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Minor Xp21 Chromosome Deletion in a Male Associated with Expression of Duchenne Muscular Dystrophy, Chronic Granulomatous Disease, Retinitis Pigmentosa, and McLeod Syndrome

UTA FRANCKE,[1] HANS D. OCHS,[2] BÉRENGÈRE DE MARTINVILLE,[1,3] JOSEPH GIACALONE,[1] VALERIE LINDGREN,[1] CHRISTINE DISTÈCHE,[4] ROBERTA A. PAGON,[2] MARTEN H. HOFKER,[5] GERT-JAN B. VAN OMMEN,[5] PETER L. PEARSON,[5] AND RALPH J. WEDGWOOD[2]

SUMMARY

We are reporting a male patient who suffered from chronic granulomatous disease associated with cytochrome b_{-245} deficiency and McLeod red cell phenotype, Duchenne muscular dystrophy, and retinitis pigmentosa. On cytogenetic analysis, he seemed to have a very subtle interstitial deletion of part of band Xp21. Since it was impossible to know whether this material was truly deleted or inserted elsewhere in the genome, somatic cell and molecular studies were carried out. In somatic cell hybrids, the deleted X chromosome was isolated on a Chinese hamster background. Southern blot analysis with 20 single-copy probes, that had been mapped to the X short arm, led to the discovery of one (probe 754) that is missing from this patient's X chromosome and also from his total DNA. This proves that he, indeed, has a deletion rather than a balanced insertion. The results provide cytological mapping information for the X-linked phenotypes present in this patient. Furthermore, probe 754 recognizes a restriction fragment length polymorphism of high frequency that makes it the

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[1] Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510.

[2] Department of Pediatrics, University of Washington School of Medicine, Seattle, Wash.

[3] Present address: Department of Laboratory Medicine, University of Minnesota, Minneapolis, Minn.

[4] Department of Pathology, University of Washington.

[5] Instituut voor Anthropogenetica, University of Leiden, The Netherlands.

most powerful probe currently available for linkage studies with X-linked muscular dystrophy.

INTRODUCTION

Deletion mapping has been a powerful tool in genetic analysis of different organisms. Studies of chromosomal deletions have proven that linkage maps do reflect chromosome maps and have led to discovery of recessive lethal genes and to the mapping of genetic loci within chromosomal segments in *Drosophila* [1, 2], in yeast [3] and in *Mus musculus* [4, 5]. In the human species, autosomal deletions, discovered by cytogenetic analysis of phenotypically abnormal individuals, have been exploited for mapping of loci for the enzymes red cell acid phosphatase [6], lactate dehydrogenase A [7], esterase D [8], and catalase [9]. For X-linked enzymes, gene-dosage studies have been limited to steroid sulfatase (*STS*), encoded by a gene in the most distal band of Xp that escapes X-inactivation [10]. In females heterozygous for partial deletions of an X chromosome, deletion mapping of expressed loci has generally been precluded by nonrandom inactivation of the structurally abnormal X. No males with visible deletions of the X chromosome have been reported previously. The X chromosome, comprising about 6% of the human haploid genome, carries many important genes [11], and nullisomy for several of them, resulting from a cytologically evident deletion, would probably be lethal.

We report here the first case of a male with a subtle interstitial deletion of part of the short arm of the X chromosome. The patient BB was affected with several clinical conditions that are known to exist as X-linked recessive Mendelian traits: chronic granulomatous disease (CGD) associated with cytochrome b deficiency and with the McLeod phenotype in the Kell red cell antigen system, retinitis pigmentosa (RP), and Duchenne-type muscular dystrophy (DMD). When his karyotype was analyzed, no major X-chromosome rearrangement was detected, but the central dark band on the short arm (Xp21) appeared slightly reduced in size. The possibility that the material missing from Xp had been inserted at a different chromosomal site could not be excluded by high-resolution chromosome banding studies.

