell layer. While these differences may seem modest, they are highly reproducible. For example in pigmented mice from different strain backgrounds, there are approximately 1422 $\pm$ 51 (SEM; $n = 8$) uncrossed ganglion cells. In albinos, there are approximately 1138 $\pm$ 30 ($n = 13$). Pigmented mice contain an average of 186 $\pm$ 20 displaced, ipsilaterally projecting ganglion cells, whereas the albinos contain 77 $\pm$ 9 of these displaced cells (Table 1). Thus, there are approximately 50% fewer displaced ganglion cells in albinos than in pigmented mice, whereas there are only about 20% fewer uncrossed ganglion cells in the ganglion cell layer.

Retinas from two different chimeras contained a total of 1666 and 1234 uncrossed ganglion cells (Figs. 4 and 5, respectively). On the basis of these numbers, these two retinas display uncrossed projections like those typical of normally pigmented versus albino animals (Table 1). As considered in the *lacZ/tyrosinase* mice, if the albino mutation acts intrinsic to the ganglion cell population, the mixture of genotypes within the neural retina would favor the pigmented strain in the former case (Fig. 4) and the albino strain in the latter case (Fig. 5). Yet in each of these chimeric retinas, the majority of the retinal cells were derived from the pigmented or blue strain (compare Figs. 4A and 5A). In the chimeric retina that contained the most uncrossed ganglion cells (Fig. 4C), the percentage of blue cells overall is 75%, whereas the albino genotype covers only 25% of the retina (Fig. 4A). However, in the region of the retina where the uncrossed ganglion cells reside, the temporal crescent (outlined in Fig. 4A), there were large clones of cells derived from the albino strain (asterisk in Fig. 4A). The percentage of cells derived from the albino and pigmented parental strains within this region was approximately 50%. In this retina there were 1477 normally positioned and 189 displaced ipsilateral ganglion cells (Fig. 4C). Hence, despite a significant proportion of the uncrossed population of ganglion cells with the albino genotype (arrows in Fig. 4D), the normal and displaced ipsilateral cells were increased by 24 and 45%, respectively, when compared to control albino mice. These numbers contrast with those in another chimeric retina with a similar representation of the albino and pigmented genotypes (roughly 50:50) in the temporal crescent (outlined in Fig. 5A). In this case, however, there were 1154 normally positioned and 80 displaced, ipsilaterally projecting ganglion cells (Fig. 5C). These numbers typify those found in the albino parental strain, despite the presence of uncrossed ganglion cells derived from the pigmented strain (arrowheads in Fig. 5D). These results suggest that the genotypic composition of neural retinal cells in the ventrotemporal retina does not affect the extent of the ipsilateral projection at the optic chiasm, consistent with the conclusions derived from the analysis of uncrossed retinal ganglion cells in the *lacZ/tyrosinase* mice described above.

The distinguishing feature between these two chimeras was the level of pigmentation in the RPE. In the chimeric retina that exhibited a large population of normal and displaced ganglion cells, the majority of the RPE was heavily pigmented, especially in the ventrotemporal retina (Figs. 4B and 4E). In the chimera that exhibited uncrossed cell counts similar to those in the albino strain, patches of pigment epithelial cells were observed in several areas of the ventral eye, but they were small and widely dispersed (Fig. 5B). Two additional chimeras contained only a few clusters of pigmented cells in the RPE and there were low percentages of neural retinal cells derived from the pigmented strain (Fig. 6; Table 1). These mice displayed uncrossed projections that were similar to the chimera shown in Fig. 5, indicating that small patches of pigmentation do not play a significant role in determining projections at the optic chiasm.

Albino animals exhibit a reduction in the relative number of ganglion cells with ipsilateral projections. Because

FIG. 2. Whole-mount preparations of tissue in the left eye of four different chimeras are shown in A–D. On the right side is the RPE, which is attached to the choroid and sclera, and on the left side is the corresponding neural retina. The whole-mounts are arranged in descending order relative to the genotypic contribution of the *lacZ* transgenic (pigmented) strain. Notice that the positioning of the blue cells in the neural retina and the pigmented cells in the RPE are sometimes but not always coincident. The whole-mount of the neural retina shown in D was photographed in dark-field illumination in order to highlight better the small number of blue cells. The ganglion cell layer and RPE are facing up. Dorsal is to the top of the figure and temporal is to the right. Scale bar is approximately 1 mm.

