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Frequent Alterations of Visual Pigment Genes in Adrenoleukodystrophy

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Summary

Both adrenoleukodystrophy (ALD) and red/green color blindness have been mapped to the distal long arm of the human X chromosome (Xq28). Color-vision defects are frequently associated with ALD, and study of the red and green visual pigment genes in eight ALD kindreds has shown frequent structural changes including deletions and possible intragenic recombinations. Such changes may reflect chromosomal events underlying both ALD and the associated visual defects and should help define both the structural gene responsible for ALD and physical genetic relationships in the Xq28 region.

Introduction

Adrenoleukodystrophy (ALD) and its adult variant adrenomyeloneuropathy (AMN) are X-linked genetic defects of a single peroxisomal function-beta oxidation of very-long-chain fatty acids (VLCFA). Although the precise gene involved has not been identified, ALD and AMN, which can occur in the same kindred, are characterized by VLCFA accumulation in neural white matter, adrenal glands, fibroblasts, and plasma (Moser et al. 1984).

Studies of ALD kindreds have shown close linkage between ALD, glucose-6-phosphate dehydrogenase (Migeon et al. 1981), and a recombinant human DNA probe (DXS52) that detects an RFLP in the Xq28 region (Aubourg et al. 1987). Thus, the ALD locus has been mapped to the subtelomeric region of the X chromosome, a region that also includes coagulation factors VIII and IX, fragile-X syndrome (FRAX-q27), Emery Dreifuss myopathy, dyskeratosis congenita, an X-linked form of bipolar affective illness, and protan and deutan color blindness (inability to see red and green, respectively; Connor et al. 1986; Mandel et al. 1986; Thomas et al. 1986; Yates et al. 1986; Baron et al. 1987).

We have been studying linkages between ALD and other regional markers and have asked whether genes known to be in the same region might show variation. No ALD or AMN patients are deficient in coagulation factor VIII or glucose 6-phosphate dehydrogenase (H. W. Moser, unpublished results). Recently, Nathans et al. (1986*b*) have isolated genomic and complementary (cDNA) human DNA clones for apoproteins of the three (blue, red, and green) light-sensitive pigments. While the blue-pigment gene is autosomal, the red- and green-pigment genes are proposed to reside in a head-to-tail tandem array in the Xq27-Xq28 region. Since ~8% of Caucasian males have defects in red/green color discrimination (Porter et al. 1962), we hoped to identify kindreds suitable for analysis of linkage between ALD and defects in color vision. When >50% of our AMN patients showed defective red/green color discrimination in screening tests (H. W. Moser, M. Murphy, M. B. Raven, and G. H. Sack, Jr., unpublished observations), we turned to direct study of the status of the red and green visual pigment genes in eight ALD/AMN kindreds.

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Subjects and Methods

Families Studied

Color-vision screening was performed on members of ALD kindreds by using the Farnsworth-Munsell 100-Hue Test (Farnsworth 1943). Owing to their mental status at the time of diagnosis, ALD patients could not be tested and only AMN family members with intact cognitive function and without ocular abnormalities were studied (H. W. Moser, M. Murphy, M. B. Raven, and G. H. Sack, Jr., unpublished observations).

We studied 16 patients (ALD or AMN), 12 heterozygotes, and 13 normal relatives from eight different kindreds. The ALD status of each member was established by assay of VLCFA levels in plasma and/or fibroblasts (Moser et al. 1984) and the hybridization pattern with the polymorphic DNA probe DXS52 (Aubourg et al. 1987).

DNA Analysis

DNA was isolated by the method of Kunkel et al. (1977) and digested with restriction endonucleases purchased from Boehringer-Mannheim. Agarose-gel electrophoresis and filter transfer were performed as described elsewhere (Southern 1975; Aubourg et al. 1987). One-percent agarose gels were used for all separations except for *Rsa*I digests, which were resolved best on 1.4% gels. Probes were labeled with ^{32}P-dATP to a specific activity of 2×10^8 dpm/μg by nick-translation (Maniatis et al. 1976). Hybridizations were performed at 42 C for 48 h in 50% formamide, 0.75 M sodium chloride, 75 mM sodium citrate, 0.06% Ficoll, 0.06% polyvinylpyrrolidone, 0.06% BSA, 20 mM HEPES, pH 7.4, and 0.12 mg denatured salmon-sperm DNA/ml. The blots were washed three times for 30 min at 60 C in 15 mM NaCl/1.5 mM sodium citrate, 0.1% SDS and autoradiographed with intensifying screens for 3 days on preflashed Kodak XAR film at −70 C.

