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Adrenoleukodystrophy: A Complex Chromosomal Rearrangement in the Xq28 Red/Green-Color-Pigment Gene Region Indicates Two Possible Gene Localizations

Robert Feil,*[,1] Patrick Aubourg,† Jean Mosser,* Anne-Marie Douar,† Denis Le Paslier,‡ Christophe Philippe,§ and Jean-Louis Mandel*

*Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, INSERM Unité 184, Institut de Chimie Biologique, Faculté de Médecine, Strasbourg; †INSERM Unité 342, Hôpital Saint Vincent de Paul, and ‡Centre d'Etude du Polymorphisme Humain, Paris; and §Laboratoire de Cytogénétique, Centre de Transfusion, Vandoeuvre les Nancy, Nancy

Summary

We have characterized a complex chromosomal rearrangement in band Xq28, in an adrenoleukodystrophy patient who also has blue-cone monochromacy. A 130-kb region upstream from the color-vision pigment genes was isolated as yeast artificial chromosome or cosmid clones. Another Xq28 sequence, not included in the above region, was obtained by cloning a deletion breakpoint from the patient. Using probes derived from the cloned sequences, we have shown that the rearrangement affects the color-pigment genes and includes two deletions, most likely separated by a large (>110-kb) inversion. One deletion encompasses part of the pigment gene cluster and 33 kb of upstream sequences and accounts for the patient's blue-cone monochromacy. If this rearrangement also caused ALD, the disease gene would be expected to lie within or close to one of the deletions. However, deletions were not detected in a 50-kb region upstream of the red-color-pigment gene in 81 other ALD patients. Two CpG islands were mapped, at 46 and 115 kb upstream from the pigment genes.

Introduction

X-linked adrenoleukodystrophy (ALD) and its milder, late-onset form adrenomyeloneuropathy (AMN) are neurodegenerative disorders that involve the same locus and may coexist within the same family. Biochemically, ALD is characterized by accumulation of saturated very-long-chain fatty acids (VLCFAs) in neural white matter, adrenal glands, fibroblasts, and plasma (Moser and Moser 1989). Although it has been shown that in the peroxisomal β-oxidation the activation of the VLCFAs to their CoA derivatives is defective (Wanders et al. 1988), the ALD gene still remains to be identified. The ALD locus was genetically mapped to Xq28, closely linked to the glucose-6-phosphate dehydrogenase (G6PD) gene (Migeon et al. 1981) and to the DXS52 marker locus (Aubourg et al. 1987). More recently, it was observed that 12 of 27 AMN patients had abnormal color vision (Sack et al. 1989). The red/green-color-pigment (R/GCP) genes, coding for opsins that mediate red and green color vision, are physically close to the G6PD gene (Arveiler et al. 1989) and to the DXS52 locus (Poustka et al., in press). Elsewhere we have shown that the frequency of pigment gene rearrangements, specific for abnormal color vision, was not significantly higher in 36 ALD patients than in normal Caucasian males (Aubourg et al. 1990). To study neighboring sequences in ALD patients, we performed a 195-kb chromosomal cosmid walk encompassing the entire R/GCP gene cluster (Feil et al. 1990*a*). Six genomic probes that flank the cluster were derived from this walk, and their presence was tested in 36 ALD and AMN patients. No patients

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Address for correspondence and reprints: Jean-Louis Mandel, Laboratoire de Génétique Moléculaire des Eucaryotes du C.N.R.S., INSERM Unité 184, 11 rue Humann, 67085 Strasbourg Cedex, France.

1. Present address: Department of Molecular Embryology, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, United Kingdom.

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were found deleted for probes located 3′ of the pigment genes. However, a single AMN patient with blue-monochromatic color vision, was found deleted for two probes located 5′ of the red-color-pigment (RCP) gene (Aubourg et al. 1990). The deletion includes a region 4 kb upstream of the RCP gene, which was shown to be frequently deleted in inherited blue-cone monochromacy (Nathans et al. 1989). As the estimated frequencies of ALD and blue-cone monochromacy in the Caucasian population are 1/25,000 and 1/100,000, respectively (Lewis et al. 1989; P. Aubourg, unpublished results), we hypothesized that a single deletion event caused both AMN and blue-cone monochromacy in this patient (Aubourg et al. 1990). We report here the study of the deleted region, in search for the ALD gene. We demonstrate that the deletion in this key patient is part of a complex chromosomal rearrangement and conclude that there are two distinct regions in which the ALD gene may be located.

Material and Methods

Genomic DNA Probes

hs7 is the cDNA of the RCP gene (Nathans et al. 1986). The two probes isolated previously from cosmid 42A1-1 are Fr6 (a 250-bp *Eco*RI-*Not*I fragment) and Fr7 (a 700-bp *Taq*I fragment); they are 8.2 and 15 kb 5′ of the RCP gene, respectively (Feil et al. 1990*a*). Fr8 (3.1-kb *Taq*I fragment) and Fr9 (830-bp *Rsa*I fragment) both derive from cosmid c5146 (fig. 1), and are 27 and 40 kb 5′ of the RCP gene, respectively. Fr10 is a 1.6-kb *Eco*RI-*Eag*I fragment that is 46 kb 5′ of the RCP gene and originates from phage B18 (fig. 1). Fr11 is a 700-bp *Hin*cII-*Eco*RI fragment derived from phage X15 (fig. 3) and is located more than 130 kb from the RCP gene. Fr15 is an 850-bp *Hpa*II fragment derived from phage X10 (fig. 4) and is 3 kb from Fr11. Fr26 is a 1.9-kb *Hin*dIII-*Sac*I fragment; it originates from cosmid 29B1-1 (described in Feil et al. 1990*a*) and is 8.5 kb 3′ of each color-vision-pigment gene. Fr14 is a 1.0-kb PCR-amplified fragment that is at the left-arm extremity of YAC 208C4, 130 kb upstream of the RCP gene. Fr14 was obtained following a PCR amplification technique adapted from Riley et al. (1990): yeast DNA of YAC208C4 (vector pYAC4) was digested with *Bst*YI and ligated to the two-strand vectorette construction (the top strand with a 5′ protruding *Bst*YI extremity). The PCR amplification was performed using primers 224 and 1089, described by Riley et al. (1990).