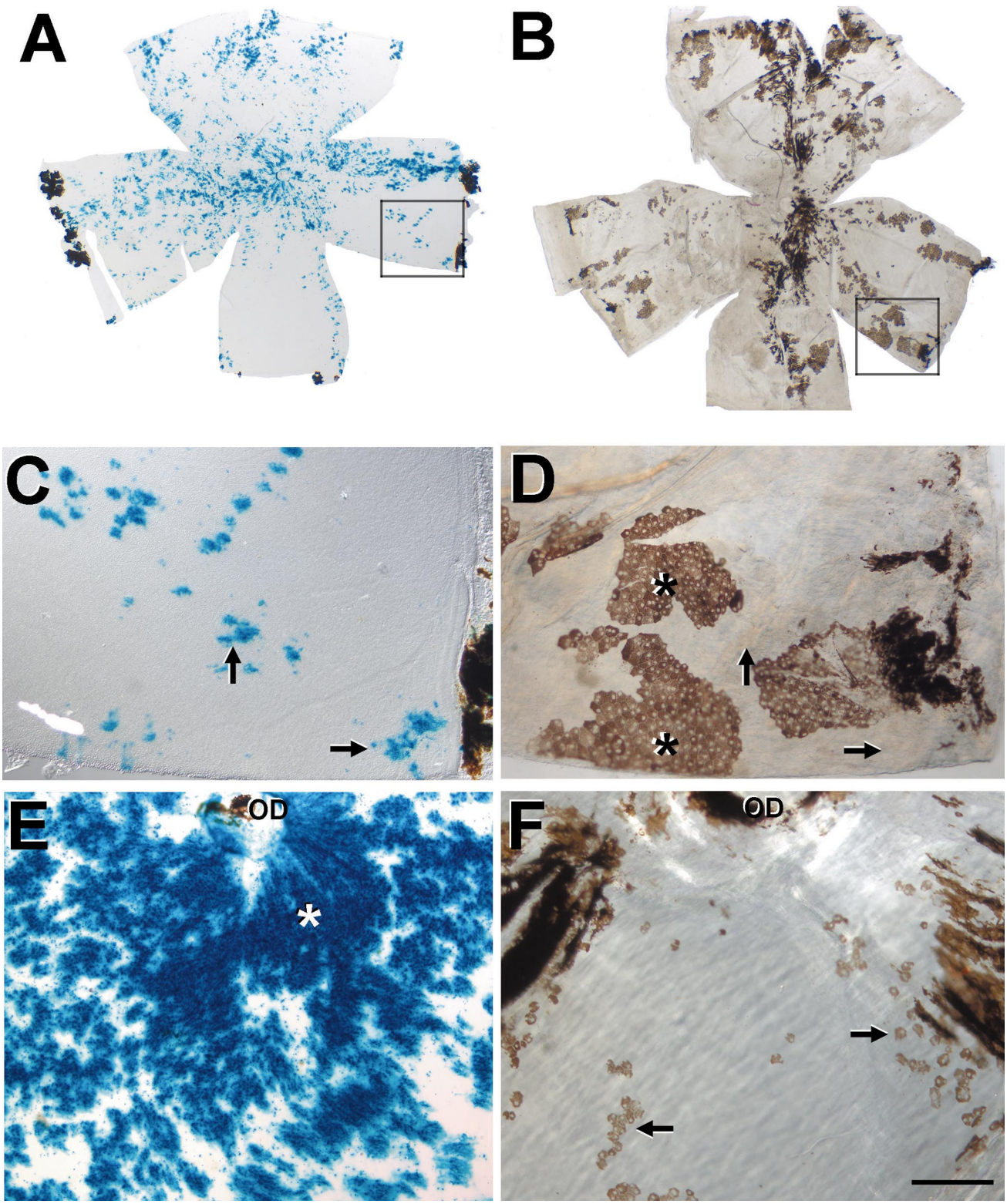


FIG. 3. Cells with similar genotypes are often misaligned in the neural retina and RPE. (A–D) Low- and high-power images of the neural retina and RPE in whole-mount view from a chimera generated between a pigmented mouse and an albino mouse. The boxes in A and B are shown at higher magnification in C and D. Although there are a few small clones of blue cells (arrows in C) in the periphery of the retina,

TABLE 1
Summary of Uncrossed Ganglion Cell Counts (±SEM) and Decussation Ratios in Pigmented, Chimeric, and Albino Mice

Genotype	Ipsilaterally projecting cells			Axons	Decussation ratio
	Normal	Displaced	Total		
Pigmented (n = 8)	1238 ± 54	186 ± 20	1422 ± 51	60,100 ± 4900	2.5 ± 0.2
Chimera 1	1477	189	1666	61,200	2.7
Chimera 2	1154	80	1234	63,400	1.9
Chimera 3	1182	115	1297	nd	nd
Chimera 4	1060	80	1140	63,200	1.8
Albino (n = 13)	1061 ± 35	77 ± 9	1138 ± 30	63,500 ± 1500	1.8 ± 0.1