Autoradiograph band intensity was quantitated by scanning with a Joyce-Loebl photodensitometer and a Numonics digitizer. Relative band intensities were determined by comparison with patterns from rehybridizing the filters to a beta-globin cDNA probe. Gene structures were analyzed by scoring each restriction-enzyme digest for bands of sizes predicted for various probes (Nathans et al. 1986*b*).

Figure 1 shows the gene structures and the probes used. Plasmid hs7 contains a 1.7-kb cDNA insert corresponding to nucleotides 455–1694 of the red-pigment mRNA and six additional adenosine residues starting at position 1694 marking the 3′ end; it is also 98% identical to the green-pigment-gene sequence. Coding regions hybridizing to probes I and II are indicated by dashed lines. Probe I (300 bp) encompasses exon 1 and the 5′ half of exon 2. Probe II (350 bp) spans the 3′ end of exon 4 and the 5′ end of exon 5. A 900-bp fragment of hs7 spanning the 3′ half of exon 2 and all of exons 3–6 also was used as probe with *Bam*HI/*Eco*RI double digests, but those results supported conclusions derived using the other probes and are not shown.

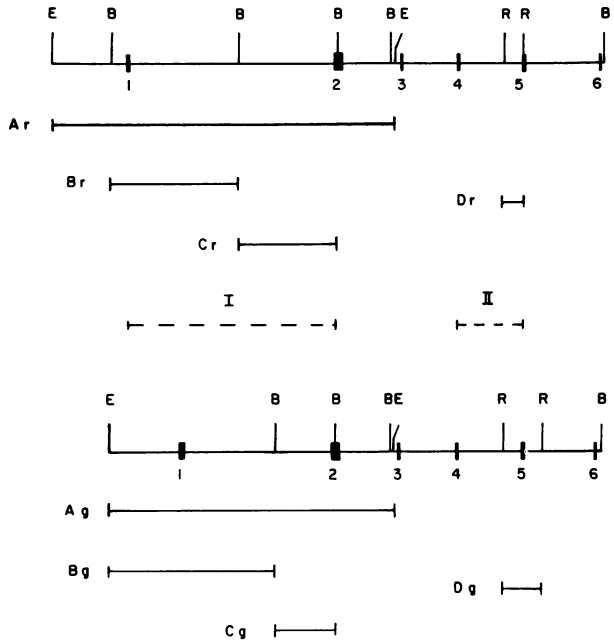

Figure 1 Map of the human red and green visual pigment genes, based on studies of Nathans et al. (1986*b*), showing exons (numbered bars) and restriction-endonuclease cleavage sites (vertical lines: E = *Eco*RI; B = *Bam*HI; R = *Rsa*I) pertinent to these studies.

Results

When hybridized to genomic human DNA digested with *Eco*RI, probe I hybridizes to 11.1-kb (Ar) and 9.2-kb (Ag) fragments derived, respectively, from the 5′ end of the red- and green-pigment genes (fig. 2*A*). Following hybridization of probe I to *Bam*HI/*Eco*RI double digests, Bg (5.4-kb), Br (4.4-kb), Cr (3.7-kb), and Cg (1.9-kb) bands appear (fig. 2*B*). While the number of copies of the green-pigment gene varies (Nathans et al. 1986*b*), the ratios Ag:Ar,