Therefore, we produced somatic cell hybrids in which the deleted X chromosome was the only human chromosome present on a Chinese hamster background. When 20 different anonymous single-copy DNA fragments that had previously been mapped to Xp were hybridized to DNA from patient BB's Epstein-Barr virus-transformed lymphoblastoid cell line and from the hybrid cells, one was discovered (probe 754) that was missing from both patient BB's X chromosome and his total DNA. However, the gene for ornithine transcarbamylase (*OTC*), previously mapped to Xp21 [12–14], was shown to be present at band Xp21 in the patient by blot and in situ hybridization.

The results of these studies enable us to characterize this patient's deletion with a precision that exceeds the resolution of the light microscope and to

postulate subband localizations of genes for several important X-linked recessive disorders. Furthermore, the work reported here should facilitate the molecular cloning of the sequences involved in these disorders.

CASE REPORT

Patient BB was born after an uneventful pregnancy to an apparently healthy mother. Birth weight was 4.4 kg, and birth length, 56 cm. Apgar scores were 8 at 1 min and 9 at 5 min. He was placed for adoption at 10 days of age. Nothing is known about his family history. During the next 15 years the following conditions were identified:

Chronic Granulomatous Disease (CGD)

Hepatosplenomegaly and lymphadenopathy were noted at 6 months of age. During the first 2 years of life, he developed recurrent pyoderma, respiratory infections, and febrile episodes. Subsequently, he presented with recurrent suppurative lymphadenitis, osteomyelitis, and pneumonia due to *serratia marcescens* and staphylococcal organisms. The diagnosis of CGD was established at age 3 by demonstrating a severe bacteriocidal defect of his neutrophils, decreased glucose utilization during phagocytosis, reduced quantitative iodination [15], failure of his neutrophils to reduce nitroblue tetrazolium (NBT) [16], and lack of cytochrome b [17].

McLeod Red Cell Phenotype

McLeod red cell phenotype of the Kell blood group system was diagnosed at age 2 by Dr. E. Giblett of the Puget Sound Blood Center during evaluation of a microcytic, hypochromic anemia. By age 3, some acanthocytic red cells were seen in the peripheral blood smear.

Duchenne-type Muscular Dystrophy

Motor milestones were delayed. He sat at age 1, stood with support at age 2, walked short distances at age $3\frac{1}{2}$,but was unable to rise from the floor without support. At age 10, neurologic examination revealed a wide-based gait and weakness of both the proximal and distal muscles of the upper and lower extremities. At age 12, he became wheelchair bound, and at age 13, became dependent on a motorized wheelchair. At age 15, he was still able to do his written schoolwork and to hold a spoon, although he could not raise his hands to his mouth. At that time, he had pseudohypertrophy of the calf muscles.

At age 2, serum CPK activity was 145 U (nl 0–30). At age 10, nerve-conduction velocities were normal, an electromyogram (EMG) was consistent with a myopathy, and CPK was 1,039 U (nl up to 95). A biopsy of a thigh muscle at age $10\frac{3}{12}$ showed diffuse loss of muscle fibers and replacement by fibrous and adipose tissue and marked variation in fiber size on cross sections. Occasional small groups of muscle fibers showed necrotic change including infiltration by histiocytes. Focal endomysial fibrosis was seen in other portions of the specimen. Infrequent inflammatory cells such as lymphocytes and occasional eosinophils were seen associated with some muscle fibers.

Retinitis Pigmentosa

At age 2, ophthalmologic examination revealed diffuse atrophy of the retinal pigment epithelium (RPE) and intraretinal pigment clumping. At age 15, his visual acuity was 20/100 OD and 20/40+ OS. Funduscopic examination showed progression of the RPE atrophy, intraretinal pigment clumping, and retinal vessel attenuation. In addition, there was atrophy of the macula with cystoid macular edema and drusen of the disc. No focal chorioretinal defects were seen. Visual field testing by Goldmann perimetry using the

largest test object (V 4e) showed severely constricted fields (15°) bilaterally. Electroretinography done under standardized conditions using a corneal electrode and a Glanzfeld full field system showed no detectable rod or cone responses. Fluorescein angiography confirmed the retinal findings. At age 2, there was no evidence of nongenetic or systemic causes of the retinal abnormalities. There was no history of prior infection with cytomegaly virus, rubella, toxoplasmosis, or syphilis; a urine amino acid screen was normal; and skull radiographs showed no intracranial calcification.

