Increase in retinyl palmitate concentration in eyes and livers and the concentration of interphotoreceptor retinoid-binding protein in eyes of vitiligo mutant mice

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Retinyl esters play an important role in the visual cycle because they are involved in regeneration of 11-*cis*-retinal for use in rhodopsin formation. In the present study, retinyl ester concentrations were significantly elevated in eyes and livers of mice homozygous for the vitiligo mutation (*mi^{vt}/mi^{vt}*). Vitiligo mice demonstrate a slowly progressing retinal degeneration characterized by gradual loss of photoreceptor cells and rhodopsin as well as uneven pigmentation of the retinal pigment epithelium (RPE). Analysis of retinoids by h.p.l.c. indicated that the retinyl palmitate level was increased fivefold in eyes of affected mice at 10 weeks postnatally and was threefold higher at 22 weeks of age. Accumulation of retinyl palmitate occurred in the RPE rather than the neural retina. Furthermore, the concentration of all-*trans*-retinol was elevated in the RPE of vitiligo mice. Levels of

interphotoreceptor retinoid binding protein (IRBP) were increased in vitiligo mice between ages 4 and 14 weeks, but returned to normal by 16 weeks. Increased IRBP levels were not due to increased protein synthesis because IRBP mRNA levels did not differ significantly between control and affected animals. To examine possible systemic involvement in vitiligo mice, retinoids were evaluated in liver and plasma. Mean hepatic total vitamin A levels in affected mice were approximately 1.7 times higher than controls. Analysis of esterified and non-esterified retinoids in liver showed that the concentration of retinyl palmitate was elevated. Plasma retinol levels were normal. This study provides the first evidence of altered systemic retinoid metabolism in vitiligo mice, which occurs, significantly, under normal dietary conditions.

INTRODUCTION

The importance of vitamin A (retinoids) to normal visual function is well established. Examples of experimentally induced retinal degeneration have been reported in animals deprived of vitamin A (Katz et al., 1993). However, in the absence of dietary deprivation, abnormalities in retinoid metabolism associated with retinal degeneration have not been reported. The present study assessed retinoid levels in a newly described model of retinal degeneration, the vitiligo mouse. Mice carrying the vitiligo mutation gradually lose their pelage pigmentation (Lerner et al., 1986), and in addition slowly lose retinal photoreceptor cell nuclei over the course of a year (Smith, 1992). Levels of rhodopsin in vitiligo mice are similar to controls at 6 weeks of age, but are reduced by about 50 % by 22 weeks (Smith, 1992). As might be predicted on the basis of the skin depigmentation, the retinal pigment epithelium (RPE) is unevenly pigmented in this animal (Lerner et al., 1986; Boissy et al., 1987; Smirnakis et al., 1991). The mutation maps to mouse chromosome 6, is an allele of the microphthalmia locus (Lamoreux et al., 1992; Tang et al., 1992) and has been assigned the strain designation *mi^{vt}*.

The gradual decrease in rhodopsin and the altered pigmentation of the RPE prompted an investigation of visual cycle retinoids in vitiligo mice. The RPE plays a major role in uptake, storage and mobilization of vitamin A for use in the visual cycle (Berman, 1991). Within the RPE, all-*trans*-retinol is converted

into retinyl ester (primarily retinyl palmitate), isomerized to 11-*cis*-retinol and subsequently converted into 11-*cis*-retinal. Within the photoreceptor cell, the 11-*cis*-retinal is covalently linked to opsin to form rhodopsin. On photolysis of rhodopsin, 11-*cis*-retinal is isomerized to all-*trans*-retinal, reduced to all-*trans*-retinol and returned to the RPE.

Retinol is unstable in its free form and membranolytic (Blomhoff et al., 1990; Meeks et al., 1981); thus a transport vehicle for retinol between photoreceptors and RPE had long been suspected and led to identification of interphotoreceptor retinoid-binding protein (IRBP) (Wiggert et al., 1978; Adler and Martin, 1982; Lai et al., 1982; Liou et al., 1982). IRBP, a 140 kDa soluble glycoprotein, is synthesized by photoreceptor cells and is a major constituent of the interphotoreceptor matrix (IPM), the extracellular material that separates the neural retina from the RPE (Fine and Zimmerman, 1963; Feeney, 1973). Two groups of investigators have shown that IRBP plays an active role in the release of 11-*cis*-retinal from the RPE to the IPM (Okajima et al., 1990; Carlson and Bok, 1992). In addition, it facilitates the transport of all-*trans*-retinol from the photoreceptor cell to the RPE after rhodopsin bleaching (Okajima et al., 1989).

