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Immunocytochemical Colocalization of Clusterin in Apoptotic Photoreceptor Cells in Retinal Degeneration Slow *rds* Mutant Mouse Retinas

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In the *rds* mutant mouse the photoreceptor cells differentiate normally for the first few postnatal days, with the inner segments projecting an extended cilium. However, outer segments fail to form and only rudimentary disks and opsin-laden vesicles assemble at the tip of the cilium. These are shed into the interphotoreceptor space where they are phagocytosed by the retinal pigment epithelial cells. In this animal model, the photoreceptors undergo a slow degeneration via apoptosis leading to eventual loss of the entire photoreceptor population. Since increased expression of clusterin has been implicated in apoptosis, we studied the expression of clusterin in the *rds* mutant mouse retina and compared it to normal BALB/c retinas. Small intestinal microvillus epithelium was used as a positive control tissue for apoptosis. Immunocytochemistry revealed the presence of clusterin in the ganglion cell, inner nuclear and outer plexiform layers and in the retinal pigment epithelium of both the *rds* and the BALB/c retinas. Interestingly, scattered clusterin-positive cells were observed in the outer nuclear layer (onl) of dystrophic retinas. Since the increased presence of clusterin protein in the onl of dystrophic retina may indicate dying photoreceptor cells due to apoptosis, we utilized a co-localization procedure for apoptotic nuclei and clusterin. For apoptosis we utilized an in situ 3′ end labeling of fragmented DNA (TUNEL) and immunohistochemistry for clusterin using brown and red colored substrates respectively. Small intestine tissue sections were also included as positive controls for apoptosis. Our results show that clusterin is co-localized with apoptotic nuclei both in the onl of *rds* mutant retinas as well as in the small intestine epithelial cells undergoing cell turnover and exfoliation. These results are of interest since overexpression of clusterin is also observed in other neuro-degenerative diseases such as Alzheimer's and Pick's disease. © 1996 Academic Press, Inc.

The *rds* mutant mouse has long been considered a model for the inherited human disease, retinitis pigmentosa (RP) (1). The *rds* mutation has been shown to affect the peripherin/rds gene which encodes a protein that localizes to the rims of the outer segment disks and is required for disk morphogenesis (2–4). In mutant retinas this protein is not expressed and the *rds* mutant mice fail to differentiate outer segments. However, the basic gene defect (i.e., the peripherin/rds gene) of the *rds* mutation does not explain the phenotype of the disease, since the failure to form outer segments does not explain the cause of photoreceptor cell death. Recently it was shown that the photoreceptors of the *rds* retina undergo apoptosis (5–7).

We have earlier shown that the expression of opsin and interstitial retinol binding protein (IRBP) mRNAs is normal in *rds* retinas as compared to normal BALB/c retinas based on a per cell basis (8–9). We concluded that these photoreceptor specific proteins are constitutive

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and their expression is not affected by the *rds* mutation, as long as functional photoreceptors with intact inner segments are present. On the other hand, the *rds* retinas lack a distinct diurnal cycle of arrestin mRNA such that the arrestin mRNA is always expressed at a high level as compared to BALB/c animals. In BALB/c retinas, arrestin mRNA is expressed at a lower level in dark adapted retinas than in the light adapted ones (10).

Clusterin is a 70–85 kDa sulfated glycoprotein containing two nonidentical polypeptides of 40 kDa linked by disulphide bonds. The polypeptides are proteolytically derived from a precursor protein translated from a single mRNA transcript (11). Clusterin is present at concentrations of 50–100 μg/ml in normal human plasma and about 10-fold higher concentrations in seminal plasma, and is expressed in a variety of tissues (11–15). The mature protein appears to be multifunctional. In blood it associates with components of the complement system (11), and it also appears to interact with apolipoprotein A1, suggesting possible involvement in lipid transport (16–17). Most significantly, an increase in the protein itself or elevated expression of clusterin mRNA has been observed in several instances of tissues undergoing apoptosis as well as in RP affected retinas (18–22). Furthermore, levels of clusterin mRNA in the rat ventral prostate rise greatly following castration when cells are dying, and are repressed by testosterone administration (23). Although clusterin is considered to be a marker of apoptosis, experiments to demonstrate its direct involvement with apoptosis are generally lacking. Here we report that in the apoptotic death of photoreceptors in the *rds* mutant retina, clusterin is co-expressed in apoptotic cells of dystrophic retinas as well as small intestinal epithelial cells, which undergo apoptosis physiologically to renew their epithelium. The results of its co-expression in the apoptotic cells suggest its possible involvement in apoptosis.