Idiopathic Intestinal Pseudoobstruction

Idiopathic intestinal pseudoobstruction was diagnosed at age 15. He had a lifelong history of chronic abdominal distention, diarrhea, and poor weight gain. A rectal biopsy showed normal ganglion cells. By age 6½, postprandial vomiting occurred frequently and barium studies revealed esophageal and duodenal hypomobility and a redundant colon. Sigmoidoscopy to 15 cm showed no evidence of granulomatous disease. Bowel transit time was decreased, and he required repeated hospitalization for functional ileus.

Mental Retardation

Both gross motor and language skills were delayed. He attended special education classes, and at age 15, he was functioning at about the 7th grade level in mathematics and 5th grade level in reading. He was an accomplished organist until age 12 when he became unable to play because of muscle weakness. He had no deterioration in intellectual ability.

Expression of Other X-linked Genes

Glucose-6-phosphate dehydrogenase activity was normal in red cells and white cells, including normal enzyme stability to heat and normal substrate affinity. The red cells were Xg^a positive. Physical examination revealed no other abnormalities known to be X-linked. Patient BB died in an automobile accident at age 16.

MATERIALS AND METHODS

Chromosome Analyses

Heparinized blood was cultured in RPMI 1640 medium with PHA and harvested after 68 hrs, or exposed to 10^{-7} M methotrexate for 17 hrs and released into medium containing either thymidine (10^{-5} M) or bromodeoxyuridine (10^{-4} M) for 5½ hrs. Chromosome preparations and GTG- or RFA-binding were carried out as described [18, 19]. Another sample was cultured in medium 199 with 2% fetal bovine serum. The cells were scored for the presence of the marker X. A lymphoblastoid cell line (LCL 119), established by EB virus transformation, was karyotyped after partial cell synchronization as described for blood cultures. This lymphoblastoid cell line has been deposited at the Human Genetic Mutant Cell Repository at the IMR in Camden, N.J.

Somatic Cell Hybrid Series 29

Chinese hamster cells V79/380-6, deficient in hypoxanthine guanine phosphoribosyltransferase (HPRT) activity, that had been grown in medium containing 8-azaguanine to select against revertants, were mixed in suspension with LCL 119 lymphoblasts at a ratio of 1 : 1. Cell fusion was obtained by exposing the mixed cell pellet to 45% polyethylene glycol (PEG 6000) in serum-free Ephrussi's modified F12 medium at 37°C for 1 min. The PEG solution was then gradually diluted with 10 times the volume of medium over 5 min. The cells were washed gently and plated at low density in complete medium containing 10% fetal bovine serum (Hyclone, Logan, Utah) and the components of the HAT selection system [20]. After 2 weeks of selection against the V79/380-6 cells, 19

actively growing colonies were isolated from 10 different plates. Chromosome analysis by trypsin-Giemsa banding (GTG) and by Giemsa 11 staining [21] revealed that 16 of them were interspecies hybrids containing between one and nine human chromosomes. Two hybrids were subcloned in both HAT and 8-azaguanine media. Five primary hybrids and two subclones were expanded for simultaneous analysis of chromosome content, enzyme markers, and DNA restriction fragments. The presence or absence of an enzyme marker for the human X chromosome, glucose-6-phosphate dehydrogenase (G6PD), was determined in hybrid cell extracts by cellogel electrophoresis [22].