Note. nd, not determined.

different mouse strains exhibit large differences in the total number of ganglion cells, this decrease is best observed by comparing the percentage of ganglion cells with uncrossed projections in albino and pigmented animals (Rice *et al.,* 1995; Williams *et al.,* 1996). The decussation ratio is the total number of uncrossed cells divided by the total number of ganglion cells in the retina. This ratio is decreased from approximately 2.5 ± 0.2% (SEM; n = 8) in pigmented mice to 1.8 ± 0.1% in albino mice (n = 13), regardless of the genetic background of the mouse strain (Table 1). We determined the total number of ganglion cells by counting the number of axons in the optic nerve ipsilateral to the site of the HRP injections. The decussation ratios for the chimeric retinas are similar to either pigmented or albino strains that we have studied. For example, the percentage of uncrossed retinal ganglion cells in the chimeric retina shown in Fig. 4 was 2.7%, whereas this percentage was 1.9% for the chimera shown in Fig. 5. The decussation ratio was determined for one of the chimeric retinas that exhibited a small proportion of neural retinal cells and RPE cells derived from the pigmented strain and that contained uncrossed cell counts similar to that for the albino strain. The decussation ratio in this case was 1.8%. These results suggest that the decussation ratio, or the extent of the uncrossed visual pathway, reflects the level of pigmentation in the RPE, not the presence of the normal *tyrosinase* allele in the ganglion cell itself.

DISCUSSION

The present study addressed the question of where in the visual pathway the albino mutation acts to influence pathway decisions made by growing axons as they traverse the optic chiasm. We considered two possible sites of action for the albino mutation. We first considered the possibility that the albino mutation acts cell-intrinsically, within the retinal ganglion cells themselves, directly rendering them defective in their ability to respond to cues at the optic chiasm. Alternatively, we considered the possibility that ganglion cells are influenced by the albino mutation in a non-cell-autonomous manner. In this case, the albino mutation impacts the growth of axons at the optic chiasm indirectly, by acting on some other cellular target that in turn alters retinal ganglion cells. We considered the possibility that this action occurs within the RPE itself.

We used two unique approaches to examine the primary site of action of the albino mutation. In *lacZ/tyrosinase* transgenic mice, we relied upon the phenomenon of random X-inactivation to establish a population of ganglion cells that either possessed or lacked a potentially functional *tyrosinase.* We then identified the population of uncrossed ganglion cells and assessed the proportion containing this potentially functional *tyrosinase* relative to those lacking it. We found that roughly identical numbers of uncrossed retinal ganglion cells possessed or lacked *tyrosinase,* indicating that tyrosinase action within the ganglion cells themselves does not play a role. Estimates of the size of the proportion of uncrossed ganglion cells affected by the albino mutation range from about 20% (Rice *et al.,* 1995) to 40% (Guillery *et al.,* 1987), so we would have expected to detect a change in the ratio of uncrossed cells with either of the two X chromosomes active if a cell-intrinsic action of *tyrosinase* contributed to the laterality decision at the optic chiasm. The same conclusion was drawn from our second approach, using aggregation chimeras derived from morulas

they are not aligned with the large clusters of pigmented cells (asterisks) in the underlying RPE. E and F show the central retina of a different chimera, in which the optic disc (OD) is used as a point of reference. There is a large polyclone of blue cells (asterisk in E) derived from the pigmented genotype near the OD. In the underlying RPE, however, the majority of cells are derived from the albino genotype (compare with F). There are only a few, scattered pigmented cells (arrows in F) in the RPE in this part of the retina. Scale bar is approximately 1 mm in A and B and 200 μm in C–F.