MATERIALS AND METHODS

The animals. The animals were housed in the UNT HSC at Fort Worth vivarium in accordance with AAALAC and NIH guidelines. The animals were housed in a controlled light environment (5–6 foot candles) on a 12h/12h light/dark cycle with lights coming on at 8:00 a.m. and going off at 8:00 p.m. The rds/rds 020 (21–30 days old) and BALB/c (30 days old) animals came from a colony of Dr. Michael Chaitin. The animals were sacrificed by carbon dioxide exposure.

Tissue fixation and preparation. Small intestine and eyes from BALB/c and *rds* mutant mice were fixed in 4% buffered paraformaldehyde and embedded in Polyffin (Triangle Biomedical Sciences, Durham, NC). Tissue sections (5–8 μm) were cut and allowed to adhered to glass slides previously treated with Vectabond (Vector Laboratories, CA). Polyfin was removed and the slides hydrated by transferring the slides through the following solutions: twice in 100% Xylene for 5 minutes, twice in 95% ethanol for 5 minutes, twice in 90% ethanol for 5 minutes, once in 80% ethanol for 5 minutes and then glass double distilled water for 5 minutes. Fresh solutions were prepared each time. All solutions and buffers were made with glass double distilled water only. In order to remove proteins from the nuclei, tissue sections were incubated with 20 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO) for 15 minutes at room temperature and then washed in double distilled water twice for 2 minutes. Endogenous peroxidase was inactivated by incubating the tissue sections in 3% hydrogen peroxide in methanol for 5 minutes at room temperature.

Immunolocalization of clusterin. Following hydrogen peroxide treatment and phosphate buffered saline (PBS) wash, tissue sections were incubated with normal rabbit serum as a blocking agent for 30 minutes at room temperature. The serum was blotted off from the tissue sections and sections incubated with the primary antibody (sheep anti rat clusterin, 1:5000) diluted in PBS containing 1% BSA. The sections were incubated overnight at 4°C in a humidified chamber. The next day, tissue sections were washed twice in PBS for 3 minutes each wash and then incubated for 60 minutes at room temperature with biotinylated rabbit anti-sheep IgG and for 60 minutes with the ABC reagent (Vector Laboratories, Inc.,). The concentrations used were as specified in the Vectastain ABC immunoperoxidase kit. Diaminobenzidine (DAB) was used as substrate. Slides were mounted with Aquamount and examined examined on a Nikon Microphot FXA photomicroscope using Nomarski optics. Selected slides were photographed using Kodak Royal Gold color print film.

In situ 3′-end labeling of DNA (TUNEL procedure) and colocalization of clusterin with apoptotic cells. Following proteinase K and hydrogen peroxide treatment, the sections were rinsed twice in double distilled water for 2 minutes followed by 3 one minute washes in PBS and then incubated in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 5 minutes. Following washing in the TdT buffer, tissue sections were circumscribed with a diamond pencil to prevent runoff of solutions. The TUNEL

reaction was performed with the modifications as described by Gavrieli et al. (1992) (24) and consisted of incubating the tissue sections in TdT (0.3 enzyme units/μl) and biotinylated dUTP in TdT buffer for 2 hours at 37°C in a humidified chamber. The reaction was ended by transferring the slides to a buffer rinse (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes at room temperature and double distilled water for 10 minutes. The sections were covered with a 2% aqueous solution of bovine serum albumin (BSA) for 10 minutes at room temperature, rinsed quickly in double glass distilled water and immersed in PBS for 5 minutes. Negative control sections consisted of all steps and washes but the TUNEL labeling reaction was ommitted. Subsequently, the Vectastain ABC kit (Vector Laboratories, CA) was used to visualize nuclei labeled with biotin. Slides were incubated in the Vectastain ABC reagents for 30 minutes at room temperature. Slides were washed 3 times in PBS at room temperature for 1 minute each wash. The generation of the color reaction product was performed using Sigma Fast (Sigma Chemical Co., St. Louis, MO) DAB peroxidase substrate procedure. After the color development of TUNEL positive cells, some tissue were processed for immunocytochemistry for clusterin using a sheep anti rat clusterin antibody (overnight at 4°C), a biotinylated rabbit anti-sheep IgG for 60 minutes at R.T. and the Vectastain ABC-AP reagent for 60 minutes at R.T. (Vector Laboratories, CA). Following incubation, the tissue sections were washed twice with PBS for 5 minutes each wash and then incubated with an alkaline phosphatase substrate consisting of Vector Red (Vector Laboratories, CA) in 100 mM Tris-HCl (pH 8.2) made in double glass distilled water. Endogenous alkaline phosphatase activity was inhibited by the addition of 0.25 mg/ml levamisole to the buffer used to prepare the substrate solution. Following incubation, the sections were rinsed in buffer for 5 minutes and mounted with Fluormount G (Southern Biotechnology Associates, Inc., Birmingham, AL). The apoptotic cells alone appeared brown, clusterin containing cells were pink, and the cells in which there was co-localization of clusterin and TUNEL appeared deep red in color.