Southern Blotting and in Situ Hybridization

Cloned fragments of the human X chromosome with or without known function were obtained from several investigators. Probes RC8, RD6, and RJ8 were from a library of sorted human X chromosomes [23]. Probes pB24, pD2, pL1, p18-55, p71-7A, and p75-42 have recently been described [24]. Probes 58HAI, 48HAI, 16BA, 33HA, and 3SA, developed by Bruns et al. [25], M2C and C7, provided by J. L. Mandel, and probe p51, from P. Szabo, were all known to hybridize to sequences on the short arm of the X. Precise mapping of these probes to one of five regions of Xp, in particular the assignments of B24, L1, M2C, and C7 to parts of band Xp21, is reported in the accompanying paper [14]. Probe L1.28 was a 1.2 kilobase pair (kbp) *Eco*RI fragment isolated from a normal female placental DNA library [26]. Probes 754 and 782 were isolated by screening the X-enriched sorted chromosome library of Kunkel et al. [27, 28]. They consist of *Hin*dIII fragments, 2.2 kb and 4.3 kb in size, that were subcloned in plasmids [28]. These latter probes recognize high-frequency restriction fragment length polymorphisms [28]. A cDNA clone of the human gene for ornithine transcarbamylase (OTC) was provided by A. Horwich [29]; localization of *OTC* to subband Xp21.1 has been reported [12–14]. Probe pDP31, which is homologous to sequences at the *DXYS1* locus, was used as a control for the presence of the X long arm [30].

Extraction of DNA from cultured lymphoblasts, from somatic cell hybrids and from BB's liver tissue frozen at autopsy, and Southern blotting were performed as described [14]. The OTC probe was hybridized in situ to normal human metaphase spreads and to LCL 119 lymphoblasts as reported [31]. Chromosome identification by quinacrine and subsequent Wright staining was performed as described [32].

RESULTS

Chromosome Analyses

Initially, we analyzed 20 GTG-banded mitotic cells from peripheral blood lymphocyte cultures at 550–850-band stages (ISCN 1981) [33]. A subtle reduction in the short arm-to-long arm ratio of the X chromosome was apparent in the more condensed metaphase spreads but was less obvious in prometaphase cells (figs. 1 and 2*A*). In higher resolution chromosomes, band Xp21 appeared reduced in size and failed to subdivide at the appropriate stages. The lightly staining flanking bands Xp11.4 and Xp22.1 appeared to be of normal size. RFA-banding patterns were consistent with these observations.

The most likely interpretation of the abnormal banding pattern on Xp was that it resulted from a deletion of subband Xp21.2 and of part(s) of Xp21.1 and/or Xp21.3 (fig. 2). Cytological analysis alone did not provide information as to how much of the remaining Xp21 band was derived from Xp21.1 and/or from Xp21.3. There was no evidence of an inversion or of a more complex rearrangement of the X chromosome. Scrutiny of all the other chromosomes failed

