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A Longitudinal Study on the Expression of the Opsin Gene in the Degenerating Retina of C3H/He Mice

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Summary. **By a combination of immunohistochemistry and *in situ* hybridization using a ^{35}S-labeled opsin cDNA probe, it was revealed in rd mutant C3H/He mice that opsin mRNA is not detectable in photoreceptor cells which still exhibit opsin-immunoreactivity at later stages of retinal degeneration. This indicates that the photoreceptor cells of rd mutant mice cease to express the opsin gene at earlier stages of retinal degeneration.**

The hereditary degeneration of retinal photoreceptor cells, first reported by KEELER (1927), is caused by an autosomal recessive gene (rd) and can be observed in several mouse strains including the C3H (DUNN, 1954). Histologically, the degeneration of the mutant retina is manifested as a retardation in the development of the rod outer segments after the 8th postnatal day (P8), followed by a rapid and extensive degradation of the photoreceptor cell layer during the second and third postnatal weeks (NOELL, 1958; LASANSKY and DE ROBERTIS, 1960; CALEY et al., 1972). The molecular mechanism responsible for this disorder is unknown. With regard to rhodopsin, it has been established that the rhodopsin content as measured by spectrophotometry parallels the length of the rod outer segments during degeneration (CARAVAGGIO and BONTING, 1963). A previous immunohistochemical study has demonstrated that opsin-immunoreactivity is persistently expressed in the plasma membranes of the remaining photoreceptor cells during retinal degeneration (ISHIGURO et al., 1987). Considering that, in the normal mouse photoreceptor cells, opsin is incorporated into the disk membranes of the rod outer segments (NIR et al., 1984) which undergo renewal in 10 days (YOUNG, 1967), it is pertinent to determine whether the opsin synthesis in the mutant photoreceptor cells continues during the entire degeneration period or ceases prior to or following the onset of degeneration. For this purpose, we attempted the combined immunohistochemical and *in situ* hybridization analysis of developmental changes in the expression of the opsin gene in the C3H mouse retina.

MATERIALS AND METHODS

Male C3H/He mice and C57BL/6 mice as normal control (Charles River) were sacrificed by decapitation at postnatal days 4, 8, 12, 14, 16 and 20. The eyes were immediately removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 4 h at 4°C. They were subsequently rinsed overnight in 30% sucrose and were snap-frozen in liquid nitrogen. Sections, 15 μm thick, were made on a cryostat and mounted on glass slides coated with 1% gelatine/0.5% chrome alum. The sections could be stored at -80°C for a few months until use.

For immunohistochemical detection of opsin in the retina, opsin was purified from the bovine retina by ConA-sepharose column chromatography and an anti-opsin antiserum was raised in white rabbits as described previously (KUO et al., 1988). The cryostat sections were incubated with the anti-opsin antiserum (1:500 dilution) in a humidified chamber for 16 h at room temperature. The immunoreaction was visualized by fluorescent immunohistochemistry using FITC-conjugated anti-rabbit IgG antiserum (Dako) as described elsewhere (NEGISHI et al., 1987).

The opsin cDNA was isolated from a cDNA library prepared from total poly[A] mRNA of bovine retina as previously described (KUO et al., 1986). A ScaI-PstI DNA fragment, about 1 Kbp in length, encoding 29 to 348 amino acid residues of opsin and containing 3' non-coding region (43 bp), was used as the opsin cDNA probe. The probe was labeled by the nick translation (RIGBY et al., 1977) with DNA polymerase

I (Böhringer) and [^{35}S] deoxycytidine 5'-(α-thio) triphosphate (New England Nuclear). Specific activities of the probe were within a range of $1-2\times10^8$ dpm/μg. For detection of opsin mRNA in the retina, *in situ* hybridization was performed according to BLOCH et al. (1986) with slight modifications. Briefly, the sections were incubated in the prehybridization mixture containing 50% (V/V) deionized formamide, 4×SSC (1×SSC=0.15 M sodium chloride, 0.015 M sodium citrate), 1×Denhardt solution (0.02% polyvinyl pyrrolidon, 0.02% Ficoll, 0.02% bovine serum albumin), 2% Sarkosyl, 2 mM 2-mercaptoethanol and 250 μg/ml of heat denatured salmon sperm DNA in 0.1 M sodium phosphate buffer (pH 7.2). After incubation for 2 h at room temperature, the sections were dehydrated in alcohol, dried and then covered with 50 μl per slide of the hybridization mixture containing 5×10^5 dpm of the haet-denatured opsin probe in the same solution as in prehybidization. After incubation in a humidified chamber for 16 h at 37°C, the sections were washed in three 10-min changes of 2×SSC at room temperature, followed by three 1-h changes of 0.3×SSC and 0.1% Sarkosyl at 37°C. After dehydration, the sections were dipped in NTB2 nuclear track emulsion (Kodak) and incubated for 7 days at 4°C for autoradiographic exposure. After development with D19, the sections were stained with hematoxylin-eosin.

