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Analysis of esterification of retinoids in the retinal pigmented epithelium of the *Mitf^{vit}* (vitiligo) mutant mouse

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Purpose: Mice homozygous for the vitiligo mutation of the microphthalmia (*Mitf*) gene have a retinal degeneration characterized by slow loss of photoreceptor cells and compromised retinal pigment epithelial (RPE) structure and function. The levels of retinyl esters, which are essential for generation of 11-*cis*-retinaldehyde for the formation of rhodopsin, were reported previously to be elevated by 6 weeks postnatally in the RPE of vitiligo mutant mice. The purpose of the present study was to determine whether this elevation was due to increased activity of lecithin:retinol acyl transferase (LRAT) the enzyme that converts all-*trans*-retinol to retinyl esters.

Methods: Retinoids extracted from the RPE and neural retina of mutant and normal mice ages 2, 4, 6 and 8 weeks were analyzed by reversed-phase HPLC. The esterification capacity of the RPE to convert ^{3}H-retinol to ^{3}H retinyl ester was determined by HPLC in mutant and normal mice at 3 and 9 weeks.

Results: Retinyl ester levels were elevated significantly in the mutant RPE as early as postnatal week 2 and were four-fold greater by 8 weeks. The esterification assay indicated no significant differences between mutants and controls at 3 weeks. At 9 weeks, the esterification activity of the mutant RPE was significantly reduced compared to controls rather than elevated.

Conclusions: The data suggest that the accumulation of retinyl esters is not due to increased LRAT activity. Alternative explanations for the retinyl ester accumulation are discussed.

The importance of vitamin A (retinoids) for normal visual function is well documented (reviewed recently by Saari [1] and by Crouch et al. [2]). Deprivation of vitamin A is known to induce degeneration of the retina [3]. Recently, an inherited model of retinal degeneration was reported in which retinoid metabolism is perturbed in the eye [4,5]. This model, the vitiligo mouse, is homozygous for the recessive *Mitf^{vit}* allele at the microphthalmia (*Mitf*) locus [6,7]. The animal was designated the vitiligo mouse because it exhibits progressive depigmentation of the skin and fur [8]. Following initial characterization of the slow photoreceptor cell degeneration and gradual loss of rhodopsin in this mutant [9, 10,11], studies of the visual cycle were undertaken.

The visual cycle requires continuous generation of 11-*cis*-retinaldehyde for use in the formation of rhodopsin [12]. To produce 11-*cis*-retinaldehyde, all-*trans*-retinol from the circulation is delivered to the RPE and is quickly esterified. The esterification is primarily catalyzed by lecithin: retinol acyl transferase (LRAT) and the primary product is retinyl palmitate [13-17].

Previous studies using high pressure liquid chromatography (HPLC) analysis of retinoid concentrations in whole eyes of *Mitf^{vit}* mutant mice indicated that the retinyl ester levels were elevated by 4 weeks postnatally. Retinyl ester levels were five-fold greater in mutants than controls by 10 weeks and remained elevated through 42 weeks [4]. Further analysis of the separated RPE and neural retina carried out at a single age (6 weeks) revealed that the accumulation of retinyl esters was occurring in the RPE as expected, not in the neural retina. These studies did not address the cause of the accumulation of the retinyl esters in this mutant. We speculated that the elevation could be due to increased activity of LRAT. The present study was designed to test whether there were differences in the esterification activity in the vitiligo mouse RPE.

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METHODS

Animal Model— The *Mitf^{vit}* mice were the offspring of our colony of breeding pairs. C57BL/6 wild-type mice were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Animals were maintained in clear plastic cages and subjected to standard light cycles (12 h light and 12 h dark). Light levels averaged 12.9 to 16.1 lux. Room temperature was 23±1°C. Mice were fed a Harlan Teklad rodent diet (minimum crude protein, 20.0%; minimum crude fat, 10.0%; maximum crude fiber, 2.0%; vitamin A, 15.7 nmol/g). Care and use of the animals followed the procedures set forth in the National Research Council publication The Guiding Principles in the Care and Use of Animals, 1996.