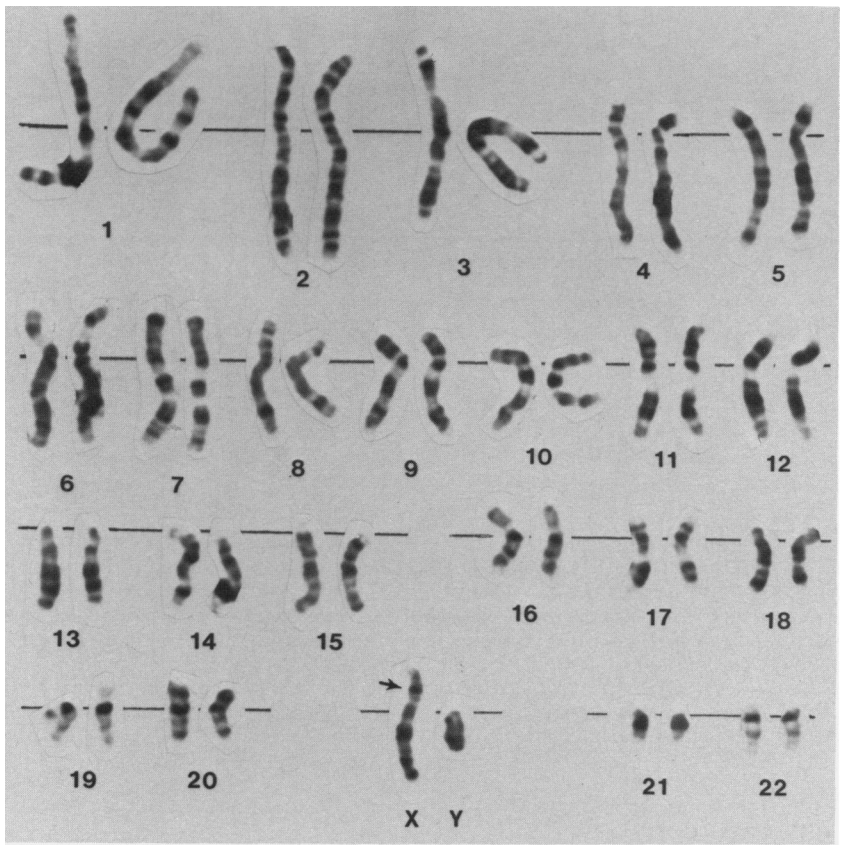

FIG. 1.—Trypsin-Giemsa-banded karyotype from PHA-stimulated lymphocyte culture: 46,Y, del(X)(pter→p21.3::p21.1→qter). *Arrow* points to site of deletion.

to reveal the presence of a translocation. However, so small a segment of Xp21, even though clearly missing from its normal position, could have escaped detection when translocated to another chromosomal site (fig. 1).

In order to distinguish between an unbalanced deletion and a balanced insertion, we turned to somatic cell hybridization and molecular analyses using the EBV-transformed lymphoblastoid line from this patient (LCL 119). GTG-banding of the LCL 119 chromosomes done on several occasions confirmed that the del(X) chromosome had remained structurally unchanged (fig. 2*B*), although other abnormalities such as a heterozygous deletion of band 8p23 were detected in LCL 119 cells (data not shown).

Because the patient was slightly developmentally delayed, we sought to exclude the possible diagnosis of mental retardation/fragile X syndrome. No marker X chromosome expressing the fragile site at Xq27 was seen in 50 cells scored from the medium 199 culture.

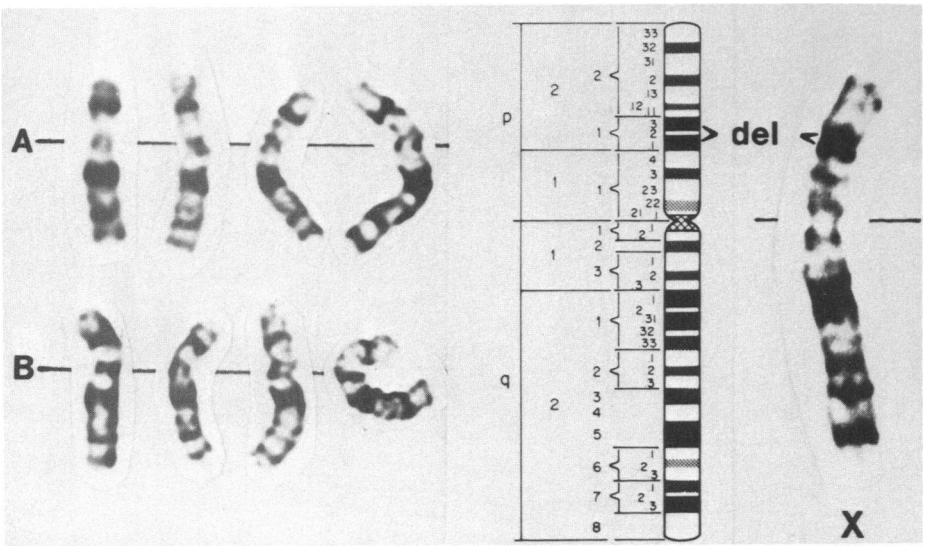

FIG. 2.—GTG-banded del(X) chromosomes from blood lymphocytes of patient BB (*A*) and from lymphoblastoid cell line LCL 119 (*B*). Ideogram of normal human X chromosome at the 850-band stage [33] with a GTG-banded normal X *on the right*. In metaphase chromosomes from the patient, the width of band Xp21 and the short arm/long arm ratio appeared to be reduced. On high-resolution X chromosomes, band Xp21.2, which normally separates the dark subbands Xp21.1 and Xp21.3, was never observed. *Brackets* (del) indicate the location and approximate size of the deletion. Because the deleted material was mostly GTG-dark, its relative size appeared larger in metaphase than in prometaphase chromosomes.