A systematic analysis of retinoids and IRBP in eyes of vitiligo mice was undertaken, which led subsequently to analysis of retinoids in liver and plasma. Elevated retinoid levels were found in livers, as well as eyes, suggesting that the vitiligo mutation may represent a systemic perturbation in retinoid metabolism.

Abbreviations used: RPE, retinal pigment epithelium; IPM, interphotoreceptor matrix; IRBP, interphotoreceptor retinoid-binding protein; LRAT, lecithin-retinol acyltransferase; 1 × SSC, 0.15 M NaCl/0.015 M trisodium citrate, pH 7; 1 × SSPE, 0.15 M NaCl/10 mM NaH₂PO₄/1 mM EDTA; 1 × Denhardt's, 0.1 g of Ficoll 400, 0.1 g of polyvinylpyrrolidone and 0.1 g of BSA/500 ml; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance.

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EXPERIMENTAL

Animals

The C57BL/6 mi^{rt}/mi^{rt} mice were the offspring from our colony of breeding pairs. Age-matched wild-type controls (C57BL/6 +/+) were obtained from Harlan Sprague-Dawley, Indianapolis, IN, U.S.A. Animals were maintained in clear plastic cages and subjected to standard light cycles (12 h light and 12 h dark). Light levels averaged 12.9–16.1 lx. Room temperature was $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Mice were fed on a Harlan Teklad rodent diet (minimum crude protein, 20.0%; minimum crude fat, 10.0%; maximum crude fibre, 2.0%; vitamin A, 15.7 nmol/g). Care and use of the animals conformed to the procedures set forth in the DHEW publication *The Guiding Principles in the Care and Use of Animals*.

Tissue and plasma collection

Retinoid levels were determined in whole eyes of affected and control animals aged 2, 4, 6, 8, 10, 14, 17, 22 and 42 weeks ($n = 24$ eyes/age group). After overnight dark adaptation in dim red light, mice were killed by CO_2 asphyxiation and eyes were removed and frozen until analysed.

The distribution of retinoids in photoreceptor and RPE cells in vitiligo and control animals was also determined. Neural retina was separated from RPE in animals at 8 weeks of age using a technique described previously (Smith et al., 1991). One set of tissues (i.e. neural retina and RPE) was fixed for histology by a method described previously (Smith, 1992) and examined microscopically for evidence of cross-contamination. In order to examine possible systemic alterations in retinoid metabolism, liver and plasma were obtained from affected and control animals 6 weeks of age. Livers were isolated, weighed, frozen, lyophilized, reweighed, purged with argon and stored at -80°C until analysed. To obtain plasma, mice were anaesthetized by intraperitoneal injection of 0.1 ml of rodent anaesthesia cocktail containing 100 mg/ml ketamine hydrochloride, 20 mg/ml xylazine hydrochloride and 10 mg/ml acepromazine. A blood sample (1–1.25 ml) was obtained via the left cardiac ventricle and transferred to a tube containing 10 μl of 0.5 M EDTA (disodium salt, pH 7.4). Samples were gently mixed and centrifuged at 325 g (2000 rev./min), at 4°C for 20 min. Plasma samples were frozen at -80°C until analysed.

Tissue and plasma extractions

All procedures involving vitamin A were performed under gold light to minimize the possibility of photoisomerization. Lipids in plasma, liver and whole eyes, neural retina and RPE/eyecups were extracted into hexane using a modification (Green et al., 1987) of the procedures of Thompson et al. (1971). Before lipid extraction, whole eyes, neural retinas and RPE/eyecups were homogenized in 300 μl of PBS, pH 7.4 at 4°C using a Duall tissue grinder (size 20; Kontes, Vineland, NJ, U.S.A.). Homogenates were transferred to 15 ml glass tubes and lipids were extracted into hexane. Retinoids in the hexane-extractable lipid phase were analysed by h.p.l.c.