Chromatographic analysis of retinyl ester and all-trans-retinol levels in the RPE— All procedures involving vitamin A were performed under gold light to minimize the possibility of photoisomerization. After overnight dark adaptation in dim red light, mice were killed by CO_2 asphyxiation. Retinoid levels were determined in RPE (and separately in neural retina) of mutant and control animals ages 2, 4, 6 and 8 weeks. To separate the RPE from neural retina, eyes were placed in 0.01 M PBS, the lens was removed, and the neural retina dissected free from the RPE and eyecup. Tissues were homogenized in

300 µl of PBS, pH 7.4 at 4 °C using a Duall tissue grinder (size 20; Kontes, Vineland, NJ). Homogenates were transferred to 15 ml glass tubes and lipids were extracted into hexane. Retinoids in the hexane-extractable lipid phase were analyzed by HPLC. To prepare samples for HPLC analysis, solvent was evaporated from portions of lipid extracts in a 37 °C water bath using a gentle stream of N_2. Samples were resolubilized in methanol before HPLC injection. Retinoids were separated by reversed-phase HPLC using a Resolve C-18 column (3.9 mm x 150 mm; Millipore) and methanol/water (9:1,v/v) as the mobile phase at a flow rate of 1 ml/min. The mobile phase was changed to 100% methanol (2 ml/min) at 10 min to elute the retinyl esters. The HPLC system included two model 501 pumps and a model 486 tunable wavelength detector (Millipore, Milford MA) set at 325 nm. For instrument control, data acquisition, and peak area integration, an IBM PC/AT and Maxima 810 chromatography software package (Millipore) system were used. For quantification of retinoids, external standard curves were developed using authentic retinoid standards obtained from Sigma Chem. Corp. (St. Louis, MO). Retinoid concentrations were calculated as nmol/eye. Calculation of the data on a per eye basis allowed comparison of the findings of the present study of retinoids in the RPE with the earlier analysis of retinoids in whole eyes [4]. Reporting the data in this manner necessitated the measurement of the eyes to determine whether there were differences in eye size between the mutant and normal mice. Analysis of the diameter of glutaraldehyde fixed eyes of normal mice (n=12) and mutant (vitiligo) mice (n=12) indicated that the mean equatorial diameter at 2 weeks was 3.0±0.23 mm and 3.1±0.5mm for normal and mutant mice, respectively. At 8 weeks the mean diameters were 3.28±0.05 mm and 3.35±0.21mm for normal and mutants respectively. There was no significant difference in eye diameter between age-matched normal and control mice ($p<0.05$). Thus, although some other *Mitf* mutant mice have markedly reduced eye size as reviewed by Moore [18], *Mitf*^{vit} mice do not. A multivariate analysis of variance was used to compare retinoid concentrations in vitiligo and control mice by age, group, and tissue type followed by a univariate analysis of variance. A value of $p<0.05$ was considered significant.

Analysis of retinol esterification: Esterification Assay—The esterification assay was conducted initially with liver microsomes as a positive control for the assay and subsequently with RPE tissue. The details for preparing liver microsomes and enriched RPE samples are provided below. Once the tissues were dissected, the esterification assay was performed according to the method of Randolph et al. [19]. Briefly, 20 µl of RPE-enriched (or liver microsome) sample was suspended in 255 µl of 0.15 M KH_2PO_4, pH 7.2 and 15 µl of 20 mM (final concentration) bovine serum albumin (BSA). To this, 10 µl DMSO-dispersed ^{3}H-retinol (DuPont NEN, 35.2 Ci/mmol) was added. The reaction mixture was incubated in a 37 °C shaking water bath for 10 min. One milliliter of cold ethanol was added to terminate the reaction. One milliliter of deionized H_2O and 4 ml of hexane were added and samples were vortexed for 1 min and centrifuged at 480 x g for 10 min. The top hexane phase was collected and 4 ml of hexane was added to the remaining lower phase, which was vortexed and centrifuged as before. The top hexane phase was collected and combined with the previous hexane phase. Samples were evaporated under a gentle stream of N_2 and resuspended in 125 µl methanol of which 90 µl was analyzed by HPLC using the Resolve C-18 column as described above. Fractions were collected at 1 min increments and suspended in Budget-solve cocktail (Research Products Intl. Corp., Mount Prospect, IL). The disintegrations per min (dpm) present in each fraction were counted using a Beckman LS 6500 Multi-Purpose Scintillation Counter (Beckman Instruments, Arlington, IL). Controls included: (1) boiling a fraction prior to the addition of ^{3}H-