Electrophoresis

Isolation of genomic DNA, electrophoresis, blotting (on diazobenzyloxymethyl paper or on Hybond N^+; Amersham), and hybridization were performed according to methods described by Oberlé et al. (1986). Yeast DNA was isolated according to a method described by Holm et al. (1986). For pulsed-field gel electrophoresis (PFGE) analysis of YAC 208C4, agarose blocks with 10^5 cells were prepared according to the method of Bellis et al. (1987) and digested to completion. Digested DNA was run at 13°C on 1% agarose gels in 0.5 × TBE (1 × TBE = 45 mM Tris base, 45 mM boric acid, 0.5 mM EDTA pH 8.3) on a pulsed-field electrophoresis unit (LKB pulsaphor with CHEF electrode array or Rotaphor type 3 apparatus) at 100 V; pulse times were 40 s (24 h), 30 s (24 h), and 20 s (16 h), respectively. After 15 min of depurination in 0.25 N HCl, size-fractionated DNA was transfered to Hybond N^+ membrane and subsequently hybridized (with Fr6, Fr10, and pYAC4 vector probes), as for the genomic Southern blots. The YAC left-arm (a 375-bp *Bam*HI-*Eco*RI fragment) and right-arm probes (an 1-kb *Ava*I-*Bam*HI fragment) derived from pYAC4 (Burke et al. 1987).

Cell Lines

The panel of somatic hybrid cell lines used for X-chromosome localization has been described by Oberlé et al. (1986). Other cell lines used were GM1202 (48XXXXY), a lymphoblastoid cell line from the Human Genetic Mutant Cell Repository (Camden, NJ); 908BK17, a hamster-human hybrid cell line retaining the human Xq28 chromosome (Schonk et al. 1989); and TU6A, a (t(X;9)(q28;q13)xA9) hybrid cell line described by Du Sart et al. (in press); Hybrid-O is a hamster-human hybrid cell line from fibroblasts of patient O.

Genomic Libraries

X15 and X18 were isolated from a bacteriophage λ library of hybrid O, and X10 was isolated from a phage λlibrary of the 908K1B17 cell line. These libraries were constructed as follows: 40 μg of *Xba*I-digested cell-line DNA was size fractionated on a 15% sucrose gradient (16 h at 105,000 *g;* Beckman SV41 rotor); fifty fractions were collected and analyzed by standard Southern blotting technique and hybridized

with the appropriate probe. Pooled DNA of four positively hybridizing fractions was precipitated and subsequently ligated to *Xba*I + *Sal*I-digested DNA of bacteriophage λ2001 (Karn et al. 1984). One microgram of ligated material was encapsidated (using GigapackII Gold kit; Strategene) and used to infect the bacterial strain P2PLK17 (P2-lysogen; Strategene) with 2×10^8 pfu/μg vector DNA. B18 was isolated from a *Bam*HI λ2001 library, made by ligating non-size-fractionated *Bam*HI-digested DNA of YAC208C4 into the *Bam*HI cloning site of bacteriophage λ2001.

Results

Two CpG Islands Detected in Cloned Sequences Upstream of the RCP Gene

To study the region affected by the deletion in the AMN patient with blue-cone monochromacy (patient O), we extended our previous 195-kb chromosomal walk (Feil et al. 1990*a*) that included the R/GCP gene cluster. Using Fr7, a probe derived from cosmid 42A1-1 (Feil et al. 1990*a*), we isolated one cosmid (c5146) from the X-chromosome reference-cosmid library, constructed in the Imperial Cancer Research Fund (ICRF) laboratories (Nizetic et al. 1991). The restriction map of *Eco*RI and *Bam*HI sites showed that cosmid c5146 extends by 26 kb the walk in the 5′ direction (fig. 1). We obtained an end clone, containing the 5′-most 4.4 kb of the cosmid insert, after *Hin*dIII digestion and religation at low DNA concentration. From this end clone we derived probe Fr9 (an 830-bp *Rsa*I restriction fragment). Two further cosmids were picked from the ICRF X-chromosome-reference cosmid library with the Fr9 probe; both were almost identical to cosmid c5146 and thus did not extend the walk (data not shown).