Liver samples were saponified before h.p.l.c. analysis. To 0.15 g portions of freeze-dried tissue, ethanol (1 ml) containing pyrogallol (1 g/l, Sigma) as an antioxidant was added. After addition of 0.5 ml of 10.1 mol/l KOH, samples were saponified for 1 h at 60°C under an atmosphere of argon. After saponification, 1 ml of water was added and lipids, including retinol, were extracted into 5 ml of hexane. The aqueous phase was twice re-

extracted with hexane; then samples of the pooled extract were taken for retinol analysis by h.p.l.c.

In addition to total hepatic vitamin A levels, the non-esterified and esterified forms of retinol in liver were determined. Using a modification (Green et al., 1987) of the method of Hara and Radin (1978), non-esterified and esterified retinol were extracted into hexane and samples were taken for h.p.l.c. analysis.

Chromatography

To prepare samples for h.p.l.c. analysis, solvent was evaporated from portions of lipid extracts in a 37°C water bath using a gentle stream of argon. Samples were resolubilized in either methanol (plasma, liver) or 5% dioxane in hexane (whole eyes, neural retina, RPE/eyecup) before h.p.l.c. injection. For plasma and livers, retinoids were separated by reversed-phase h.p.l.c. using a Resolve C18 5 μm column (3.9 mm $\times$ 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. For whole eyes, neural retina and RPE/eyecups, retinoids were separated using a LiChrospher Si-60 5 μm column (4 mm $\times$ 250 mm; EM Science, Gibbstown, NJ, U.S.A.) and a mobile phase consisting of 5% dioxane in hexane (v/v) for the first 10 min at a flow rate of 2 ml/min. After 10 min, dioxane was increased to 10%. The h.p.l.c. system included two model 510 pumps and a model 490 multiwavelength detector (Millipore, Milford, MA, U.S.A.) set at 325 and 370 nm and a model 1046A fluorescence detector (Hewlett-Packard, Waldbronn, Germany) with the excitation and emission wavelengths set at 310 and 470 nm respectively. For instrument control, data acquisition and peak area integration, an IBM PC/AT and Maxima 820 chromatography software package (Millipore) were used. For quantification of retinoids, external standard curves were developed using authentic retinoid standards. The 11-*cis*-retinal was a generous gift from Dr. Rosalie Crouch. All other retinoids were obtained from Sigma (St. Louis, MO, U.S.A.).

Determination of IRBP levels

The amount of IRBP was determined in whole eyes of mi^{rt}/mi^{rt} and control mice from postnatal day 3 until 22 weeks. Two mice per age group were killed by CO_2 asphyxiation. Eyes were removed and frozen at -80°C until used. For each assay, two eyes were homogenized in 300 μl of Tris-buffered saline, centrifuged at 100 000 g (35 000 rev./min), for 1 h at 4°C . The supernatant was analysed for total protein (Bradford, 1976) and IRBP. All samples were analysed in triplicate using the slot-blot analysis technique described previously (van Veen et al., 1988). Because of the increase in IRBP observed beginning at 4 weeks, an additional group of affected and control mice was used to determine IRBP levels on each day between 4 and 5 weeks of age.

Determination of IRBP mRNA levels

Northern-blot analysis

Total RNA was extracted from mouse eyes using RNazol B (Tel-test Inc., Friendswood, TX, U.S.A.). The ethanol-precipitated RNA was electrophoresed on 1.2% agarose gels in the presence of 1.2% formaldehyde. The RNA was capillary-blotted on to an Immobilon N membrane (Millipore Corp., Bedford, MA, U.S.A.), u.v. cross-linked for 5 min and hybridized with a ^{32}P -labelled mouse IRBP gene probe (gel-purified 1.9 kb *Ss*I fragment entirely from the protein-coding region of the first exon of the mouse IRBP gene). Prehybridization and hybridization

were carried out at 42 °C for 16–18 h in solution containing 5 × SSPE, 5 × Denhardt's, 0.5 % SDS, 50 % formamide, 10 % dextran sulphate and 0.1 mg/ml salmon sperm DNA. After hybridization, membranes were washed three times in 1 × SSC containing 1 % SDS for 20 min at room temperature, then twice in 0.1 × SSC containing 0.1 % SDS for 15 min at 65 °C, and exposed to Kodak X-AR film. Membranes were subsequently stripped and reprobed with human β -actin probe (Clontech, Palo Alto, CA, U.S.A.).