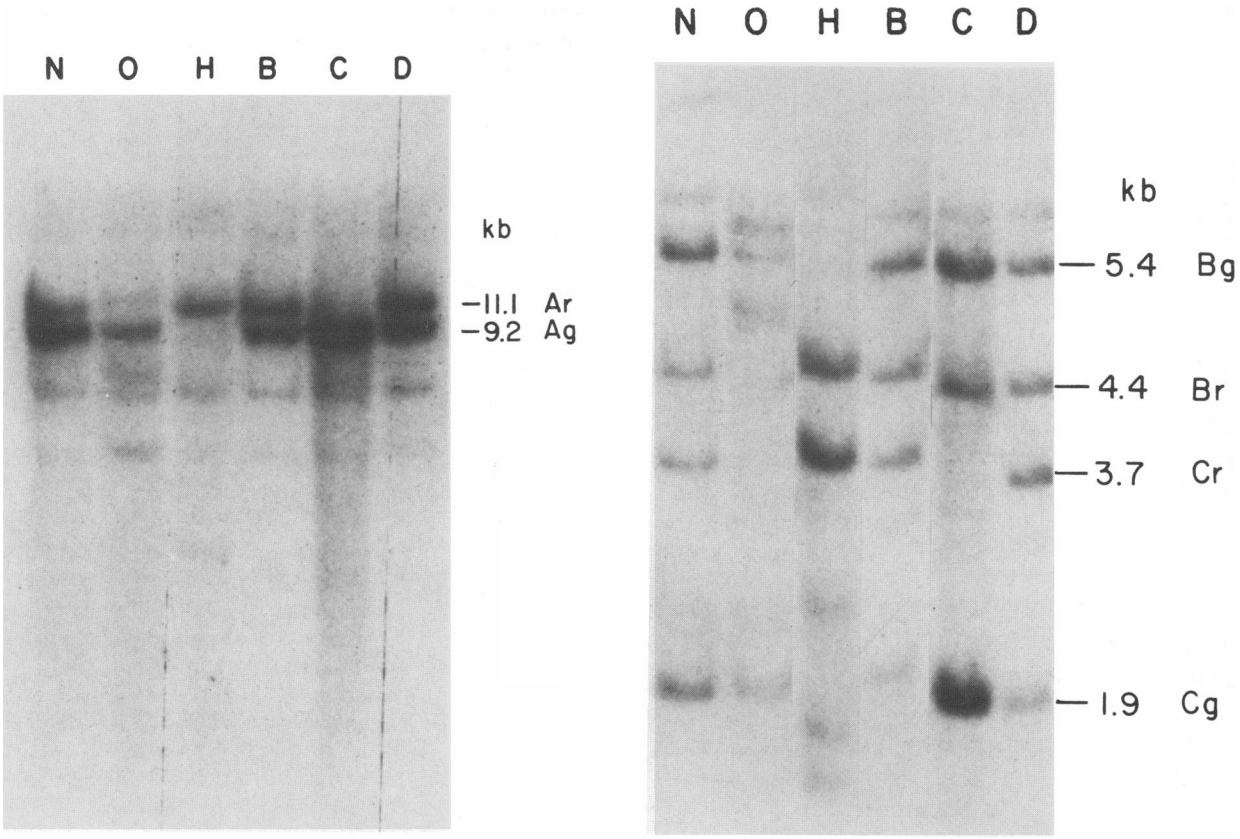

Figure 2 Autoradiograms of probe I hybridized to human DNA from ALD (or AMN) patients. Kindreds are indicated by letters as noted in text; N = normal control. *A*, Probe I hybridized to *Eco*RI digests with Ar and Ag band lengths (predicted from fig. 1) indicated. *B*, Probe I hybridized to *Eco*RI/*Bam*HI digests showing Br, Bg, Cr, and Cg bands.

Bg:Br, Cg:Cr, and Dg:Dr are 2:1 in ≥80% of individuals with normal color vision.

When probe II is hybridized to *Rsa*I digests, it reveals Dg (1.2 kb) and Dr (0.8 kb). These fragments share a common 5′ border but differ at their 3′ borders because the green-pigment gene lacks the *Rsa*I site in exon 5 present in the red-pigment gene (figs. 1, 3).

Probe hybridization patterns are shown in figures 2 and 3, with models in figure 4. Owing to the 98% sequence identity between red- and green-pigment genes (Nathans et al. 1986*b*), our interpretations, based only on band size and copy number, ultimately must be confirmed by sequence analysis. Some faint background bands can be seen, which, as noted (Nathans et al. 1986*b*), most likely correspond to cross-reacting sequences in autosomal blue visual pigment genes. In five of the eight ALD families we have examined, affected males showed a deletion

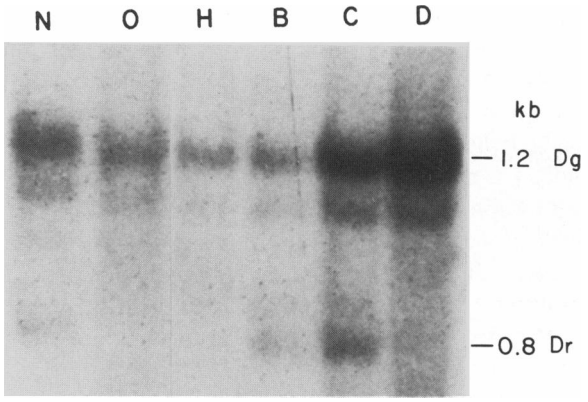

Figure 3 Autoradiogram of probe II hybridization with *Rsa*I digests of normal (N) and ALD patient DNAs as described for fig. 2 and showing D fragments.