Somatic Cell Hybrids

From the fusion of LCL 119 with Chinese hamster cells 380-6, 16 primary hybrids (series 29) were obtained. Under HAT-selective pressure, all had retained the del(X) chromosome, as determined by GTG-banding and by expression of human G6PD enzyme. In hybrid clone 29-11B, the del(X) was the only human chromosome; no other human material was detected by Giemsa 11 staining. The 22 Chinese hamster V79/380-6 chromosomes [34] appeared structurally unaltered (fig. 3, lower part). The independent hybrid 29-1F contained the human del(X) and one copy of chromosome 21 in each of the 45 cells examined (fig. 3, upper part). Ten subclones isolated in 8-azaguanine medium had lost the del(X) but still retained chromosome 21. Subclone 29-1F-3a aza, which contained chromosome 21 at a frequency of 1.0 copy/cell, and primary hybrids 29-1F and 29-11B were chosen for Southern blot analysis. Any DNA sequences present in LCL 119 but absent in hybrids containing only the X chromosome would indicate that in patient BB's genome, an X-chromosome fragment had been translocated elsewhere. Other hybrids of series 29 could then be used, in conjunction with in situ hybridization, to determine which chromosome had received the insertion of Xp21 material. Sixteen of the autosomes, in various combinations, were represented in the 14 remaining series 29 hybrids.

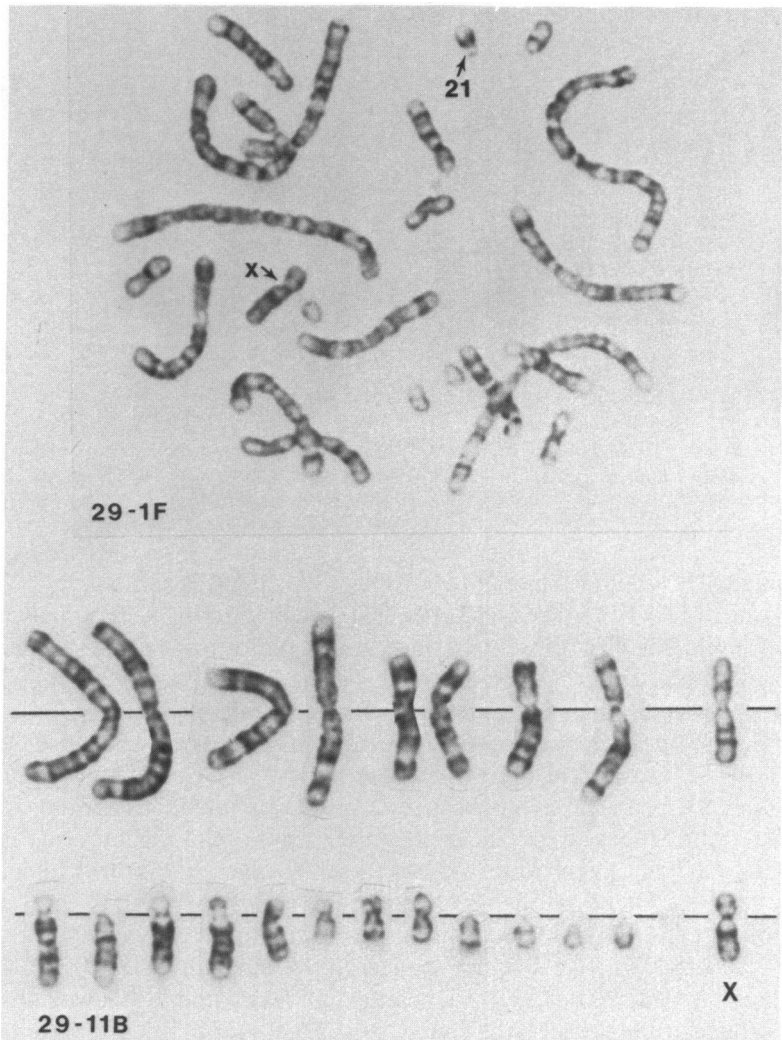

FIG. 3.—GTG-banded chromosomes of LCL 119 × Chinese hamster V79/380-6 hybrid cells. Hybrid clone 29-1F (*top*) had retained only human chromosomes X and 21 (*arrowed*). In clone 29-11B (*bottom*), the X was the only human chromosome present. The V79 chromosomes are all structurally unchanged; they are arranged as reported [34].