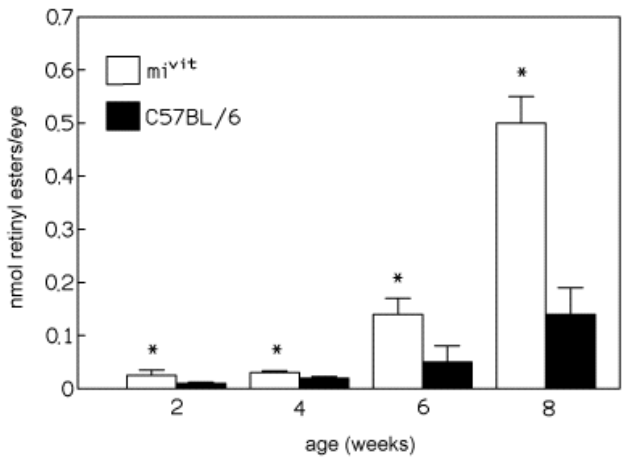

Figure 1. Bar graph of retinyl ester concentrations in the RPE/choroid of vitiligo mice (unshaded boxes) compared to control mice (shaded boxes) at ages 2, 4, 6 and 8 weeks. Each assay reflects data collected from 4 eyes. Four assays were completed at 2, 4 and 6 weeks and three at 8 weeks. Error bars represent the standard error. *Significantly different from controls (Univariate Analysis of Variance, $p<0.001$, $F=20.389$).

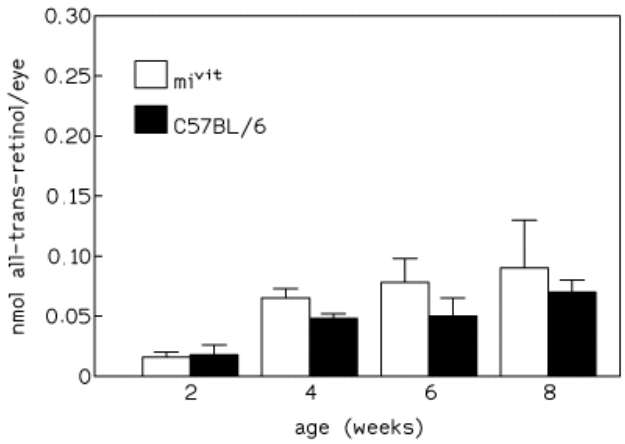

Figure 2. Bar graphs of retinol concentrations in the RPE/choroid of vitiligo mice (unshaded boxes) compared to control mice (shaded boxes) at ages 2, 4, 6 and 8 weeks. Each assay reflects data collected from four eyes. Four assays were completed at 2, 4 and 6 weeks and three at 8 weeks. Error bars represent the standard error. There were no significant differences at the ages examined. ($p=0.214$).

retinol as a control to correct for background radioactivity; (2) not adding the tissue sample to the reaction mixture; and (3) not adding ^{3}H-retinol to another reaction mixture. These controls were used to demonstrate background and inhibition of esterification activity. The esterification activity was calculated from the amount of radioactivity present in the retinyl palmitate fraction as compared to the total concentration of ^{3}H-retinol detected by HPLC and counted by scintillation. Esterification activity was expressed as the ratio of retinyl esters to protein content in the case of liver microsomes and to phospholipid content in the case of the RPE. The results from 3 and 9 week old vitiligo mouse samples were compared to age-matched control mouse samples and a student's t-test value of $p<0.05$ was considered significant.

Liver microsome preparation— As a positive control for the RPE esterification assay, mouse liver microsomes were prepared and analyzed for their ability to esterify retinol to retinyl palmitate. Microsome preparation was conducted per the method of Ong et al. [13]. Mice were killed by CO_2. The livers were removed, rinsed twice with cold KH_2PO_4, 0.15 M, pH 7.2, minced into small pieces and homogenized in a Dounce tissue grinder containing 4 ml KH_2PO_4. The homogenate was centrifuged at 20,000 x g for 15 min at 4°C. After centrifugation, the floating fat was removed and the supernatant saved. The pellet was rehomogenized in 2 volumes of KH_2PO_4 and centrifuged at 20,000 x g, 4 °C, for 15 min.