RESULTS

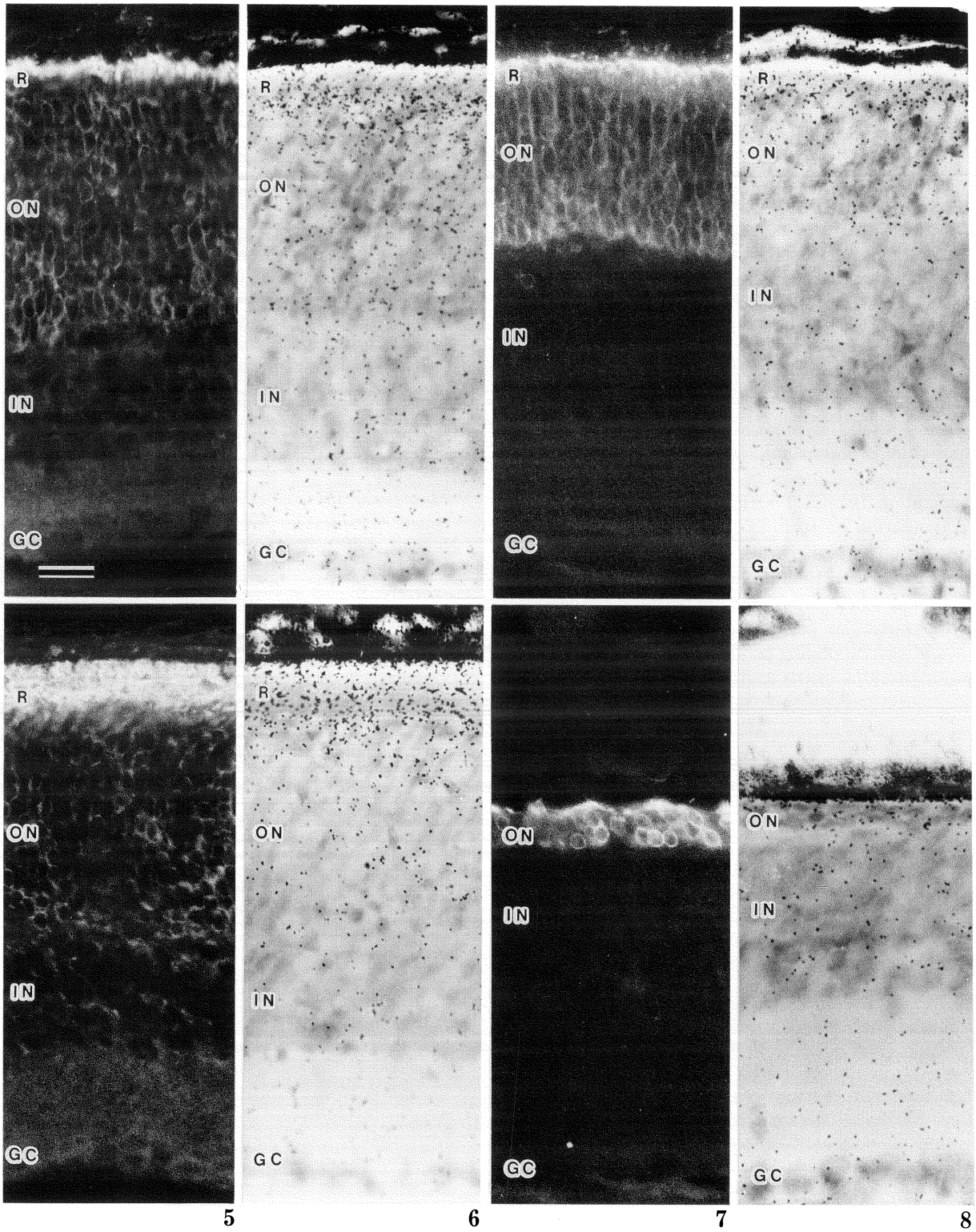
Since the gradient of differentiation in the retina is known to be arranged concentrically with respect to the optic nerve head (BERKOW and PATS, 1964; KONDO et al., 1984, 1988), the present observations were confined to the central portion of the retina in both the control and mutant mice. By P8, all the cell layers observed in the normal adult retina became recognizable and the immunoreactivity for opsin was localized in the outer nuclear layer, without any difference in appearance between the control and C3H retinas. The C3H retina continued to develop thereafter, while the development of the outer nuclear and photoreceptive rod layers was retarded as compared to that in the control retina. At P12, the outer nuclear layer of the control retina was weakly immunoreactive for opsin and the rod layer was apparent as a distinct layer external to the outer nuclei and intensely immunoreactive for opsin (Fig. 1). Both of the layers were thinner in the C3H than in the control retina (Fig. 3). The autoradiographic silver grains representing opsin mRNA were concentrated in the rod layer in both the control and C3H retinas (Figs. 2, 4).