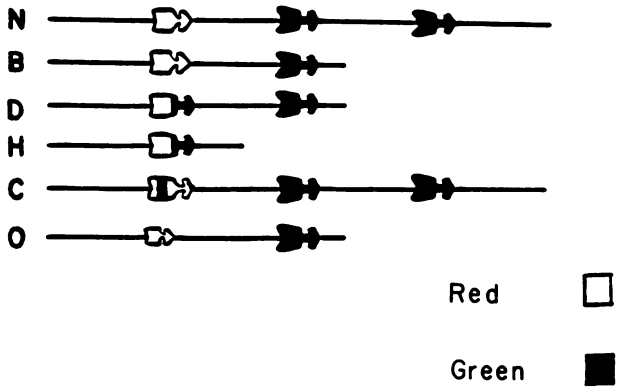

Figure 4 Proposed structure of red and green visual pigment genes consistent with the hybridization studies described. The arrows indicate 5′-to-3′ directions. Most normal individuals (N) have the organization indicated. Deletions and reorganizations in the ALD kindreds shown are based on size and copy number of hybridizing patterns as initially described (Nathans et al. 1986*a*, 1986*b*).

(with or without rearrangement) of at least one green-pigment gene. In four of these five families, the deletions involved both red- and green-pigment genes. The genotypes are different in each of the five families.

In three families (M, T, and G; see table 1), hybridization patterns in ALD or AMN patients were the same as in normal males with two intact green-pigment genes and one intact red-pigment gene. The same pattern was observed for both X chromosomes of heterozygous and normal females in these kindreds. All kindred members who were tested had normal color vision by the screening test.

In family B affected males have one copy each of Ag, Ar, Bg, Br, Cg, Cr, Dg, and Dr and should thus have one intact red-pigment and one intact green-pigment gene. In our screening tests these males do not show reduced green or red color sensitivity, a result that is in agreement with the data of Nathans et al. (1986*a*), which show such a situation in 20% of normal males. However, their normal brothers have one red-pigment and two green-pigment genes and thus deletion of one green-pigment gene has occurred in association with ALD.

Affected males in family D have one copy each of Ag, Ar, Bg, Br, Cg, and Cr but two copies of Dg and none of Dr. This suggests that they have a single green-pigment gene and a hybrid gene (5′ red–3′ green) on the chromosome with the ALD gene.

In family C, affected males have three copies of Cg; two copies each of Ag, Bg, and Dg; one copy each of Br and Dr; and no Ar or Cr, suggesting that they have one hybrid gene (5′ red–middle green–3′ red) and two intact green-pigment genes.

Affected males in family H have one copy each of Ar, Br, Cr, and Dg but no Ag, Bg, Cg, or Dr. This pattern suggests that they have only one hybrid gene (5′ red–3′ green) and no intact green- or red-pigment gene.

In family O, the affected male has only one copy each of Ag, Bg, Cg, and Dg, suggesting that he has only one green-pigment gene. We are uncertain of the significance of the extra band at 12 kb seen in figure 2*A* and the two extra bands (4.8 and 5 kb) in figure 2*B*, but these most likely reflect a novel rearrangement of the other gene. We propose that this gene might produce a nonfunctional opsin or not even be transcribed. Interestingly, color-vision testing suggested a blue-cone monochromatic pattern, consistent with the patient's combination of poor visual acuity (apparently unrelated to his AMN) and monochromacy (Alpern et al. 1971) and suggesting that even his apparently intact green-pigment gene may not function normally.

Figure 4 shows our proposed arrangement of red- and green-pigment genes in the five families with changes. Normal male and female members of each of these families have green-/red-pigment-gene ratios of 2:1. The relative band intensities in heterozygotes (data not shown) conform to a model in which one X chromosome is normal and the other X chromosome has the same arrangement of green- and red-pigment genes seen in ALD males of that kindred. No crossing-over has been found between ALD and the visual pigment gene cluster in the five families with altered pigment genes (28 meioses). The green- and

Table 1

Ratios of Southern Blot Bands in AMN and/or ALD Males in Eight Families

Family	Ag:Ar	Bg:Br	Cg:Cr	Dg:Dr	Cg:Bg
M	2.15	2.25	2.05	2.20	0.85
T	1.85	1.95	1.85	2.15	0.90
G	1.90	2.15	1.95	2.05	0.80
C	...[a]	1.75	...[a]	2.20	1.90
D	1.04	1.03	0.80	...[a]	0.81
B	0.97	1.25	0.82	1.25	0.89
H	0	0	0	...[a]	...
O	...[a]	...[a]	...[a]	...[a]	0.95

[a] The denominator is zero since no identifiable red-pigment-gene fragment was present.

red-pigment-gene defects accompany six ALD as well as five AMN patients.