Reverse transcription PCR analysis

Following the method of Kutty et al. (1993), ethanol-precipitated RNA was reverse-transcribed using oligo(dT) primers (Invitrogen Corp., San Diego, CA, U.S.A.). A reaction mixture (50 μ l) containing the first-strand cDNA (corresponding to 1 μ g of total RNA), IRBP and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) primers (0.5 μ M each), Tris/HCl (10 mM, pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, gelatin (0.001 %), deoxynucleotide triphosphates (200 μ M each), [α -³²P]dATP (5 μ Ci, specific radioactivity 1930 Ci/mmol; Dupont NEN, Boston, MA, U.S.A.) and 2.5 units of *Taq* polymerase (Perkin-Elmer-Cetus) was kept at 94 °C for 1 min and then subjected to 16 cycles at 94 °C for 30 s, at 55 °C for 30 s, at 72 °C for 1 min, followed by 72 °C for 10 min (Gene Amp PCR system 9600, Perkin-Elmer-Cetus). Primers, 5'-CAGAGGATGCCAAAGACCGA and 5'-GAATCTCAAGTAGCCAATGT, were designed from the genomic sequence of mouse IRBP and span an intron. They were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer and purified using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA, U.S.A.). G3PDH primers 5'-TGAAGGTCGGTGTGAACGGATTTGGC and 5'-CATGTAGGCCATGAGGTCCACCAC were obtained from Clontech. Portions of PCR reaction mixtures (25 μ l) were electrophoretically separated on polyacrylamide gels (4–20 %, Novex, Encinitas, CA, U.S.A.) using Tris/borate/EDTA buffer. The gels were dried and scanned for 16 h using an AMBIS imaging system to determine the radioactivity associated with the amplified products.

Data analysis

Statistical analysis utilized multifactor analysis of variance (ANOVA) to determine if levels of retinoids in eyes, plasma and liver were significantly different between affected and control groups, and if levels of IRBP differed significantly between groups. A *P* value of < 0.05 was considered significant. Tukey's paired comparison test was the *post-hoc* test.

RESULTS AND DISCUSSION

Retinoid analysis in whole eyes and in RPE versus neural retina

After extraction of retinoids from whole eyes into hexane, h.p.l.c. analysis revealed that levels of retinyl palmitate differed significantly between affected and control animals. Figure 1 illustrates the differences in retinyl palmitate levels in eyes of *mi^{vit}/mi^{vit}* mice and C57BL/6 controls. At 2 weeks of age, levels were similar in the two groups (about 0.025 nmol of retinyl palmitate/eye). In controls, retinyl palmitate levels ranged between 0.18 nmol/eye and 0.23 nmol/eye throughout the 42 weeks studied. In affected animals, however, the level of retinyl palmitate was higher than in controls at 4 weeks of age and increased sharply at 6 weeks. By 10 weeks, the level was more than threefold higher than in controls. At 42 weeks, retinyl

palmitate concentration in *mi^{vit}/mi^{vit}* eyes had decreased somewhat compared with that at 22 weeks, but it was still considerably higher than in controls. The levels of retinol, 11-*cis*-retinal and all-*trans*-retinal in whole eyes did not differ significantly between *mi^{vit}/mi^{vit}* and control mice (*F* = 0.557, *P* = 0.731).

In the normal eye, retinyl palmitate is present primarily in the RPE. In order to determine whether retinyl palmitate accumulated in this tissue or in the neural retina of affected mice, these tissues were dissected free from each other. The microscopic evaluation indicated minimal cross-contamination between rod outer segments and RPE in both *mi^{vit}/mi^{vit}* and C57BL/6 retinas. H.p.l.c. analysis of retinoids in these two tissues revealed that retinyl palmitate accumulated in RPE rather than neural retina (Table 1). The levels in affected *mi^{vit}/mi^{vit}* mice were threefold higher than in controls. Retinyl palmitate levels in

Figure 1 Increase in retinyl palmitate levels in vitiligo mouse eyes

Retinoid levels were determined by h.p.l.c. as described in the Experimental section. For each age studied, eyes from 12 affected (□) and 12 control (■) animals were used. Four eyes were used per assay and each assay was performed three times. The variation in data was less than 5%. ANOVA indicated that levels of retinyl palmitate differed significantly between vitiligo and control mice (*F* = 5.57, *P* = 0.0011).