Between P12 and P16, extensive degeneration of the C3H retina occurred. In contrast to the progressive thickening and enhanced opsin-immunoreactivity of the rod layer in the control retina (Fig. 5), the rod and outer nuclear layers of the C3H retina were reduced in thickness. At P16, the rod layer was no longer recognizable and the outer nuclear layer was composed of only a few rows of cells. However, they remained intensely immunoreactive for opsin (Fig. 7). In the control retina the silver grains were well localized in the rod layer and further increased in number by P16 (Fig. 6), while the silver grains in the C3H retina decreased in number and eventually no significant concentration of the silver grains above the background level was detectable in the remaining outer nuclear layer at P16 (Fig. 8). At P20, the control retina was mature in appearance and the radioactive label was well localized in high density to the inner segments of the rod layer. In the C3H retina, in contrast, only a few solitary opsin-immunoreactive cells were dispersed in a single row in the outer nuclear layer, where the radioactive labels were no longer detectable.

DISCUSSION

The chronological changes in morphology and expression of opsin-immunoreactivity in the photoreceptor cells of the mutant retina has been described

Figs. 1-8. Fluorescent micrographs showing opsin-immunoreactivity (**Figs. 1, 3, 5, 7**) and autoradiographs showing *in situ* hybridization for opsin mRNA (**Figs. 2, 4, 6,** 8) in the retinas from the C57BL control (**Figs. 1, 2, 5, 6**) and the C3H mutant mice (**Figs. 3, 4, 7,** 8). At P12, a thin but distinct rod layer (*R*) exhibits intense immunoreactivity for opsin in both the control (**Fig. 1**) and mutant (**Fig. 3**) retinas. The outer nuclear layer (*ON*) is also weakly immunoreactive. The concentration of the silver grains for opsin mRNA is detectable in the rod layer of both the control (**Fig. 2**) and mutant (**Fig. 4**) retinas. At P16, the immunoreactive rod layer thickens in the control retina (**Fig. 5**). In contrast, the rod layer is no longer apparent and a few rows of cells composing the attenuated outer nuclear layer exhibit intense immunoreactivity in the mutant retina (**Fig. 7**). The concentration of silver grains in the rod layer is augumented in the control (**Fig. 6**) while no grains are detectable in any layers above the background level in the mutant retina (**Fig.** 8). *GC* ganglion cell layer, *IN* inner nuclear layer. ×450 (Bar: 20 μm)



Figs. 1-8. Legends on the opposite page.

by several authors (NOELL, 1958; LASANSKY and DE ROBERTIS, 1960; CARAVAGGIO and BONTING, 1963; ISHIGURO et al., 1987). The present immunohistochemical findings are basically in accord with their results: After attaining a peak in the development of the outer nuclear and rod layers at P12, the photoreceptor cells of the C3H retina degenerate progressively and only a few rows of outer nuclei immunoreactive for opsin remain without apparent recognition of the rod layer at P16. However, the present *in situ* hybridization analysis demonstrated that the radioactive labels for opsin mRNA can be detected at P12 but are no longer detectable in the remaining rod cells at P16. This finding indicates that expression of the opsin ceases at earlier stages of retinal degeneration. On the other hand, the finding that opsin-immunoreactivity persists in the remaining photoreceptor cells throughout the degeneration period suggests that the turnover time of opsin is longer than that of opsin mRNA. The loss of rod outer segments and consequent cessation of normal opsin renewal in the C3H retina may contribute to the accumulation of opsin in the outer nuclear layer as shown by the higher intensity of opsin-immunoreactivity in the C3H than in the control retina. It remains to be seen whether the time lag between the cessation of the particular gene expression and the disappearance of corresponding protein exists in cases of other major proteins in the mutant retina.

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