RESULTS

Immunolocalization of clusterin. Immunocytochemical localization of clusterin in the *rds* mutant mouse and control BALB/c retinas was done using sheep anti-rat clusterin polyclonal antibody. The results demonstrated that clusterin is localized to the ganglion cells, the inner nuclear layer, and to the retinal pigment epithelium in both the BALB/c (Figure 1A) and *rds* retinas (Figure 2). The clusterin expression was not observed either in the photoreceptor cell layer or in the cell bodies of the photoreceptor cells in the BALB/c retinas. Interestingly, some of the photoreceptor cell bodies in the outer nuclear layer of *rds* retinas were stained positive for clusterin (Figure 2). In the small intestine, clusterin was localized in epithelial cells within the apical villi with some expression in the connective tissue (Figure 3 B).

In situ 3′-end labeling of DNA. We studied apoptosis in small intestinal epithelium as well as in the *rds* mutant and normal BALB/c retinas using the TUNEL procedure as described by Gavrieli et al. (1992). Since small intestinal epithelium has been shown to renew its epithelium via apoptosis, it was included as a positive control. As expected apoptotic nuclei were detected in the epithelial cells associated with apical villi (Figure 3A) as well as in the cell bodies of the photoreceptor cells of *rds* mutant mouse retinas (Figure 3C). The control BALB/c retinas did not show any TUNEL positive nuclei in their onl (Data not shown).

Colocalization of clusterin with apoptotic cells. Since clusterin positive nuclei were observed in both the epithelium of the intestinal villi as well as in the onl of the *rds* mutant mouse retinas and these nuclei were the same nuclei undergoing apoptosis, we co-localized clusterin (pink color substrate) with apoptotic nuclei (brown color substrate) using double labeling procedure. The results show that clusterin is co-localized with the apoptotic nuclei as shown by the deep red colored nuclei (arrows) in figure 3B of small intestine and for *rds* mutant mouse retinas (Figure 3D).

DISCUSSION

The series of events which lead from the initial molecular defect to the final stages of photoreceptor cell death in retinal degenerations are not known. The causes of photoreceptor loss in inherited retinal dystrophies have been intensively studied both in patients with retinitis pigmentosa and in various animal models. In the last few years, molecular defects in the diseased photoreceptors were identified in the mutant *rd* mouse and *rds* mouse, and recently in human retinitis pigmentosa. The recent discovery of point mutations in the opsin gene in