Table 1 Retinyl palmitate concentrations in RPE and neural retina of vitiligo and control mice

Retinyl palmitate was determined in RPE and neural retina of 6-week-old *mi^{vit}/mi^{vit}* and control mice by h.p.l.c. as described in the Experimental section. Four eyes were used per assay and each assay was performed three times. Results are means ± S.D. *Significantly different from controls (ANOVA *F* = 12.58, *P* = 0.0075).

Tissue	Retinyl palmitate (nmol/g)	
	C57BL/6	<i>mi^{vit}/mi^{vit}</i>
RPE	0.118 ± 0.007	0.386 ± 0.070*
Neural retina	0.014 ± 0.004	0.015 ± 0.003

Table 2 Concentrations of all-*trans*-retinol in RPE and neural retina of vitiligo and control mouse eyes

Levels of all-*trans*-retinol were determined in RPE and neural retina of 6-week-old mi^{vit}/mi^{vit} and control mice by h.p.l.c. as described in the Experimental section. Four eyes were used per assay and each assay was performed three times. Results are means $\pm$ S.D. *Significantly different from controls (ANOVA $F = 18.69$, $P = 0.0035$).

Tissue	All- <i>trans</i> -Retinol (nmol/g)	
	C57BL/6	mi^{vit}/mi^{vit}
RPE	0.023 $\pm$ 0.012	0.091 $\pm$ 0.005*
Neural retina	0.054 $\pm$ 0.010	0.048 $\pm$ 0.017

Figure 2 IRBP levels in eyes of vitiligo (□) and control (■) mice

Each point represents the mean $\pm$ S.D. of two to four assays. In each assay, two eyes were homogenized in 300 μ l of Tris-buffered saline, ultracentrifuged at 100 000 g (35 000 rev./min) (4 °C, 1 h). The supernatant was analysed for total protein using slot-blot analysis (van Veen et al., 1988) in which samples were applied in triplicate to Immobilon poly(vinylidene fluoride) membrane. The primary antibody was a 1:150 dilution of goat anti-(bovine IRBP) incubated 4 °C overnight, and the secondary antibody was affinity-purified horseradish peroxidase-conjugated rabbit anti-goat IgG (H + L) (Kirkegaard-Perry). Purified bovine IRBP was used as the standard at 1–75 ng concentrations. Quantification was accomplished using an LKB ultrascan XL laser densitometer and 2400 gel scan software package. ANOVA indicated that between 4 and 16 weeks of age, IRBP levels differed significantly from controls ($F = 26.83$, $P = 0.0003$).

neural retinas did not differ significantly between the two groups ($F = 0.568$, $P = 0.644$).

The increase in retinyl palmitate levels in the vitiligo eye precedes the onset of photoreceptor cell loss. Retinyl palmitate is the primary storage form of vitamin A in the retina. Retinyl esterification is considered a detoxifying reaction, because it converts retinol, which is known to be membranolytic, into an

insoluble relatively non-toxic form (Saari et al., 1993). One possible explanation for accumulation of retinyl palmitate might be that it is a compensatory reaction for an excess amount of all-*trans*-retinol. All-*trans*-retinol is the precursor for retinyl palmitate in the visual cycle and is converted into retinyl palmitate in the presence of lecithin–retinol acyltransferase (LRAT) (Saari and Bredberg, 1988, 1989; Barry et al., 1989; Rando, 1991; Saari et al., 1993). Although all-*trans*-retinol levels did not appear to be elevated when whole eyes were analysed, closer scrutiny of retinoid concentrations in RPE separated from neural retinal revealed an increase in all-*trans*-retinol concentration in the former (Table 2). In fact, retinol levels were four times higher in RPE of affected animals than in controls. Levels of all-*trans*-retinol in neural retina were similar between the two groups ($F = 1.310$, $P = 0.294$). An increase in all-*trans*-retinol had not been detected when whole eyes were assessed, suggesting that differences were masked when the entire eye was studied. Levels of 11-*cis*-retinal and all-*trans*-retinal were similar between the two groups regardless of whether retina or RPE was analysed ($F = 0.568$, $P = 0.644$).

All-*trans*-retinol is a potentially toxic compound, and long-term exposure of membranes to it could result in a lytic episode that could severely compromise retinal integrity. The mi^{vit}/mi^{vit} retina may shuttle the more toxic compound toward the storage form, retinyl palmitate, to decrease the membranolytic consequences of excessive amounts of it. The observation that all-*trans*-retinol was increased in these mutant mice led to experiments to assess levels of IRBP, which could bind excess retinol, in the interphotoreceptor space.