We then screened by colony hybridization with Fr6 (see fig. 1), a yeast artificial chromosome (YAC) library of a male genome, constructed at the Centre d'Etude du Polymorphisme Humain (Albertsen et al. 1990). One 380-kb YAC clone (YAC 208C4) was picked. We compared this YAC with cosmids 42A1-1 and c5146. Hybridization with several genomic DNA probes showed that the YAC did not contain sequences located 3′ of the pigment genes. Hs7 (the

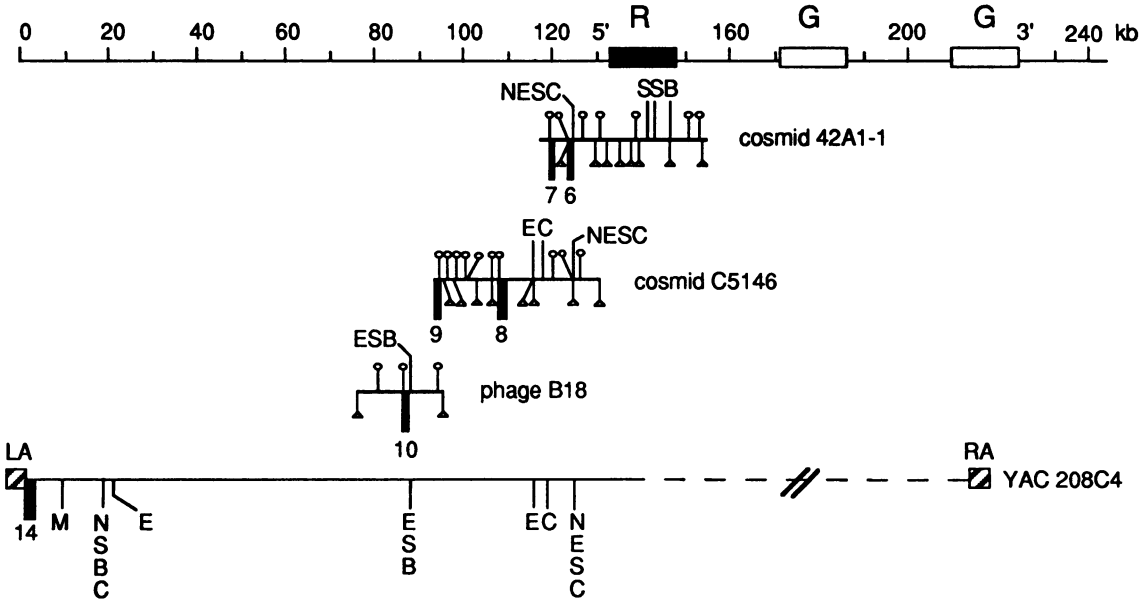

Figure 1 Map of 130-kb cloned region. Cosmids 42A1-1 and c5146, bacteriophage λ clone B18, and YAC 208C4 are shown relative to the red (blackened rectangle; R) and the green (unblackened rectangles; G) color-pigment genes. In the cosmids and the lambda clone, the *Eco*RI (↑) and *Bam*HI (▲) restriction sites are indicated. Sites for rare-cutting restriction enzymes were mapped in all four clones: B = *Bss*HII; C = *Cla*I; E = *Eag*I; S = *Sac*II; M = *Mlu*I; and N = *Not*I. The left-arm (LA) and the right-arm (RA) side of the pYAC-4 vector are shown as hatched squares; the part of the YAC insertion indicated by the interrupted line (240 kb) is not contiguous to the left-arm part. Probes Fr6, Fr7, Fr8, Fr9, Fr10, and Fr14 are indicated as vertical black bars in the clone from which they derive and are further described in Materials and Methods.

cDNA clone of the RCP gene; Nathans et al. 1986) did not detect the *Bam*HI restriction fragments in YAC 208C4, specific for either the RCP or the green-color-vision-pigment (GCP) gene. However, it detected an abnormal 9-kb *Bam*HI fragment (fig. 2*A*), indicating that only the first exon of the RCP gene is present in YAC 208C4. The other tested sequences (hybridized by c5146 and Fr9), all located 5′ of the RCP gene, are entirely present in the YAC insert. The 5′-most probe, Fr9, detects an 18-kb *Bam*HI fragment in YAC 208C4, a fragment which is of the same size as the *Bam*HI fragment detected by Fr9 in genomic DNA. Using a recently developed technique (vectorette PCR amplification; Riley et al. 1990), we isolated a 1.0-kb end fragment of YAC 208C4 (Fr14), located at the left-arm-vector side (see Material and Methods). Fr14 hybridized to the same restriction fragments in *Cla*I-, *Bss*HII-, *Eag*I-, *Mlu*I-, *Not*I-, and *Sac*II-digested DNA of YAC 208C4 as did a pYAC4 left-arm-vector probe. Fr14 was mapped to Xq28 by using an appropriate panel of somatic cell hybrids (data not shown). From these data we conclude that the sequences 5′ of the RCP gene up to the left-arm extremity of the YAC insert, covering 130 kb, are a faithful copy of human genomic DNA. The remaining 250 kb of the YAC insert originate from elsewhere in the genome.

Rare CpG-rich restriction sites were mapped in YAC 208C4 by PFGE analysis. This permitted the positioning of the YAC insert relative both to the pigment genes and to the cosmid clones (fig. 1). A cluster of *Eag*I, *Bss*HII, and *Sac*II sites were mapped 46 kb 5′ of the RCP gene, and a cluster of *Bss*HII, *Cla*I, *Not*I, and *Sac*II sites was found 115 kb from the RCP gene. The 18-kb *Bam*HI fragment (B18) containing the clustered CpG-rich sites 46 kb 5′ of the RCP gene was isolated from a *Bam*HI bacteriophage λ2001 library of the yeast clone, after being screened with Fr9 (fig. 1). From B18, a 1.2-kb *Eco*RI-*Eag*I fragment (Fr10) located directly 5′ of the CpG island (fig. 1) was derived. Location of the two clusters of CpG-rich sites was confirmed by PFGE analysis (hybridizing with probes Fr6 and Fr10) of male leukocyte DNA, in which these sites were unmethylated (data not shown).