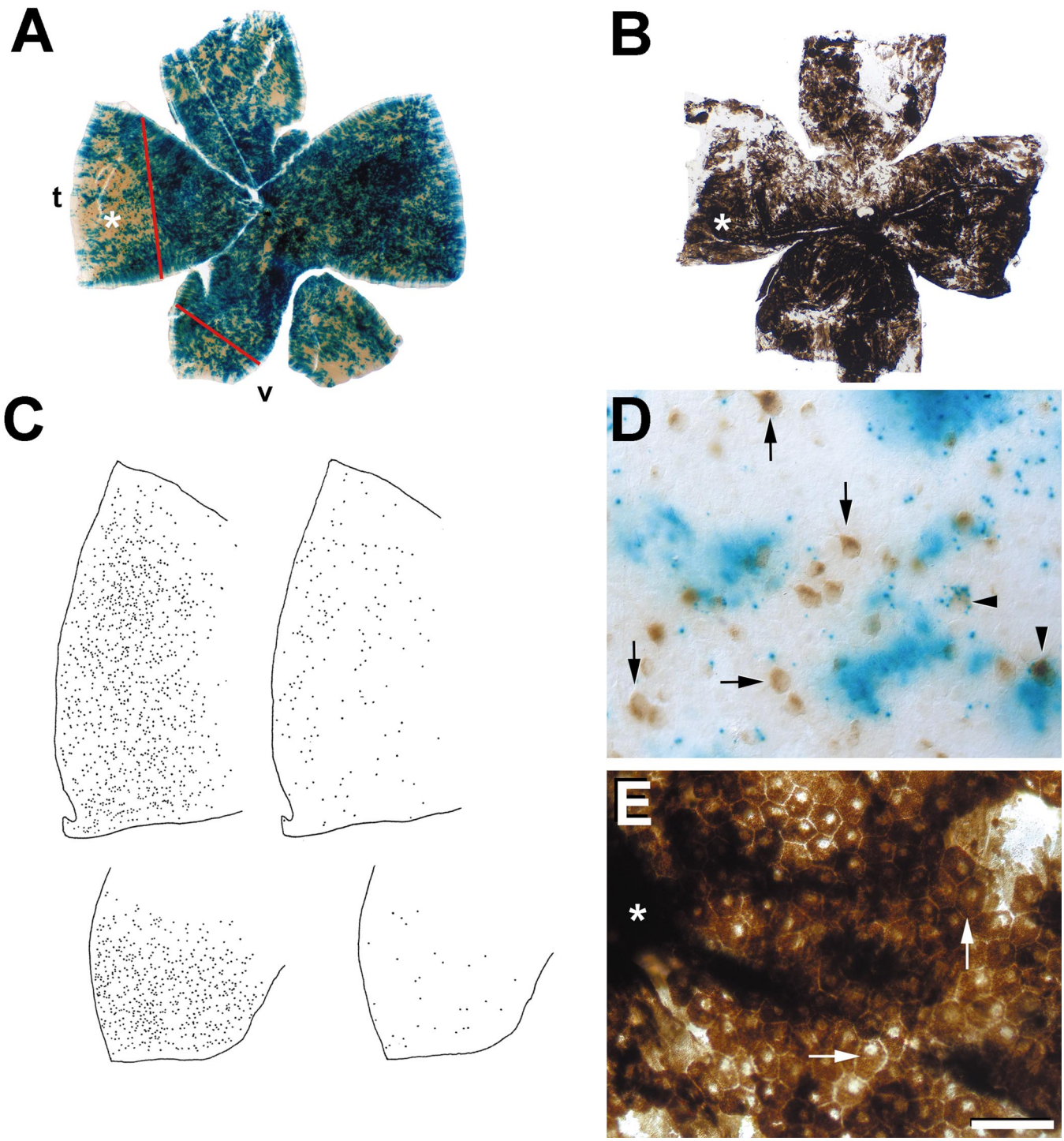


FIG. 4. Chimeric retina with a pattern of decussation at the optic chiasm like that of a normally pigmented mouse. (A) Whole-mount preparation of the neural retina ipsilateral to the HRP injection in the brain. Cells derived from the *lacZ* (pigmented) strain appear blue, whereas those derived from the albino strain are white. Overall, this chimera contains a majority of cells derived from the pigmented strain. However, in the temporal crescent (outlined in red), which is located in the ventral (v) and temporal (t) retina and is the site of origin of the ipsilaterally projecting ganglion cells, there are large clones of cells derived from the albino strain (asterisk). The area demarcated by the asterisk is shown at higher magnification in D. (B) Whole-mount preparation of the corresponding RPE that is heavily pigmented in the area of the crescent (asterisk in B is shown at high magnification in E). (C) The number and topography of normally positioned (left), ipsilaterally projecting ganglion cells (each dot represents one cell) are similar to those in the pigmented strain, despite a majority of ganglion cells that

of albino and pigmented genotypes. We demonstrated that the extent of the ipsilateral projection was unrelated to the proportion of neural retinal cells that descended from either the albino or the pigmented genotype. Collectively, these results provide strong evidence that the albino mutation acts extrinsically to the retinal ganglion population.

Other recent evidence had implicated the eye, rather than the chiasm itself, as the most likely site of the albino gene action. Despite the fact that albinos have fewer uncrossed axons, the spatial and temporal patterns of their trajectories through the chiasmatic region are similar to those observed in normally pigmented animals (Chan *et al.*, 1993; Marcus *et al.*, 1996). Early enucleation of one eye (before any optic axons have reached the chiasm) results in a decrease in the size of the uncrossed projection of the remaining eye, appearing superficially like the abnormality observed in albinos (Godement *et al.*, 1987; Guillery, 1989) and suggesting that axonal interactions at the chiasm may be related to the misprojection of axons. However, early enucleations performed in albinos result in an exacerbation of the albino phenotype (Chan and Guillery, 1993), suggesting that the retina is intrinsically altered in these mutants before the axons reach the optic chiasm.