DNA Hybridization

DNA extracted from LCL 119 and from at least two other human cell lines was cut to completion with various restriction enzymes, transferred to nitrocellulose filters, and hybridized with 22 different X-derived low copy number human probes. Eighteen of them had been shown to map to one of five distinct regions of the X chromosome short arm [14]. With the exception of 754, all of the probes hybridized to LCL 119 in a fashion indistinguishable from control DNA. Thus, these sequences were present in LCL 119 and were not obviously

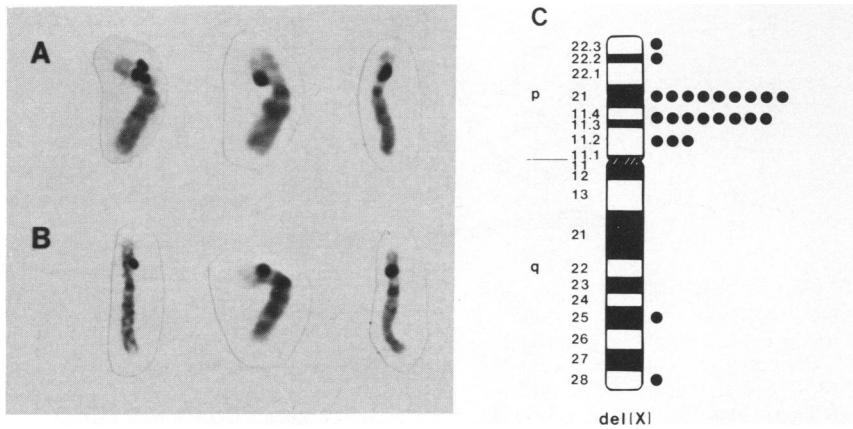

FIG. 4.—In situ hybridization of human OTC probe to X chromosomes of LCL 119 (*A*) and to normal X chromosomes (*B*). In LCL 119, 15 of 66 X chromosomes were labeled at Xp11.3-Xp21; the distribution of silver grains is shown *next to* the ideogram of the del(X) chromosome (*C*).

rearranged. This includes five of the probes (B24, L1, C7, M2C, and OTC) that were localized to band Xp21 [12–14]. They hybridized to DNA, not only of LCL 119, but also of hybrids 29-1F and 29-11B, indicating that these sequences were present on the del(X) chromosome. Examples are shown in figure 3, lane 10, and in figure 6, lanes 3, 9, and 10, of the accompanying paper [14].

To rule out an intrachromosomal rearrangement of the X chromosome, we carried out in situ hybridization with one of the DNA sequences located at Xp21, the OTC probe. When hybridized to LCL 119 metaphase chromosomes, the OTC probe produced specific label at the center of the X short arm (fig. 4*A*). In 72 cells analyzed, there were, on average, 3.71 silver grains on chromosomes per metaphase. Of all grains, 6.1% were in region Xp11.2-p21 (fig. 4), and 22.7% of all cells were labeled at this site. As reported previously, in normal female cells (46,XX), 10.3% of grains were at this site, while in 48,XXXX cells, the percentage was 20.6 [12]. No label above background levels was observed at any other chromosomal site. These data suggest that the OTC gene has not been deleted or disrupted by the Xp deletion in this patient, and, further, that at least part of the remaining Xp21 band must consist of Xp21.1 material to which *OTC* has been mapped.

As mentioned above, probe 754 failed to hybridize to DNA of LCL 119 and of series 29 hybrids. The samples in figure 5 were hybridized simultaneously with 754 and with probe 782, which maps distal to the deletion. Positive hybridization with probe 782 and absence of the band generated by probe 754 was seen in the lymphoblastoid cells and in the hybrids derived from them and was also observed in a DNA sample extracted from patient BB's liver tissue frozen at autopsy. These results allow the conclusion that patient BB did indeed have a deletion of part of band Xp21.