An 88-kb Deletion in Patient O: Part of a Complex Chromosomal Rearrangement

We previously observed that AMN patient O does not have a RCP gene and has only one copy of the GCP gene: in *Eco*RI, *Eco*RI + *Bam*HI, and *Rsa*I digests only fragments specific for the GCP gene were detected with hs7, with an intensity corresponding to one gene copy

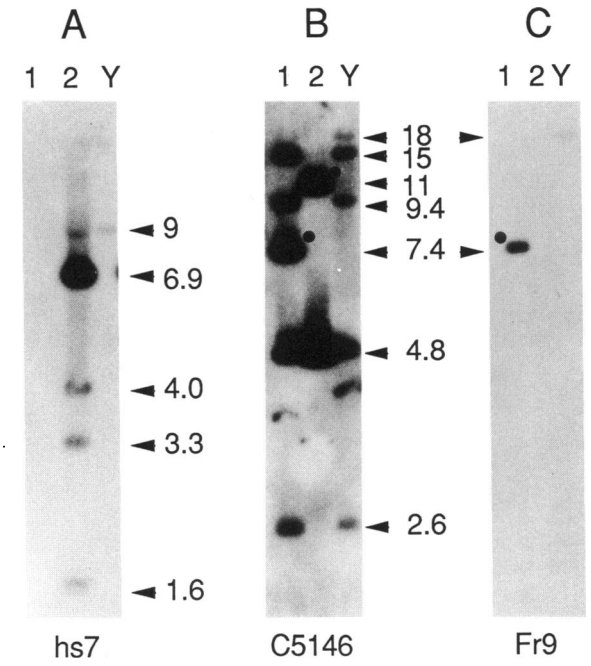

Figure 2 Comparison between YAC 208C4 and cosmids 42A1-1 and c5146. A Southern blot with 10 μg of *Bam*HI-digested DNA of YAC 208C4 (lane Y) and with 0.1 μg of *Bam*HI-digested DNA of cosmids c5146 (lane 1) and 42A1-1 (lane 2) was hybridized with hs7 (*A*), cosmid c5146 (*B*), and Fr9 (*C*), respectively. All four exposures are after final washing in 0.5 × SSC, 0.1% SDS at 65°C. The small black dots indicate fragments partly consisting of vector DNA. Fragment sizes are in kb.

(Aubourg et al. 1988). Probes Fr6 and Fr7 were shown to be deleted in patient O (Aubourg et al. 1990). The 3.1-kb probe Fr8 (fig. 1), derived from cosmid c5146 and located 27 kb 5′ of the RCP gene, was also found to be deleted (not shown). These data indicate that the deletion includes the RCP gene and at least 27 kb of upstream sequences.

In order to obtain probes located on both sides of patient O's deletion, we set out to detect and isolate deletion junction fragments. Several fragments located 3′ of both the RCP and the GCP genes and within the 37-kb repeat unit were used to study patient O and three male controls. One of these fragments, Fr26, located 8.5 kb 3′ of each pigment gene, detected an abnormal 15-kb *Xba*I restriction fragment in patient O, as well as the normal 18-kb fragment (fig. 3). This 18-kb fragment is also detected by hs7. The 9-kb *Xba*I fragment, specific for the RCP gene, is absent in patient O; the intensity of the other bands confirms that only one pigment gene unit is present. Fr26 also detected an abnormal *Kpn*I fragment in patient O (not