Analysis of IRBP and IRBP mRNA

Using the slot-blot analysis technique, IRBP levels were determined in mi^{vit}/mi^{vit} and control mice. As shown in Figure 2, the concentration of IRBP in control mice ranged between 0.5 and 1.5 μ g/mg of protein. In the mi^{vit}/mi^{vit} mouse, the levels were similar to controls between postnatal day 3 and 4 weeks. After 4 weeks, IRBP levels increased abruptly and remained high until about 12–14 weeks of age, although they had returned to control values by 16 weeks. The abrupt increase in IRBP just after 4 weeks prompted a second set of experiments to examine IRBP levels on each day between 4 and 5 weeks of age. There was a steady increase in IRBP concentration between 4.0 weeks (1.6 μ g of IRBP/mg of protein) and the peak at 4.4 weeks [4 μ g of IRBP/mg of protein in mi^{vit}/mi^{vit} mice, a value that was 68 % higher than in controls (1.31 μ g of IRBP/mg of protein)]. Western blotting of IRBP from normal and vitiligo mouse eyes showed no alteration in the size of the protein in the mutant mouse (results not shown).

The marked increase in IRBP at 4 weeks coincides with the increase in retinyl palmitate in the retina of affected animals. IRBP levels remain high for an extended period of time and return to normal levels only by 16 weeks of age, the time at which the number of rows of photoreceptor cell nuclei is reduced by about 50 %. This is intriguing because the photoreceptor cell synthesizes IRBP, and a decrease in cell number would be expected to result in a decrease in protein synthesis, not the retention of normal levels. One possible explanation for the increase could be increased protein-synthetic rates. Evaluation of IRBP mRNA levels, however, did not support this explanation. Quantification of IRBP mRNA using reverse transcription PCR analysis is presented in Table 3 and indicates that the amount of IRBP mRNA in mi^{vit}/mi^{vit} animals is not significantly different from that in controls ($F = 1.368$, $P = 0.2598$). Northern-blot analysis (Figure 3) also shows that the size and amount of IRBP

Table 3 Reverse transcription PCR quantification of IRBP mRNA in *mi^{vit}/mi^{vit}* and C57BL/6 mouse eyes

The radioactivity in the IRBP band and in the G3PDH band was determined using the AMBIS system described in the Experimental section, and the ratio $\pm$ S.D. of radioactivity was determined from six assays per age per group. No significant differences were detected.

Age (weeks)	Radioactivity in IRBP band/ radioactivity in G3PDH band	
	C57BL/6	<i>mi^{vit}/mi^{vit}</i>
1	0.053 $\pm$ 0.009	0.052 $\pm$ 0.007
2	0.066 $\pm$ 0.007	0.054 $\pm$ 0.008
4	0.073 $\pm$ 0.020	0.068 $\pm$ 0.010
6	0.068 $\pm$ 0.010	0.068 $\pm$ 0.004
8	0.060 $\pm$ 0.010	0.070 $\pm$ 0.010

Table 4 Analysis of esterified and non-esterified retinoids in the livers of vitiligo and control mice

Each data point represents the mean $\pm$ S.D. retinoid level (nmol/g) (eight assays). The esterified and non-esterified forms of retinol were extracted from the livers of 6-week-old mice into hexane and analysed by h.p.l.c. using a Resolve C18 5 μ m column as described in the Experimental section. *Significantly different from controls (ANOVA $F = 6.314$, $P = 0.031$).

Retinoid	Retinoid extracted from liver (nmol/g)	
	C57BL/6	<i>mi^{vit}/mi^{vit}</i>
Retinyl palmitate	194.73 $\pm$ 79.00	382.00 $\pm$ 165.00*
Retinol	54.73 $\pm$ 32.41	37.08 $\pm$ 3.11

Figure 3 Northern-blot analysis of total RNA isolated from whole mouse eyes

Hybridization was with a ^{32} P-labelled gel-purified 1.9 kb *Sst*I fragment entirely from the protein-coding region of the first exon of the mouse IRBP gene. The membrane was stripped and rehybridized with a ^{32} P-labelled human β -actin probe. Lane 1, normal C57BL/6 mouse eyes; lane 2, *mi^{vit}/mi^{vit}* mouse eyes. The amount of total RNA applied in each lane was 18 μ g.

mRNA in 8-week-old vitiligo mice are identical with controls. These data suggest that increased expression of IRBP mRNA is not the cause of the increased amount of protein. Perhaps, the increase in IRBP is the result of a profound decrease in turnover of the protein. In an earlier study of normal mice in which the effects of light deprivation on IRBP were assessed, the protein message was markedly decreased and yet IRBP levels remained normal or were elevated (Kutty et al., 1994).