Further support for a retinal abnormality has been provided by studies in which retinal and chiasmal tissues derived from either albino or pigmented embryos were cocultured in order to assay the site of action of the albino mutation. Retinal neurite outgrowth was indistinguishable when presented with chiasmal tissue derived from either normally pigmented animals or albinos (Marcus *et al.*, 1996). Indeed, anatomical and immunohistochemical studies of the optic chiasm in pigmented and albino animals failed to detect any differences in the molecular composition and distribution of chiasmal cells (Marcus *et al.*, 1996; Mason and Sretavan, 1997). The optic chiasm, therefore, appears similar in both the pigmented and the albino animals. By contrast, the extent to which neuritic outgrowth from the ventrotemporal retina was inhibited by chiasmal cells was dependent on whether the retinal tissue was derived from albino or pigmented retina (Marcus *et al.*, 1996; Wang *et al.*, 1996). In explants derived from albino eyes, many neurites of ganglion cells in the ventrotemporal retina exhibited growth parameters similar to those observed for neurites with normally crossed projections, suggesting that there is a respecification of ganglion cells in the ventrotemporal retina of albinos. Although the abnormality in the retinofugal pathway of albinos is not apparent until the optic chiasm, where fewer ganglion cell axons extend into the ipsilateral optic tract, the conclusion from these studies is that the eye is the site of the albino gene action (Chan *et al.*, 1993).

The misalignment of neural and pigmented epithelial cells that are derived from the same parental strain in chimeric eyes is due to the extensive intermixing of clonally distinct cells prior to the formation of the optic cup, followed by the differential growth of the inner and outer layers of the cup as they form the neural retina and RPE layers, respectively (Pei and Rhodin, 1970; Sanyal and Zeilmaker, 1977; Bodenstein and Sidman, 1987). This mismatch in clonally related cells between the two retinal layers provided a convenient way to pinpoint the RPE as the most likely candidate for the site of the albino gene action. The RPE is known to express tyrosinase at high levels adjacent to the neural retina (Beermann *et al.*, 1992b), and the expression of tyrosinase occurs during the period when ganglion cells are generated and projecting their axons to central targets in the brain (Dräger, 1985a; Colello and Guillery, 1990; Marcus *et al.*, 1996). Analysis of uncrossed projections in aggregation chimeras in the present study provided evidence that the RPE is indeed the site of the albino gene action. For example, the chimeric eye shown in Fig. 4 exhibited an uncrossed visual pathway that was indistinguishable from that observed in normally pigmented mice, despite the fact that large clones of retinal cells, which contained many uncrossed ganglion cells, were derived from the albino strain. This chimera exhibited a decussation ratio like that of a normally pigmented mouse and the total number of uncrossed ganglion cells was similar to that in pigmented mice. Examination of the distribution of pigmented and nonpigmented cells in the whole-mount of the RPE of this same case showed that the majority of cells under the area of the uncrossed ganglion cells (i.e., the temporal crescent) contained melanin. The extent of pigmentation in the RPE of this chimera was relatively high when compared to the chimera depicted in Fig. 5, which contained a similar contribution of parental genotypes in the neural retina. In this latter case, however, the pigmentation appeared mostly in the dorsal sector of the eye, whereas the ventrotemporal region contained only scattered populations of melanin-positive RPE cells. In this case, the decussation ratio and total number of uncrossed ganglion cells were indistinguishable from those in the albino mice, suggesting that the positioning of melanin in

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are derived from the albino strain (arrows in D). Similarly, the number of displaced (right), ipsilaterally projecting ganglion cells is similar to that found in the normally pigmented strain. (D) Higher magnification view of the area in A indicated by an asterisk. This field contains many ipsilaterally projecting ganglion cells (arrows; brown) that are derived from the albino strain (that is, which are also not blue). There are only a few ipsilateral cells that are derived from the pigmented strain (and so are also blue; e.g., arrowheads). (E) The majority of the pigment epithelium beneath the temporal crescent is populated by pigmented cells (arrows). The asterisk indicates an area that contains melanin in the RPE as well as the choroid. In A–C, dorsal is up and temporal is to the left. Scale bar in E is approximately 1 mm in A and B, 600 μm in C, and 100 μm in D and E.