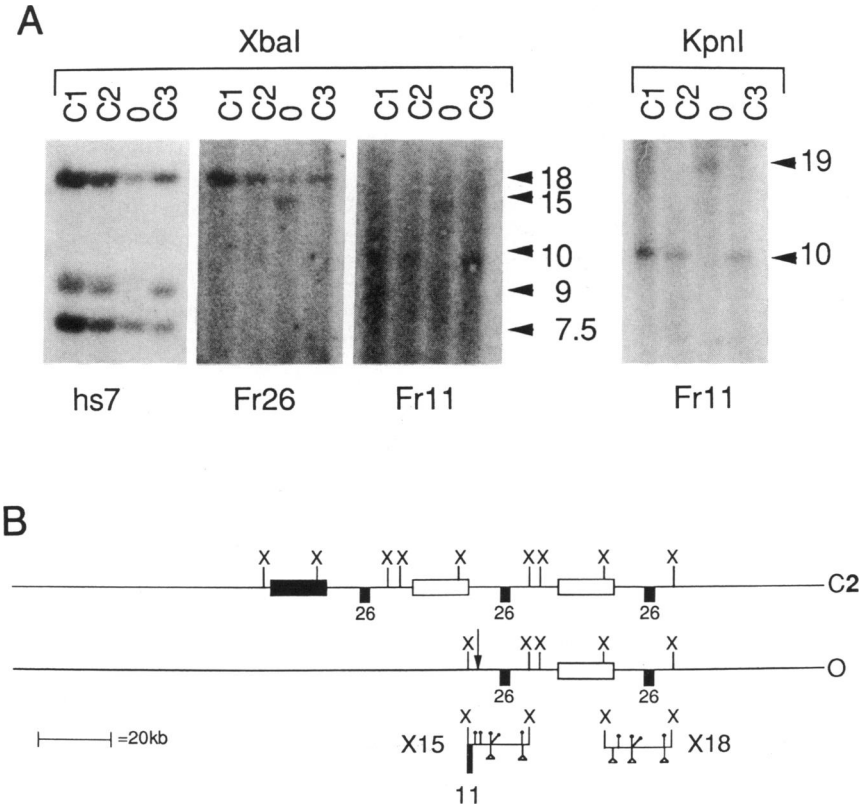

Figure 3 Detection and cloning of breakpoint junction in patient O. *A*, Two Southern blots with genomic DNA of AMN patient O (O) and of three male control individuals (C1, C2, and C3), hybridized in 50% formamide mixture at 42°C, as described by Oberlé et al. (1986). Since both Fr11 and Fr26 contain repetitive sequences, these probes were first prehybridized to an excess of human placental DNA. All exposures were after final washing in 0.5 × SSC, 0.1% SDS at 65°C. Fragment sizes are in kb. *B*, Map of normal DNA (C2), showing position of three copies of Fr26 fragment relative to red (blackened rectangle) and two green (unblackened rectangles) color-pigment genes. A similar map is given for O, who has two copies of Fr26. In both maps the *Xba*I sites (X) present within the gene cluster are indicated. In bacteriophage λ clones X15 and X18, both derived from O's DNA, the *Eco*RI ( ) and *Bam*HI ( ) restriction sites are indicated. The vertical arrow indicates the breakpoint junction detected by probes Fr26 and Fr11, both indicated as numbered vertical bars.

shown). The abnormal 15-kb *Xba*I fragment was cloned by screening, with Fr26, an *Xba*I bacteriophage λ2001 library, constructed from a hamster-human hybrid cell line containing the X chromosome of patient O. Several recombinant phages were obtained, containing either the 15- or the 18-kb *Xba*I fragment. We compared one of the clones containing a 15-kb insert (X15) with one containing an 18-kb insert (X18), mapping *Bam*HI and *Eco*RI restriction sites (fig. 3). This demonstrated that, at most, 2.5 kb at one end of X15 is absent from X18. A 700-bp fragment (Fr11) was isolated from these 2.5 kb. We verified that Fr11 is located on Xq28, by hybridizing a panel of somatic hybrid cell lines. Fr11 detected the 15-kb *Xba*I fragment, from which it originates, in patient O and detected a 10-kb fragment in the three control males. Fr11 hybridized to the same breakpoint junction in *Kpn*I-digested DNA of patient O (fig. 3).

We found Fr11 to be absent from cosmids 42A1-1 and c5146, as well as from YAC 208C4. The fact that Fr11 is not within the 130 kb covered by these three clones suggested that patient O was deleted for at least the 130 kb of sequences 5′ of the RCP gene. In apparent contradiction with this observation was our finding that probes Fr9 and Fr10, both located less than 50 kb 5′ of the RCP gene (fig. 1), are present in patient O (fig. 4). The presence of Fr10 and Fr9, in conjunction with the absence of Fr7 and Fr8, indicated the existence of another breakpoint, located between Fr9 and Fr8 (fig. 5). We searched for a corresponding junction fragment. Fr9 detected in *Apa*I-digested DNA of patient O an abnormal 14-kb fragment, while an

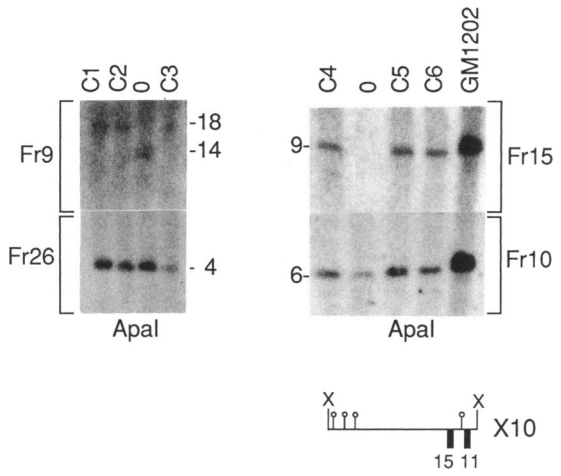

Figure 4 Detection of second Xq28 region deleted in patient O. Probe Fr9, prehybridized to an excess of human placental DNA, detects an abnormally sized (14-kb) *Apa*I restriction fragment. Bacteriophage λ cloneX10 contains the normal 10-kb *Xba*I fragment, detected by probe Fr11; *Eco*RI (↟) and *Xba*I (X) sites are indicated. Probe Fr15 was isolated from X10 and is deleted in patient O. C1, C2, C3, and O are as in fig. 3. C4, C5, and C6 are three other male controls. GM1202 is a 49XXXXY hybrid cell line (see Material and Methods).

18-kb was observed in control males (fig. 4). As both Fr11 (in Xq28 and more than 130 kb from the pigment genes) and Fr9 (38 kb 5′ of the RCP gene) detect junction fragments, the deletion in patient O is part of a more complex chromosomal rearrangement in which at least four chromosomal breakpoints occurred. To further study this chromosomal rearrangement, we isolated a single-copy genomic DNA fragment, located next to the Fr11 fragment on normal genomic DNA, and studied its presence in patient O. A recombinant bacteriophage clone containing the normal 10-kb *Xba*I fragment (X10) detected by Fr11 was isolated from a λ2001 library constructed from the hamster-human hybrid cell line 908K1B17 only retaining the human Xq28 (Schonk et al. 1989). The insert contains no *Bss*HII, *Sac*II, or *Eag*I sites, indicators of CpG islands. The extremity of X10 containing Fr11 is identical to the corresponding extremity of X15. A single-copy fragment (Fr15) located 3 kb from Fr11 was isolated from X10. It detected Xq28 fragments that were absent in patient O, indicating that a second region is also deleted (fig. 4).