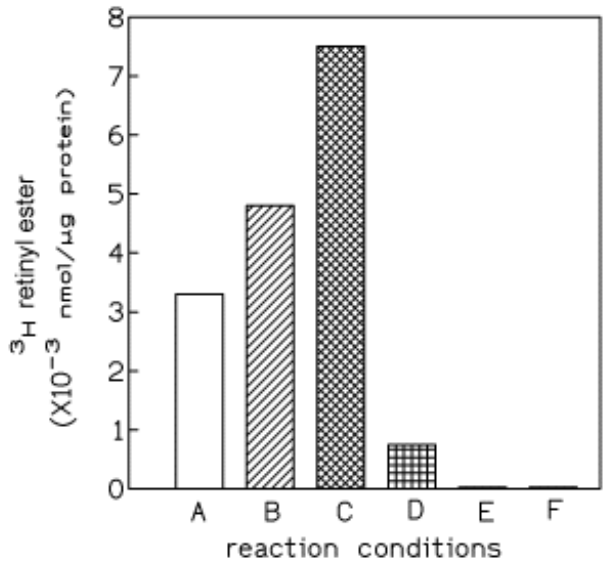

Figure 3. Bar graph of ^{3}H-retinol esterification to ^{3}H-retinyl esters in liver microsomes. As a positive control, liver microsome samples were prepared from vitiligo mice and subjected to various reaction conditions. (A) The esterification reaction was allowed to proceed for 10 min in the presence of liver microsomes prior to terminating the reaction with cold ethanol. (B) 20 min reaction. (C) 30 min reaction. (D) Liver microsome sample was boiled for 3 min prior to being subjected to a 10 min reaction. The low level radioactivity represents the residual tritium (background radioactivity) found in the assay. (E) No ^{3}H-retinyl esters were produced when the reaction was attempted for 10 min without the addition of liver microsomes. (F) No ^{3}H-retinyl esters were produced when the reaction was attempted for 10 min without the addition of ^{3}H-retinol.

The floating fat was removed and the second supernatant combined with the original supernatant. Following the addition of KH_2PO_4 to the supernatant, samples were centrifuged at 113,000x g, 4 °C, for 60 min. The pellet was resuspended in KH_2PO_4 and centrifuged as before. The resulting pellet was resuspended in KH_2PO_4 of which 0.6 ml was used for the esterification assay. The remaining 0.2 ml of liver microsome sample was analyzed for protein content using a Bio-Rad Protein Assay kit, Biorad Corp., Hercules, CA according to the method of Bradford [20].

RPE-enriched tissue isolation for the esterification assay— Mice were killed by CO_2. Eyes were rinsed in cold Ca^{2+}-free Hank's EDTA (CFHE), to remove extraneous debris, and then placed in a nine well dish also containing CFHE. Using a Zeiss stereomicroscope, the lens and neural retina were dissected free from the eye cup. The cornea, optic nerve, and muscle tissue were trimmed from the eye cup leaving the sclera,