ALD or AMN males in the C, D, and H families have dichromatic genotypes. Although these three genotypes differ, they all predict a protan phenotype. The correlation between genotype and phenotype has been performed extensively only in family H, in which four AMN patients demonstrate a moderate to severe degree of protan status while two of their normal male relatives had normal green and red color discrimination in accordance with their visual pigment genotype. Recent studies of this kindred have shown a crossover between the RFLP marker DXS52 and ALD/AMN (P. R. Aubourg and G. H. Sack, Jr., unpublished results); there is no crossover between ALD/AMN and protan color blindness.

Discussion

Our screening studies with the Farnsworth-Munsell test have shown color-vision defects in 13 of 22 unselected ALD kindreds (H. W. Moser, M. Murphy, M. B. Raven, and G. H. Sack, Jr., unpublished observations). This incidence is at least five times as great as the 8% encountered in the general population (Haupt 1926; Miles 1929). For the gene studies reported here, we studied ALD kindreds selected only for the availability of DNA samples and found visual pigment–gene reorganizations in four of eight kindreds, as well as another kindred in which deletion of the second green-pigment gene (recognized in 20% of normals) was linked to ALD. Finding four of eight kindreds with these defects, as opposed to the 8% expected, is unlikely to have occurred randomly ($P < .00149$). While we are extending our studies of color vision to other ALD kindreds, these observations indicate that the frequency of this association is likely to be quite high—and certainly much greater than the population average.

The high incidence of red- and green-pigment-gene defects in the ALD/AMN families has several implications. First, the absence of recombination between the altered pigment genes and the ALD locus in 28 meioses indicates close linkage between these loci in the Xq28 region. The identification of RFLPs recognized by the hs7 probe should permit making a numerical estimate of this genetic distance. In the course of these studies, we have found our first evidence for crossing-over between DXS52 and ALD (in family H; data not shown). Despite the close linkage to DXS52 that we have reported elsewhere (Aubourg et al. 1987), this finding suggests that the ALD locus may be closer to the color-vision-gene cluster than to DXS52.

Second, deletions seen in the red/green-pigment-gene cluster of ALD/AMN patients may extend into the ALD gene itself. Although our previous study with the polymorphic DXS52 probe (Aubourg et al. 1987) showed no evidence for such a deletion in the region spanned by the probe (since none of the constant hybridizing bands recognized by the probe were missing in affected males), this study tested only a region of ~50 kb; furthermore, we have now observed a crossover between DXS52 and ALD. Eventually the deletions described here, as well as those that occur in blue monochromatic individuals without ALD, should be useful in creating a deletion map to clarify the structure of this important chromosomal region.

The varied patterns encountered in the four kindreds described here suggest that a family of overlapping deletions and reorganizations may be present in ALD patients. Probes from the extreme ends of the deletion breakpoints should be useful in identifying the ALD gene itself. Our observations indicate that genetic lesions of varying structure may underlie ALD in different kindreds, a situation recognized in other X-chromosomal disorders, including Duchenne muscular dystrophy (Kunkel et al. 1986) and factor VIII deficiency (Youssoufian et al. 1987).

The 98% sequence homology between red and green visual pigment genes (Nathans et al. 1986*b*), as well as their tandem arrangement, provide a situation in which aberrant crossing-over may be increased. Since color-vision defects are not selected for or against, the high frequency of their association with ALD suggests that common mechanisms such as deletions or inversions may underlie both conditions. This immediately suggests that the loci are close together and that strategies for cloning the ALD locus can profitably begin with the already cloned color-vision genes.

Three of the kindreds studied showed normal color vision and the common three-gene red/green/green organization of visual pigment genes. Another kindred, which also had normal color vision, had the recognized variant two-gene pattern of red/green; however, this pattern was linked to ALD. It is unlikely that deletions underlie ALD in these patients, but other gene changes such as point mutations could

explain their disease without involving neighboring loci; these changes should become more clear with further study.

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