To account for all the above data, we propose that the complex chromosomal rearrangement in patient O results from a deletion-inversion-deletion event involving four chromosomal breakpoints (indicated as BP1-4 in fig. 5). BP1 and BP3 were localized by comparing the *Eco*RI and *Bam*HI restriction sites in clones X15, X18, and X10 (figs. 3 and 4). BP1 is 2 kb 3′ of the second-to-last GCP gene. The exact localization of BP2 between Fr8 and Fr9 was obtained by mapping *Apa*I and *Xba*I restriction sites in cosmid c5146 and phage B18 (not shown). BP3 lies in the 3 kb separating Fr15 from Fr11. The size of the deleted region that includes Fr15 has not yet been established; the localization of the (assumed) fourth breakpoint (BP4) is therefore unknown. The deleted region comprising part of the pigment gene cluster is 88 kb in size, if one assumes that, apart from the RCP gene, one GCP gene is deleted. We propose that a large region located between this 88-kb deletion and Fr11 is inverted. Since Fr11 is not contained in YAC 208C4, it must be located more than 130 kb from the R/GCP gene cluster. The inverted segment is thus more than 110 kb in size and contains at least two CpG islands (fig. 5).

Analysis of Two Chromosomal Regions near the R/GCP Gene Cluster in 81 ALD patients

If the complex chromosomal rearrangement caused AMN in patient O, the expression of the ALD gene may be affected by one of the two deletions, by the inversion, or by a combination of these events. We searched for deletions in other ALD patients by studying the following two chromosomal regions: (1) the 55 kb of sequences that is 5′ of the RCP gene and that contains probes Fr6–Fr8 (all three deleted in patient O) as well as probes Fr9 and Fr10 (both present in patient O) and (2) the region, around Fr11, that contains probe Fr15 and is deleted in patient O. Probes Fr6–8 and Fr9–11 were studied in 79–81 independent patients (which include the 34 patients previously studied by Aubourg et al [1990]), while Fr15 was tested only in a subsample of 24 patients. Figure 6 shows the results of hybridization—with probes Fr6, Fr8–Fr11, and Fr15—of *Taq*I-digested DNA from 24 of the ALD patients. Other patients were studied in *Eco*RI, *Bam*HI, or *Hin*dIII digests. Probe Fr10 detects four different *Taq*I fragments, of which two are on Xq28 and two are autosomal. No patients were found to be deleted for any of the seven probes.

Discussion

The peroxisomal VLCFA-CoA synthetase activity was found to be diminished in ALD (Wanders et al. 1988). A gene coding for this enzyme is thus a candidate gene for ALD. Recently, Suzuki et al. (1990)

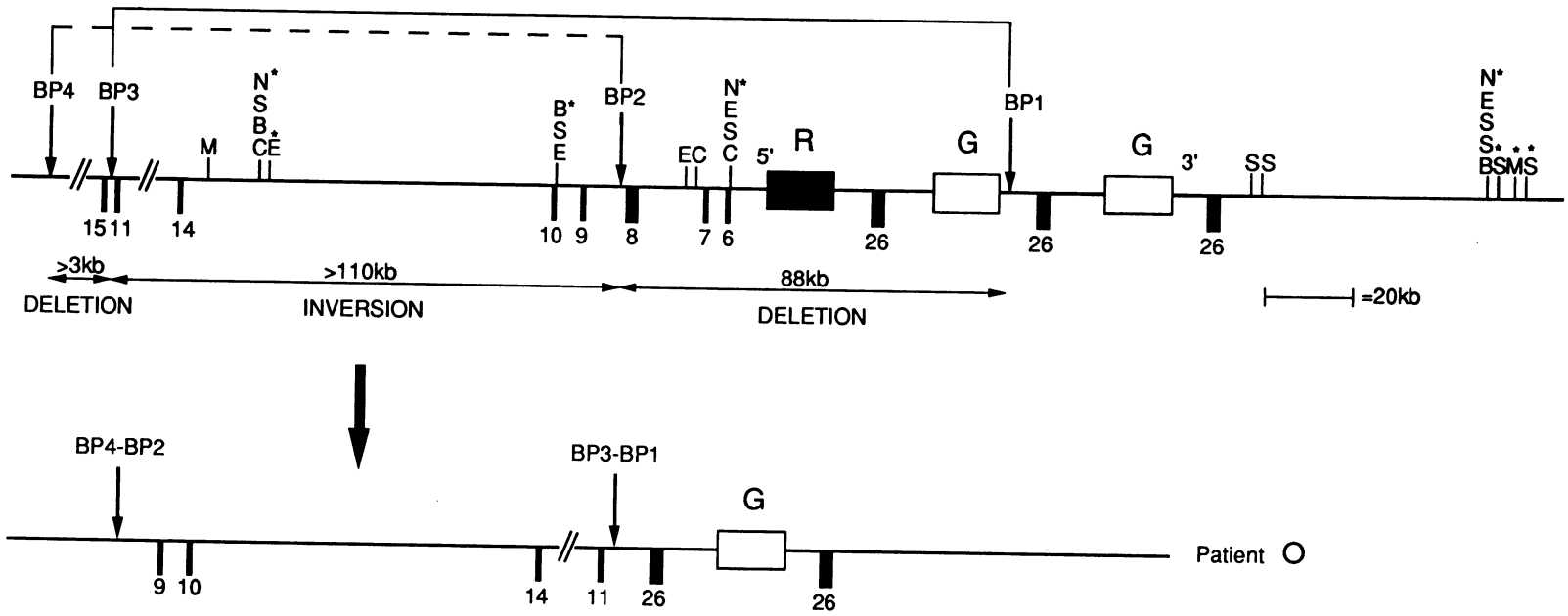

Figure 5 Summary map of region containing R/GCP genes, and localization of breakpoints characterized in patient O. Sites for rare-cutting restriction enzymes, mapped by means of PFGE in the different recombinant clones and in genomic DNA, are indicated: B = *Bss*HII; C = *Cla*I; E = *Eag*I; S = *Sac*II; M = *Mlu*I; and N = *Not*I. The rare-cutter sites within the pigment gene repeat unit are not marked; those 3′ of the pigment genes are from Feil et al. (1990*a*). Fr-probes discussed in the text are indicated as numbered vertical bars. RCP gene (blackened rectangle; R), GCP genes (unblackened rectangles; G), CpG islands unmethylated on the active X chromosome (asterisks [*]), and breakpoints (BP) are shown. BPs could be precisely mapped, except for BP4. BP1 and BP3 are joined in a single junction fragment. The joining of BP2 and the expected BP4 breakpoint has not been formally demonstrated by cloning of the corresponding junction fragment. The extents of the deletion, as well as the proposed inversion, are indicated.