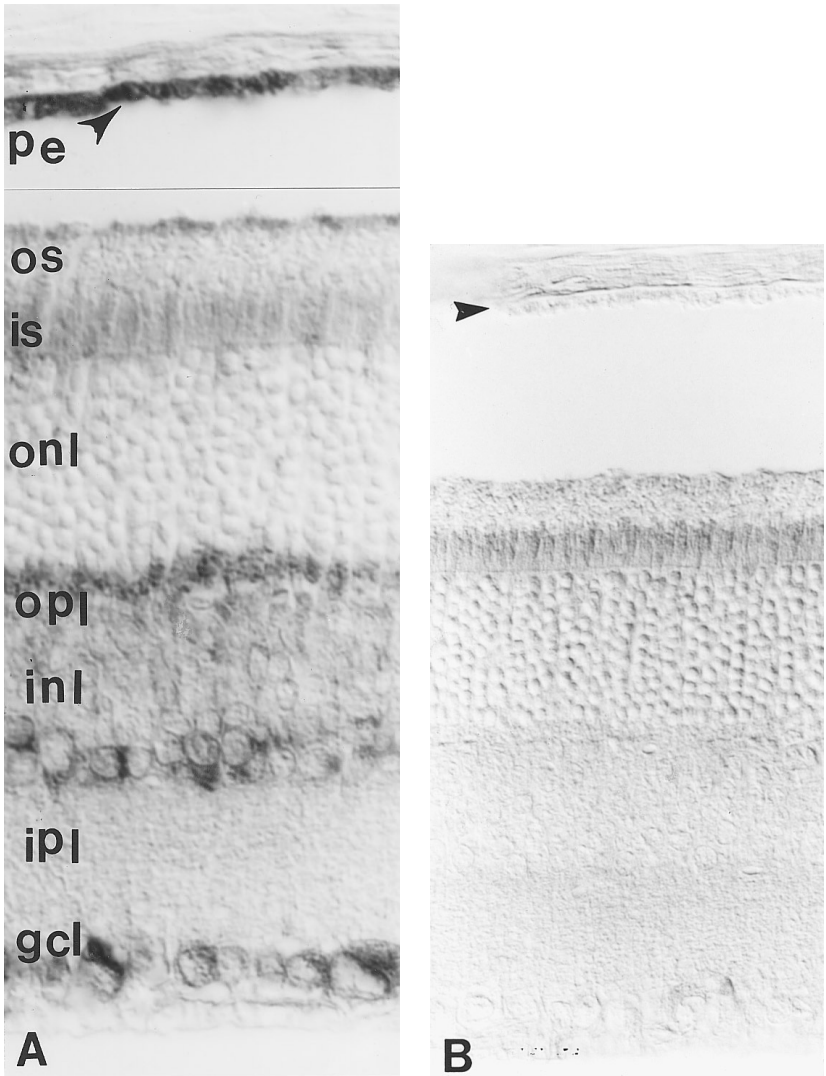

FIG. 1. (A) Immunoperoxidase labeling of clusterin in the BALB/c mouse retina. Positive label was observed in the pigment epithelium (pe), outer plexiform layer (opl), inner nuclear layer (inl), and ganglion cell layer (gcl). os, outer segments; is, inner segments; and ipl, inner plexiform layer. (B) On a control section, no label was seen when non-immune serum was used in place of the anti-clusterin antiserum. Note that the pe (arrowhead) is detached.

some patients with autosomal dominant retinitis pigmentosa (25) does not explain the cause of the eventual photoreceptor cell death, especially in adjacent cone photoreceptor cells which do not express the rhodopsin gene. Recent reports have identified apoptosis as a significant final cause of photoreceptor cell death in *rds* as well as *rd* mice and in opsin transgenic mutant mice (5–6, 26). The mechanism(s) of the photoreceptor cell death via apoptosis is not understood in retinal dystophies. We are investigating the role of other genes which might be altered in the photoreceptors of the *rds* dystrophic retinas, to understand how these cells undergo apoptosis. Since clusterin mRNA overexpression has also been observed in a number of other neurodegenerative states, including Alzheimer's and Pick's disease (27–29), as well as in tissues undergoing apoptosis (30), it is possible that the abnormal presence and/or

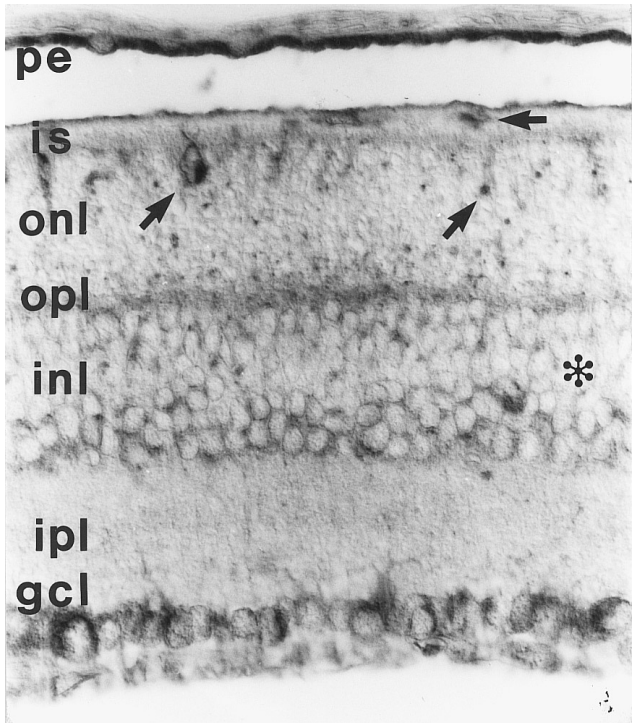

FIG. 2. Immunoperoxidase labeling of clusterin on sections of paraffin embedded *rds* mouse retinas. Positive label was observed in the pigment epithelium (pe), outer plexiform layer (opl), inner nuclear layer (inl), and ganglion cell layer (gcl). Labeled cell bodies and structures (arrows) were also seen in the inner segment (is) layer and outer nuclear layer (onl). ipl, inner plexiform layer. *, inl.