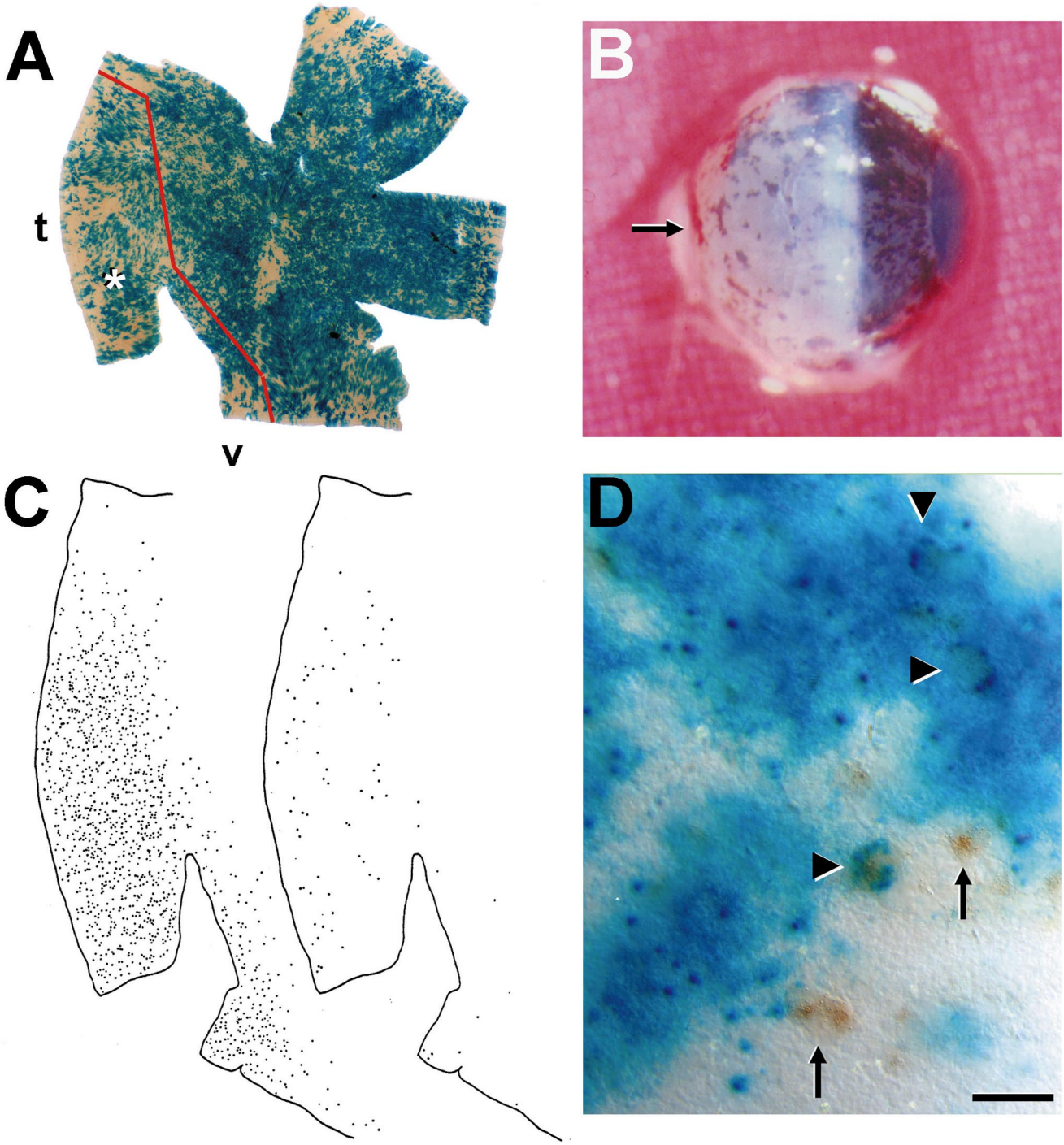

FIG. 5. Chimeric retina with a pattern of decussation at the optic chiasm typical of that found in albino animals. (A) Whole-mount preparation of the neural retina ipsilateral to the HRP injection in the brain. Cells derived from the *lacZ*, pigmented strain and the albino strain are evenly represented in the temporal crescent (outlined). (B) Ventral view of the whole eye before dissection. The RPE, as well as the choroid, is only sparsely populated with pigmented cells. The arrow indicates the posterior pole of the eye near the optic nerve head. (C) The number of normally positioned (left), ipsilaterally projecting ganglion cells is similar to that in the parental albino strain, as is the number of displaced (right), ipsilaterally projecting cells. (D) This is true despite the fact that there are frequent ipsilaterally projecting cells derived from the pigmented genotype in this chimera, evidenced by their double-labeling for HRP and β-galactosidase (arrowheads). The arrows indicate uncrossed ganglion cells derived from the albino genotype. Scale bar is approximately 800 μm in A, 1.5 mm in B, 600 μm in C, and 20 μm in D.