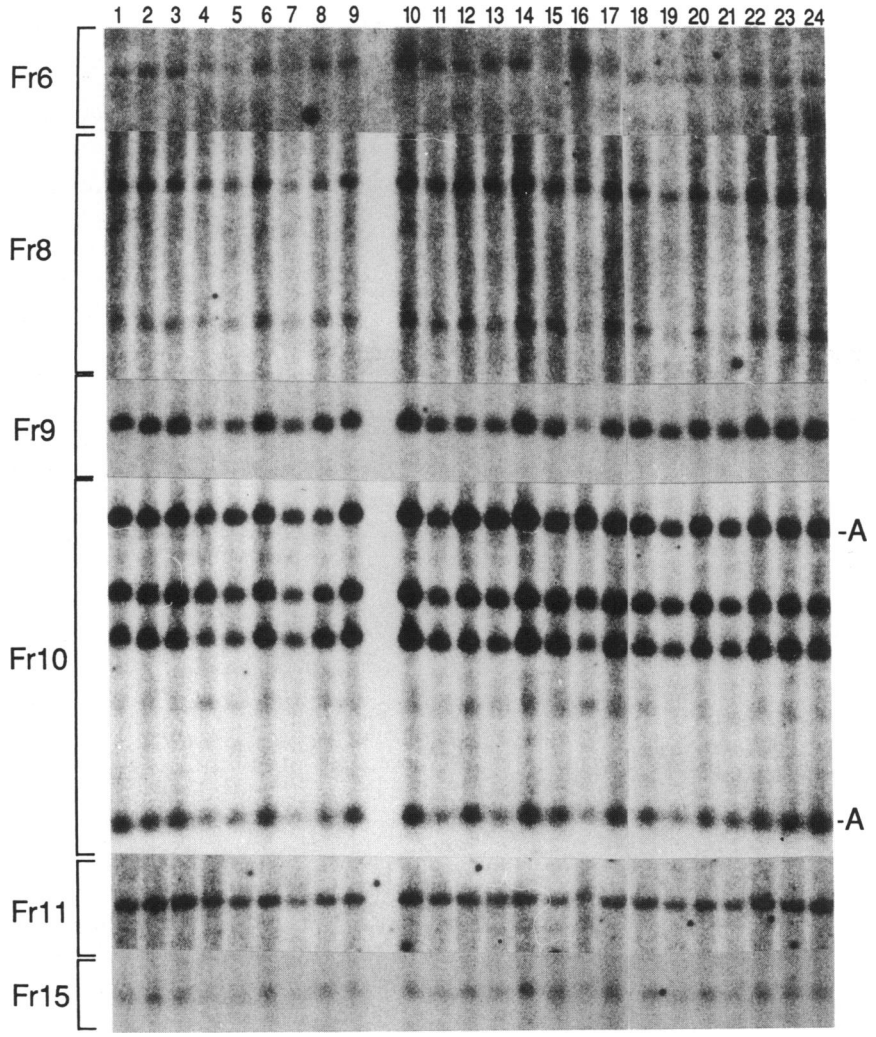

Figure 6 Search for deletions upstream of RCP gene in ALD patients. A Southern blot with *Taq*I-digested DNAs from 24 independent ALD patients (lanes 1–24) was hybridized with probes Fr6, Fr8–11, and Fr15; hybridizing fragments are 1–4 kb in size. Hybridization and washing were performed as described in the legend to fig. 2. Probes Fr8, Fr9, Fr11, and Fr15 were hybridized after competition with human placental DNA. Probe Fr10 detects two fragments on Xq28, as well as two autosomal fragments (*A*). For description of probes, see Material and Methods.

cloned a gene coding for a rat long-chain-fatty-acid CoA synthetase. We hybridized human DNA, as well as DNA of mouse-human hybrid cell lines, with the corresponding cDNA clone (pRACS15). As we did not find evidence for cross-hybridization on the X chromosome (P. Aubourg and F. Feil, unpublished results), it is not likely that the homologous human gene corresponds to the ALD gene. We are thus trying to isolate the ALD locus by positional cloning.

We chose to study the Xq28 subregion containing the R/GCP genes, since abnormal color vision was observed in 12 of 27 patients with the late-onset form of the disease, suggesting that microdeletion may account for the simultaneous presence of the two phenotypes (Sack et al. 1989). Genetically, ALD is tightly linked to the DXS52 locus: compilation of lod scores from five studies (Aubourg et al. 1987; Nezarati et al. 1989; Del Mastro et al. 1990; Willems et al. 1990; van Oost et al. 1991) yields a maximum lod score of 35.1 at a recombination fraction of .01 (lod − 1 confidence limits of recombination fraction are 0 − .03). One recombination event would place the ALD gene distal to DXS52 (van Oost et al. 1991). A recombination fraction of .03 (lod score at .03 =

43.8) has been reported between DXS52 and F8C (Nezarati et al. 1989; Brown et al. 1991), suggesting that the ALD locus is closer to DXS52 than to F8C. However, confidence intervals on these genetic map distances are too large to allow one to draw firm conclusions.