expression of clusterin in the onl of *rds* mutant mouse retinas is closely linked to the apoptotic process causing photoreceptor cell death via apoptosis.

Overexpression of clusterin mRNA in the retinas of *rds* mutant mice as well as in dystrophic retinas of patients with advanced RP (21, 31, 32) and for *rd* mutant mouse retinas (22) has been shown implicating it to photoreceptor cell death in various forms of retinal dystrophies. Increased levels of clusterin mRNA coincided with photoreceptor cell death in whole eyes of the *rds* mutant mouse (33). In situ hybridization studies, using normal and RP human retinas, show that clusterin mRNA is not expressed in photoreceptor cells (32).

In BALB/c retinas, we detected clusterin in the ganglion cell, inner nuclear, and retinal pigment epithelium layers and in the outer plexiform layer which contains processes and synaptic junctions of the photoreceptors and inner retinal neurons. In contrast, in *rds* retinas the clusterin labeling was essentially the same as in the BALB/c except that a number of clusterin positive cells were also present in the onl, representing the cell bodies of photoreceptor cells. Interestingly, in normal human retinas, clusterin was localized at the inner limiting membrane, and in the inner and outer plexiform layers and photoreceptor outer segments, with no obvious labeling in the pigment epithelium (34). Jomary et al., (1993b) (34) utilized a different clusterin antibody than the one we used which may explain the differences in its localization. However, the results of in situ hybridization for clusterin mRNA expression in human retinas (32) support our results with clusterin immunohistochemistry. Clusterin message was detected in the margins of the inner nuclear layer and in the ganglion cell layer. Possible label in the pigment epithelium was obscured by melanin and lipofuscin in the human retina.