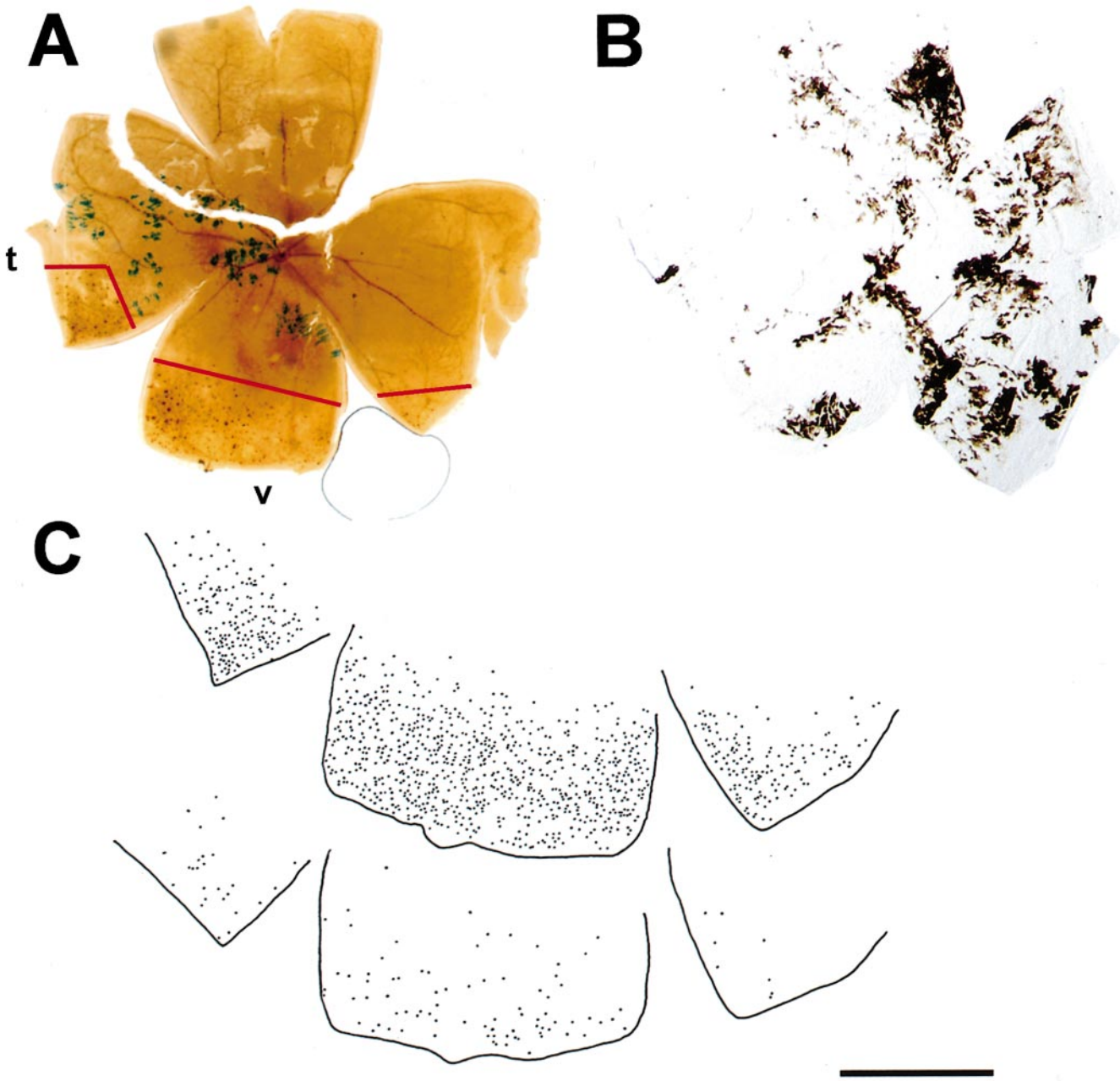

FIG. 6. Chimeric retina with a pattern of decussation at the optic chiasm like that found in albino animals. (A) Whole-mount preparation of the neural retina ipsilateral to the HRP injection in the brain. Cells derived from the *lacZ*, pigmented strain are sparsely represented in the retina. The temporal crescent (outlined) contains uncrossed ganglion cells derived from the albino strain. (B) Whole-mount preparation of the corresponding RPE, as well as the choroid, demonstrates that only scattered populations of pigmented cells are present in this case. (C) The number of normally positioned (top), uncrossed retinal ganglion cells is similar to that in the parental albino strain, as is the number of displaced (bottom) cells. In A–B, dorsal is up and temporal is to the left. The scale bar is approximately 2 mm in A and B and 800 μm in C.

the RPE is correlated with the extent of the uncrossed visual pathway in mosaic mice.