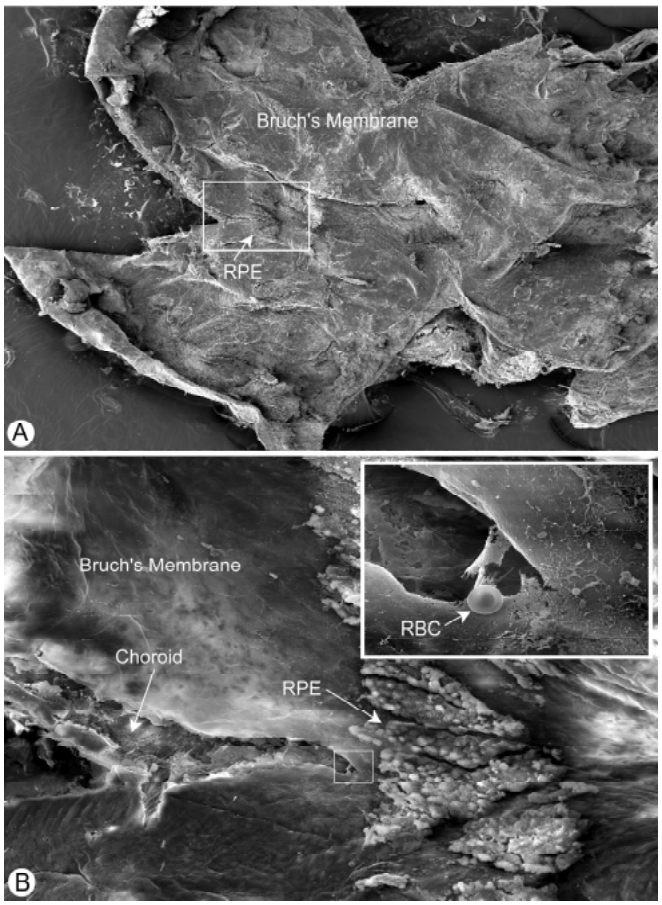

Figure 4. Scanning electron photomicrograph of a vitiligo mouse eye cup demonstrating that samples obtained for the esterification assays were enriched for RPE. (A) A 9 week old vitiligo mouse eye cup seen at 28X magnification, showing a few patches of remaining RPE cells and a largely intact Bruch's membrane after gentle brushing with a small artists brush. (B) An enlargement to 225X magnification of the boxed area in (A) reveals a patch of RPE cells and an intact Bruch's membrane. A small portion of choroid is revealed following clipping with dissecting scissors when the eye cup is laid flat during brushing. The inset, at 3628X magnification reveals minimal disruption to Bruch's membrane and the underlying choroid.

choroid, and retinal pigment epithelium (RPE). RPE cells were separated from the remaining eye cup following a modified procedure described by Wang et al. [21]. Briefly, the eyecups were rinsed twice in CFHE and were incubated at room temperature for 30 min in CFHE. The RPE was carefully brushed away from Bruch's membrane with a small, medium-bristle artist's brush. RPE-enriched tissue from 30 eyes was pooled from both vitiligo and control mouse samples and was suspended in 650 µl CFHE of which 530 µl was centrifuged at 13,800 x g for 60 min at 4 °C. The supernatant was removed and the pellet resuspended in 100 µl CFHE and assayed for esterification as described above. The remainder of the sample was used for phospholipid analysis to normalize the enzymatic activity. (Protein concentration of the RPE sample was not as reliable because melanin pigment interfered with several colorometric assays.) Phospholipid concentration was determined following the procedure of Folch et al. [22]. To verify the completeness of the RPE dissection used for the esterification assay, eyecups remaining after brushing away the RPE were selected randomly from mutant and control mice and were fixed in 1.5% glutaraldehyde and cacodylate buffer for 3 h and then incubated in cacodylate buffer overnight. The eyecups were dehydrated through serial alcohol and critical point dried using a Samdri-790 Critical Point Dryer (Tousimis Research Corp., Rockville, MD) mounted on an SEM viewing stage and gold plated using a Technics Hummer II Sputter Coater (Technics Corp., Alexandria, VA). Eyecups were inspected for the extent of RPE removal using a Philips XL-30 FEG scanning electron microscope (Eindhoven, The Netherlands).