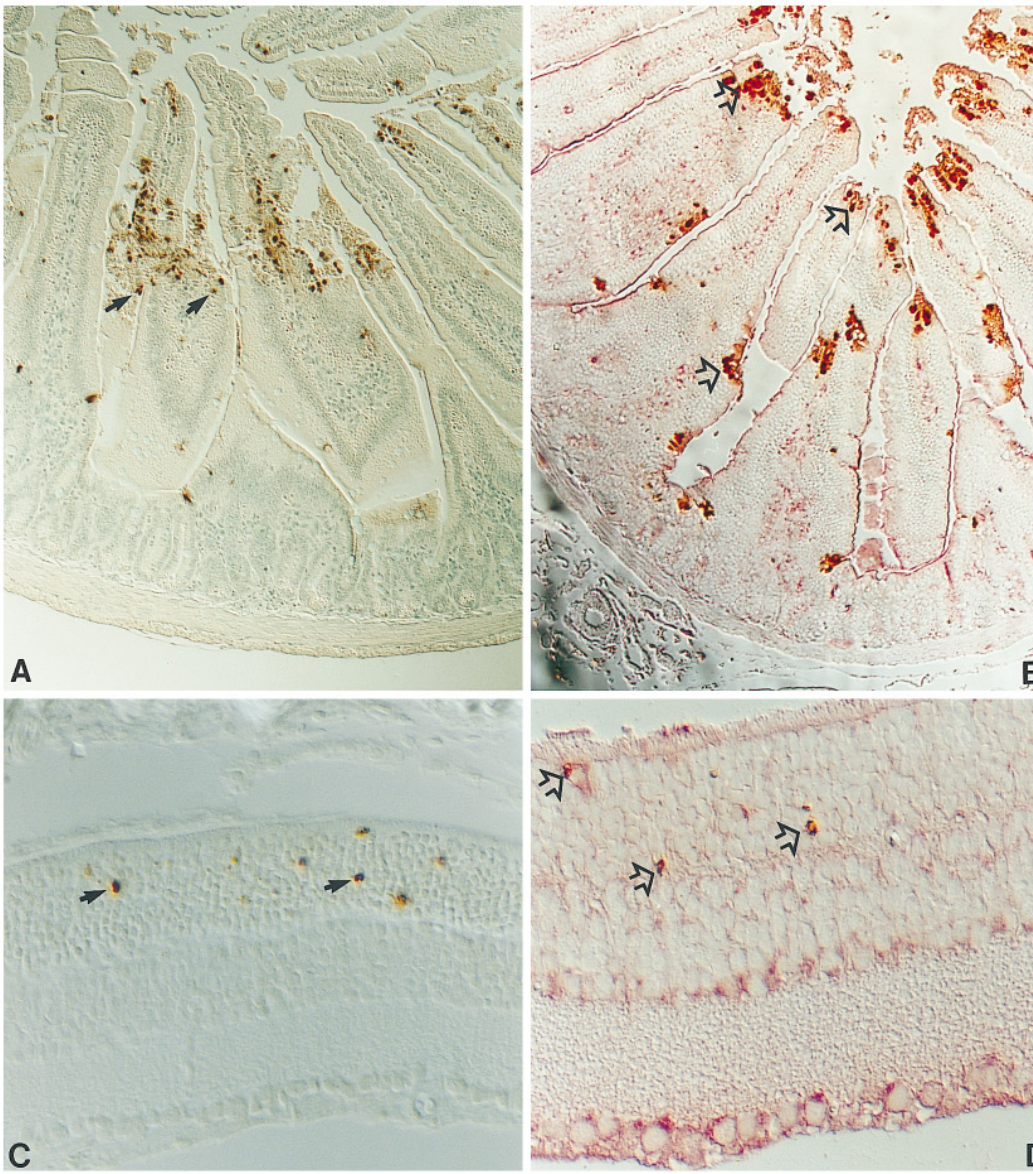

FIG. 3. Co-localization of clustrin with TUNEL positive cells in the small intestine and *rds* mutant mouse retinas. Paraffin sections of small intestine, *rds* mutant and BALB/c retinas were stained for DNA fragmentation by detecting the incorporated biotinylated dUTP by ABC kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine giving a brown color (A and C arrows) and then immunostained for clusterin using sheep anti-rat clusterin antibody as described in the methods section. The clusterin positive cells were identified with a secondary antibody rabbit anti-sheep IgG conjugated with alkaline phosphatase and developing with pink/red color with Vector Red Kit (Vector Laboratories, Burlingame, CA) as per their instructions. The deep red colored nuclei on the microvilli of the intestinal epithelium and in onl indicate the co-localization of clusterin with apoptotic cells (B and D, open arrows).

The presence of clusterin in the cell bodies of photoreceptor cells of dystrophic retinas indicated its possible direct involvement in photoreceptor cell death. Clusterin immunoreactivity in *rds* photoreceptors could indicate localization of clusterin protein in the cell bodies of the dying photoreceptors. The results of double immunolabeling studies using the terminal deoxyribo-

nucleotidyl transferase (TdT) assay for apoptosis (24) and immuno-localization of clusterin, confirm this. A cell protective role for clusterin has also been suggested in the literature (35). If clusterin is involved in apoptosis then the presence of clusterin in high amounts in the ganglion and PE cell layers raises the question as to why these cells do not undergo apoptosis. In the present study, since clusterin is not detected in onl of the control retinas, one may argue that it is the abnormal presence of clusterin in the dystrophic onl which may be responsible for photoreceptor cell death.

The mechanism through which clusterin is involved in apoptosis remains to be elucidated. It was recently shown that *c-fos* mRNA levels are increased in cells undergoing apoptosis (36). Interestingly, two AP-1 sites have been shown to be located, respectively, at positions −167 to −152 and −25 to −19 relative to the single transcription initiation site in avian clusterin gene (37). *c-fos* has been shown to form a heterodimer with another immediate early gene, *c-jun* and together they bind to the DNA regulatory element AP-1. The induction in *c-fos* mRNA preceding apoptosis (36) may be involved in regulation of clusterin mRNA transcription through the AP-1 binding site. Indeed, an induction of *c-fos* immunoreactivity was observed in rds mutant retinas (38) and this increased *c-fos* immunoreactivity was presumed to be in the dying photoreceptor cells in *rd* sretinas. These results suggest that clusterin may be associated with the apoptotic cell death of photoreceptors.

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