Mutations disrupting other genes that encode proteins involved in either the production or the packaging of melanin also result in abnormal projections from the retina to the brain, such that axons originating from a subset of ganglion cells located in the temporal part of the retina and normally extending into the ipsilateral optic tract are re-

routed into the contralateral optic tract (Sanderson, 1975; La Vail *et al.*, 1978; Balkema and Dräger, 1990). These mutations affect the displaced population of uncrossed ganglion cells even more severely than the uncrossed ganglion cells located in the ganglion cell layer (Dräger and Olsen, 1980; Balkema and Dräger, 1990; Rice *et al.*, 1995). In chimeras generated between pigmented and albino mice, we observed that the number of displaced retinal ganglion cells with uncrossed projections reflected the level of pigmentation in the RPE, as did the decussation ratio and the overall number of retinal ganglion cells. The displaced, ipsilaterally projecting ganglion cells are generated at times similar to those for the normally positioned uncrossed ganglion cells and they do not appear to represent a distinct morphological type of cell (Dräger, 1985a), so the underlying cause of the differential effect of mutations affecting pigmentation on these ganglion cells is unknown, although the positioning of these cells closer to the RPE may be more than coincidental. Based on our results, it is likely that these displaced ganglion cells are affected by the RPE in a manner similar to the other uncrossed ganglion cells.

There are several issues that remain to be addressed regarding the action of the albino mutation and the role of tyrosinase during retinal development. While it is clear that the RPE has a strong influence over the development of the neural retina, the route of communication between these layers is unclear. Gap junctions do exist between the neural retina and RPE during the earliest stages of retinal development and these may provide a conduit by which key molecules move from one cell type to another (Fujisawa *et al.*, 1976; Hayes, 1976; Townes-Anderson and Raviola, 1981). Alternatively there may be diffusable factors produced by the RPE that are capable of affecting the neural retina (Raymond and Jackson, 1995; Sheedlo and Turner, 1996; Sheedlo *et al.*, 1998).

Regardless of the means by which information is relayed, we do not yet know whether melanin is controlling the specification of retinal ganglion cell projections at the optic chiasm or whether there is some agent associated with melanin production that is responsible for the phenotypes found in the neural retina in hypopigmentation mutants (Jeffery, 1997, 1998). Tyrosinase catalyzes the oxidation of tyrosine to 3,4-dihydroxyphenylalanine (L-dopa), which has been suggested to affect the rate of the cell cycle in the retina (Akeo *et al.*, 1994). Indeed, albinos exhibit excessive mitotic profiles followed by increased numbers of pyknotic nuclei (Ilia and Jeffery, 1999), which are likely to relate to the deficits observed in rod photoreceptors in these mutants (Jeffery and Kinsella, 1992; Jeffery *et al.*, 1994). In addition, the regulation of calcium levels is disrupted in albinos due to the strong buffering capacity of melanin (Dräger, 1985b). This could also affect the cell cycle or the frequency of cells withdrawing from it in the neural retina. These abnormalities do not easily relate to the misrouting of ganglion cell axons at the optic chiasm, however, because uncrossed retinal ganglion cells are generated before these defects in cell mitosis and cell death become apparent in albinos (Dräger, 1985a; Reese and Colello, 1992). There is a spatial and temporal delay in the time at which neurons in the ganglion cell layer exit the cell cycle in albinos (Ilia and Jeffery, 1996). One possibility is that fewer cells in the albino retina are therefore exposed to cues controlling the specification of uncrossed ganglion cells, as the latter are suspected to decrease as the retina matures (Dräger, 1985a; Reese *et al.*, 1992; Baker and Reese, 1993). This may explain why the albino phenotype affects a relatively small proportion of uncrossed ganglion cells. The implication then is that these cues, currently undefined, and their transient nature are unaffected in albinos.

ACKNOWLEDGMENTS

This research was supported by grants from the NIH (EY-8868 and EY-9586 to R.W. and D.G.; EY-11087 to B.E.R.) and from the NH and MRC (to S.S.T.). We thank Dr. Bruce Cattanach for providing the founder mice used to breed subjects for Experiment 1 and for his further comments and advice and Dr. Ray Guillery for his insightful comments during the design stages of the experiments involving the *lacZ/tyrosinase* mice. We also thank Drs. Gary Baker and Glen Jeffery for critically reading the manuscript.

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Received for publication June 29, 1999
Revised August 23, 1999
Accepted August 23, 1999