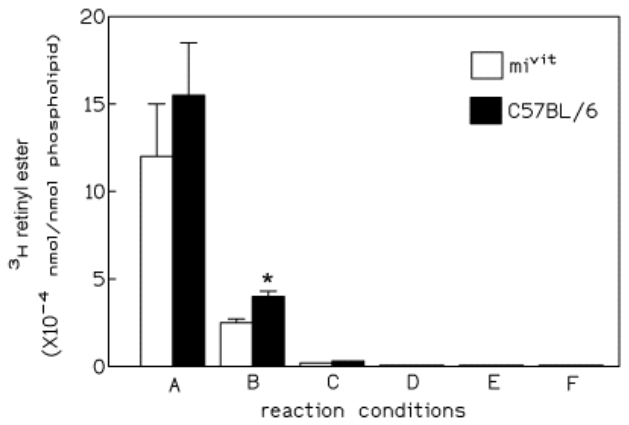

Figure 5. Bar Graph of ^{3}H-retinol esterification to ^{3}H-retinyl esters in RPE-enriched tissue. Each assay included 20 µl RPE-enriched tissue from 15 animals at the indicated age, 10 µl ^{3}H-retinol, 15 µl BSA. The reaction was terminated after 10 min. (A) RPE-enriched tissue from 3 week old mice, the amount of ^{3}H-retinol esterified to ^{3}H-retinyl esters for vitiligo mouse samples (unshaded boxes) was not significantly different from that of the control mouse samples (shaded boxes). p=0.055, t=-5.733. (B) RPE-enriched tissue from 9 week old mice, ^{3}H-retinol esterification to ^{3}H-retinyl esters in the vitiligo mouse samples was only 50% and was significantly different (marked by *) from control samples (p<0.001, t=-44.529). Each esterification assay was performed using RPE-enriched tissue obtained from 30 eyes (per mouse group). Each assay was performed twice. (C) Samples were boiled for 3 min prior to the assay, the low level radioactivity represents the residual tritium (background radioactivity) found in the assay. (D) RPE-enriched tissue was omitted from the reaction mixture, no esterification occurred. (E) ^{3}H-retinol was omitted, esterification did not occur. (F) A sample of sclera/choroid tissue was also assayed, after having brushed away the RPE, no esterification was detected.

RESULTS AND DISCUSSION

Figure 1 provides the levels of retinyl esters extracted from the RPE of mutant and normal mice. There was a significant elevation of retinyl esters in the mutant RPE compared with controls as early as 2 weeks and by 8 weeks the levels were four-fold greater than controls. This finding confirms the observation of retinyl ester accumulation in the RPE at 6 weeks [4] and further indicates that retinyl palmitate accumulation is occurring in the mutant RPE at a time preceding marked loss of retinal photoreceptor cells, which begins by 8 weeks postnatally [9,23]. Although it is unlikely that the accumulation of retinyl esters at this early age is destructive to the RPE (retinyl esters are the primary storage form for retinoids, particularly the more toxic all-*trans*-retinol), it is noteworthy that alterations in retinoid metabolism in the RPE are occurring much earlier than the onset of photoreceptor cell loss. Regarding analysis of levels of retinyl esters in neural retina, the levels were minimal in mutants and controls (less than 0.01 nmol/eye, data not shown). Figure 2 provides the levels of all-*trans*-retinol extracted from the mutant and normal RPE. At 4, 6 and 8 weeks levels of retinol were slightly greater in the RPE of mutant mice than controls, but the differences were not significant. Similarly, there were no significant differences in the levels of all-*trans*-retinal in the neural retina between the two strains of mice (data not shown).

The assay to determine esterification of all-*trans*-retinol to retinyl esters was established first in liver microsomes. This was to done to document the validity of the esterification assay in a more abundant tissue before using the RPE samples. Microsomes were subjected to various reaction conditions. As shown in Figure 3, the time dependence of the reaction was demonstrated in a 10 min reaction (Figure 3A), a 20 min reaction (Figure 3B) and a 30 min reaction (Figure 3C) under standard conditions (255 µl KH_2PO_4, 20 µl tissue sample, 15 µl BSA and 10 µl ^{3}H-retinol). Boiling the liver microsomes markedly reduced enzymatic activity (Figure 3D; the dpm of this sample reflects the background radioactivity of the assay). When the reaction was performed without the addition of liver microsomes, esterification did not occur (Figure 3E). Likewise, when ^{3}H-retinol was omitted from the reaction, no esterification activity was detected (Figure 3F).

After establishing the esterification assay in liver microsomes, the assay was carried out with mouse RPE tissue. The completeness of the RPE dissection used for the esterification assay was assured by scanning electron microscopic analysis of eyecups remaining after RPE removal. Figure 4 shows a representative scanning electron micrograph of a mutant eyecup following the removal of the RPE. It is evident that the dissection and brushing procedure removed nearly all of the RPE. Figure 4B shows an enlargement of the

boxed area in (Figure 4A) where a small patch of RPE cells remained attached to Bruch's Membrane. SEM analysis of both vitiligo and control mouse eyecups revealed very complete removal of the RPE for the esterification assay thus assuring that any LRAT activity detected was from RPE cells.