Retinoid analysis in liver and plasma

The increase in retinyl palmitate levels in eyes of *mi^{vit}/mi^{vit}* mice prompted experiments to examine possible systemic increases in retinoids. Retinoid levels were determined in liver and plasma of affected and control animals at 6 weeks of age, a time at which there was a significant increase in retinoid concentration in the eye.

The mean total hepatic vitamin A levels in *mi^{vit}/mi^{vit}* mice were approximately 1.7 times higher than, and differed significantly from, controls ($F = 15.75$, $P = 0.003$). In 6-week-old *mi^{vit}/mi^{vit}* mice, the level of vitamin A was 477.43 ± 63.5 nmol/g ($n = 6$), whereas in controls it was only 279.1 ± 104.55 nmol/g ($n = 6$). A comparison of esterified and non-esterified retinoids (Table 4) showed that the increase was in retinyl palmitate levels. Unlike in RPE, there was no increase in retinol concentration ($F = 2.33$, $P = 0.214$). This could be a reflection of the large capacity for storage of retinyl ester by the liver (Blomhoff et al., 1991).

In plasma from *mi^{vit}/mi^{vit}* mice, the level of retinol averaged 1.04 ± 0.10 μ mol/l ($n = 4$), and, in that from controls, the levels averaged 1.18 ± 0.17 μ mol/l ($n = 4$). These values did not differ significantly ($F = 1.78$, $P = 0.230$). Previous studies in normal animals have shown that plasma retinol levels are usually maintained within a normal range despite wide variations in dietary and hepatic vitamin A levels (Underwood et al., 1979; Duncan et al., 1993). Thus it is not surprising that plasma retinol levels were similar in mutant and normal mice.

The central question is then why are vitamin A levels increased in both RPE and liver of the vitiligo mouse when fed on a normal diet. In RPE, there are two possible sources for excess retinol. One is from the circulation via the choroidal blood supply. The present assessment of plasma levels of retinol demonstrated, however, that the levels in the *mi^{vit}/mi^{vit}* mouse were very similar to controls. The second source is from the eye itself via the visual cycle in which the 11-*cis*-retinal is rapidly isomerized to all-*trans*-retinal in the photoreceptor cell after bleaching of rhodopsin and is then reduced to all-*trans*-retinol and transported to the RPE by IRBP. The significant increase in retinyl palmitate in the eye might reflect a compensatory mechanism for the excessive levels of retinol in the RPE. However, the finding that retinyl palmitate concentration is elevated in the liver as well may point instead to a defect in retinoid metabolism that is common to RPE and liver, possibly in one or more factors that regulate the turnover and storage of vitamin A.

Our results showing increased retinoid levels in both the liver and RPE of the *mi^{vit}/mi^{vit}* mouse represent the first evidence of biochemical malfunction in this mutant mouse. It is not known,

however, if the abnormal retinoid metabolism is the direct cause of the retinal degeneration or the consequence of some other abnormality. One possible scenario is that increased retinyl palmitate and retinol levels in the RPE create stress in the retina which leads to photoreceptor cell degeneration. The increase in the retinoid-binding protein, IRBP, presumably as a result of decreased turnover of this protein, may also be the consequence of the increased retinoids in the RPE. Little is known about the factors that regulate the turnover of IRBP; hence this mutant mouse is potentially useful for such studies.

The levels of retinyl palmitate in the liver, although elevated, are not in the toxic range for this organ. Thus the *mi^{vit}/mi^{vit}* mouse may prove to be of considerable interest as an animal model for abnormal retinoid metabolism. To our knowledge, this is the first time that an increase in retinoid levels in the liver and RPE has been observed in an animal maintained on a normal diet. Future studies will include examination of LRAT and retinol ester hydrolase, enzymes that regulate the level of retinyl palmitate in the liver and RPE. Additional studies are planned to assess retinoid turnover in liver and RPE, as well as the consequences of manipulation of dietary vitamin A, on levels of IRBP and retinoids.

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