A physical map of Xq28, recently proposed by Poustka et al. (1991), shows R/GCP 1.5 Mb distal to DXS15, with the order DXS15–R/GCP–G6PD–F8C–telomere. DXS15 is at about 450 kb from the multiallelic polymorphism at DXS52 (Feil et al. 1990*b*). The orientation of the R/GCP-F8C cluster is in agreement with a study of the mouse-human hybrid cell line TU6A (t(X;9)(q28;q13)xA9), in which DXS15, DXS52, and R/GCP were present, while G6PD and F8C were absent (Du Sart et al., in press). Interphase in situ hybridization indicated that the RCP gene is farther from the G6PD gene than is the GCP gene(s) (Trask et al. 1991). The following map can be proposed: centromere–(DXS52/DXS15)–RCP–GCP–GdX–G6PD–F8C–telomere. It places RCP closer to DXS52 than is F8C; the rearrangement in patient O would lie between (DXS52/DXS15) and RCP, at a position compatible with the genetic mapping results for ALD. However, it should be noted that an alternative orientation of the R/GCP-F8C cluster has been proposed by Trask et al. (1991).

We continued studying patient O by cloning a deletion junction fragment and by performing a chromosomal (YAC and cosmid) walk encompassing sequences upstream of the RCP gene. Unexpectedly, patient O was found to have not a single deletion but a more complex chromosomal rearrangement comprising two deletions. One deletion contains part of the pigment gene cluster and 33 kb of sequences upstream of the RCP gene. A deletion junction fragment was isolated by screening with a probe that is within the pigment gene cluster. Probe Fr11 on the other side of this junction was localized on Xq28 but was absent from the 130 kb of cloned sequences upstream of the RCP gene. It flanks a second region, deleted in patient O. Finally, fragment Fr9, 40 kb upstream of the RCP gene, detected an abnormally sized *Apa*I fragment in patient O. These data can be explained by two different mechanisms:

1. In the deleted region, comprising part of the pigment gene cluster, a large Xq28 segment containing Fr11, is *inserted*. At the original location of this large fragment, sequences flanking Fr11 are deleted. Such a chromosomal rearrangement would have involved two different Xq28 regions and at least three recombination events, implying six chromosomal breakpoints.
2. Two Xq28 deletions are separated by a large chromosomal fragment (with Fr9 and Fr11 at its extremities) which is *inverted* in patient O. This second mechanism seems most likely to have occurred, because it may consist of two (intrachromosomal) recombination events within the same chromosomal region. We propose that recombination both between BP1 and BP3 and between BP2 and BP4 gave rise to patient O's rearrangement (fig. 5).

Recombination events leading to large DNA inversions occur physiologically during assembly of functional immunoglobulin genes (Weichhold et al. 1990). Illegitimate recombination with inversion seems to be very rare in human DNA. One deletion + inversion was reported to occur in the β-globin gene cluster in the Indian type of εγβ-thalassaemia (Jennings et al. 1985). This deletion + inversion includes an inverted fragment of 15.5 kb, flanked on both sides by deleted regions (of 1 and 7.5 kb). A second inversion, of 6 kb, was described for the cluster of apolipoprotein-encoding genes (Karathanasis et al. 1987). Patient O's rearrangement differs from these two in that it affects a chromosomal region of more than 200 kb. If his disease is caused by the complex rearrangement, the ALD gene should lie either in one of the two deleted regions or in sequences flanking these deletions.

In 81 patients, we studied both the deleted region comprising the RCP gene and its flanking sequences. Using five genomic probes that span the 50 kb of sequences (except for 10 kb between Fr8 and Fr9) upstream of the RCP gene, we did not detect any additional deletions. This does not, however, entirely exclude the possibility that the ALD gene lies upstream of the RCP gene. ALD is not lethal in all affected males, and a significant proportion of these have only adrenal insufficiency—or are even asymptomatic—and may thus have a normal reproductive fitness. The proportion of new mutations is thus likely to be less than that in other severe X-linked diseases, and therefore our panel of ALD patients may represent fewer than 81 independent mutations. Furthermore, the frequency of deletion varies widely in X-linked diseases, from less than 5% in hemophilia A and B (Youssoufian et al. 1987; Koeberl et al. 1990) to 80% in steroid sulfatase deficiency (Yen et al. 1987). No deletions larger than 3 bp were observed in the numerous mutations associated with G6PD deficiency (MacDonald et

al. 1991), possibly because complete deletion of the gene would be lethal. It could be that in ALD the frequency of deletion is low—or that deletions are small, so that they could have been missed. On the other hand, our starting hypothesis was that the high level of color-vision anomaly in ALD may be caused by deletions affecting both the ALD gene and upstream control regions for the pigment genes. Our present study of sequences upstream of the RCP gene in 81 ALD patients does not support this hypothesis.

The second deleted region and its flanking sequences have been less thoroughly studied, and we do not yet know its size. If the ALD gene is located within this region, it cannot easily be genetically related to color-vision anomaly in ALD, an anomaly which would than have to be considered as a secondary effect of the biochemical defect of the disease.

Because of a possible position effect, the inverted segment could also be a candidate location of the ALD gene. We identified two CpG islands, 46 and 115 kb upstream of the RCP gene, respectively, which are unmethylated on the active X chromosome. These could be markers for expressed genes—including those for ALD or other diseases—genetically mapped to the Xq28 region (Davies et al. 1990).

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