Figure 5 provides the results of the esterification assays conducted using RPE-enriched tissue samples. Esterification assays were carried out initially with mutant and control mice at 3 weeks. Although retinyl ester levels were elevated in the mutant RPE by 2 weeks (as shown in Figure 1), the esterification activity in mutants did not differ significantly from the control levels at this age (Figure 5A). By 9 weeks the esterification activity had decreased markedly in normals as shown in Figure 5B. Surprisingly, the esterification activity of the mutants was significantly reduced compared to normal mice rather than being elevated as predicted. That is, at 9 weeks the RPE of vitiligo mice possessed only half the esterification activity of normal mice. Boiling RPE tissue, which reduces enzymatic activity, revealed a very low level of background radioactivity (Figure 5C). When the RPE tissue was omitted (Figure 5D) and when ^{3}H-retinol was omitted from the reaction (Figure 5E) esterification was not detected. As an additional control, a sample of sclera/choroid tissue sample was obtained after brushing away the RPE and assayed for its esterification activity; no esterification was detected (Figure 5F).

The finding that the esterification activity of the mutant RPE is reduced compared with controls by 9 weeks, rather than elevated, indicates that retinyl esters are not accumulating in the mutant RPE due to hyperactive esterification of retinol by LRAT. The reduced LRAT activity may reflect an attempt on the part of the mutant RPE cell to retard the accumulation of retinyl esters either by down regulation of enhancers of esterification or by upregulation of inhibitors of esterification, such as apo-CRBP [24]. If, as our data suggest, increased LRAT activity is not the underlying cause of retinyl ester accumulation, what alternative explanations are there for the accumulation of this retinoid? One possibility is that isomerohydrolase activity in the mutant RPE is decreased, however this is unlikely because the levels of the enzymatic product of this reaction, 11-*cis*-retinol are normal in the mutant eye [4]. Similarly, in the subsequent enzymatic reaction in which 11-*cis*-retinol is converted to 11-*cis*-retinaldehyde by pro S dehydrogenase, the levels of 11-*cis*-retinaldehyde are also normal [4] in the mutant. 11-*cis*-retinaldehyde typically is carried in the presence of interphotoreceptor retinoid binding protein (IRBP) to the photoreceptor cells for regeneration of rhodopsin [25, 26]. Recent preliminary studies by Duncan and Wiggert [27] have shown a 2.5-fold elevation of retinoic acid in the eyes of vitiligo mutant mice by 10 weeks postnatally. These data may be related somehow to the accumulation of retinyl esters, but that remains to be tested. An accumulation of retinoic acid could play a role in photoreceptor cell degeneration because retinoic acid has been shown to accelerate photoreceptor cell death by apoptosis in another strain of mutant mice [28]. This information is relevant to the vitiligo mouse retinopathy in which photoreceptor cells have been shown to die by an apoptotic mechanism [29].

The results of the present study confirm an elevation of retinyl esters in the mutant RPE and the data indicate that this elevation begins by at least two weeks postnatally. Our analysis of the esterification of all-*trans*-retinol suggests the retinyl ester accumulation is not due to an increased LRAT activity. It is not likely that the accumulation of retinyl palmitate is provoking disruption of the RPE in the mutant eye. It is more likely that the accumulation reflects a group of cells whose biological machinery is malfunctioning. The observations raise intriguing questions about the possible involvement of retinoic acid in the retinyl ester accumulation as well as in the retinal degeneration in the vitiligo mutant mouse. It remains to be determined whether the mutation of the microphthalmia gene in the vitiligo mouse directly affects retinoid metabolism or whether this is secondary to deregulation of some other gene target of this transcription factor. It has been documented that in vitro the microphthalmia transcription factor regulates the transcription of genes encoding proteins in the pigmentation pathway [30]. Indeed, the protein products of these genes (the enzymes tyrosinase and tyrosinase related protein-1) are decreased in the vitiligo mutant mouse [31]. It is noteworthy that retinoic acid has been shown to decrease the activity of at least one of these enzymes (tyrosinase) in cultured cells [32] and hence the changes observed in retinoid levels of the vitiligo RPE may have a direct effect on these enzymes as well. It remains to be determined whether the alteration in retinoid metabolism, the alterations in pigmentation proteins or some combination of these alterations directly triggers the retinopathy in the vitiligo mutant mouse.

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