In the course of identification of probe 754, it was localized proximal to the DMD translocation breakpoint of the t(X;11)(p21;q13) translocation and distal

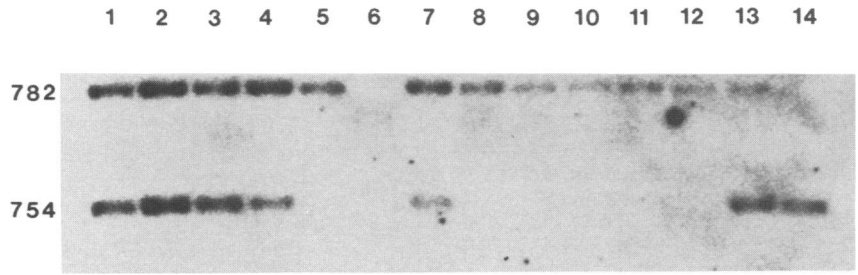

FIG. 5.—Nitrocellulose filter hybridized simultaneously with ^{32}P-labeled probes 782 and 754. In the *Hin*dIII-digested DNA samples (5–8 μg/lane), the hybridizing restriction fragments were equal in size to the human *Hin*dIII plasmid inserts: 4.3 kb for 782 and 2.2 kb for 754. *Lanes 1–5:* Human lymphoblastoid cell lines; with 46,X,t(X;9)(p21;p22) translocation GM 6007 (*lane 1*); with 48,XXXX complement GM 1415 (*lane 2*); with 46,XX (*lane 3*); with 46,X,del(X)(p21.3p21.1), female patient KC (*lane 4*); with 46,del(X)(p21.3p21.1) LCL 119, male patient BB (*lane 5*). Whereas the relative intensities of the bands generated by 782 and 754 are the same within samples in *lanes 1–3*, the 754 band is weaker than the 782 band in *lane 4*, consistent with the conclusion that the 754 sequence is missing from the del(X) chromosome in this heterozygous female while the 782 sequence is not. In *lane 5* (patient BB), no hybridization with 754 and a positive signal with probe 782 indicate that 754 maps within and 782 maps outside of patient BB's deletion. *Lane 6:* Chinese hamster V79/380-6, rodent parental cell line of the human × hamster hybrids in *lanes 7–14*. While no cross-hybridization was observed with probe 782, a single weakly hybridizing fragment was seen with 754 (not shown) that was larger than the human bands. *Lanes 7–10:* Hybrids of series XVIII with female deletion patient KC as human parent [13]; the hybrid in *lane 7* contains her del(X) and her normal X chromosome; the three independent hybrids in *lanes 8, 9,* and *10* contain the del(X) in the active or the inactive state and have lost the normal X. Hybrids in *lane 11* (clone 29-1F) and *lane 12* (clone 29-5A) are derived from LCL 119 and contain the del(X) of patient BB in addition to one or more autosomes. *Lanes 13* and *14:* hybrids of series 28 with sample in *lane 1* as human parent. Both hybrids had retained the der(X),t(X;9)(9pter→9p22::Xp21→Xqter) chromosome and had lost the reciprocal der(9) translocation product. The normal X was present in the hybrid in *lane 13* (positive with probe 782) and absent in *lane 14* (negative with 782).

to Xp11.3 [28]. To verify that probe 754 maps proximal to other DMD translocation breakpoints as well, it was tested against somatic cell hybrids (series 28) generated with cells from a female with DMD and a de novo X/autosome translocation t(X;9)(p21;p22) [14, 35]. As expected, probe 754 hybridized to clones with the der(X) chromosome isolated on a Chinese hamster background (fig. 5, lane 14). Thus, probe 754 clearly maps proximal to the *DMD* locus as defined by the X/autosome translocation breakpoints [35–37] (fig. 6). As *OTC* lies outside patient BB's deletion, the polymorphic DNA locus recognized by probe 754 must be closer to *DMD* than *OTC* is. It appears likely, although no formal proof exists, that the deletion in patient BB affects the same sequences that are altered or inactivated by the translocation events in DMD translocation females.

DISCUSSION

Evidence for a Deletion

We are reporting a male patient (BB) who manifested three different disorders and one rare red blood cell phenotype, all of which can be caused by mutations of X-linked genes: Duchenne muscular dystrophy (DMD), retinitis pigmentosa (RP), chronic granulomatous disease (CGD), and the McLeod

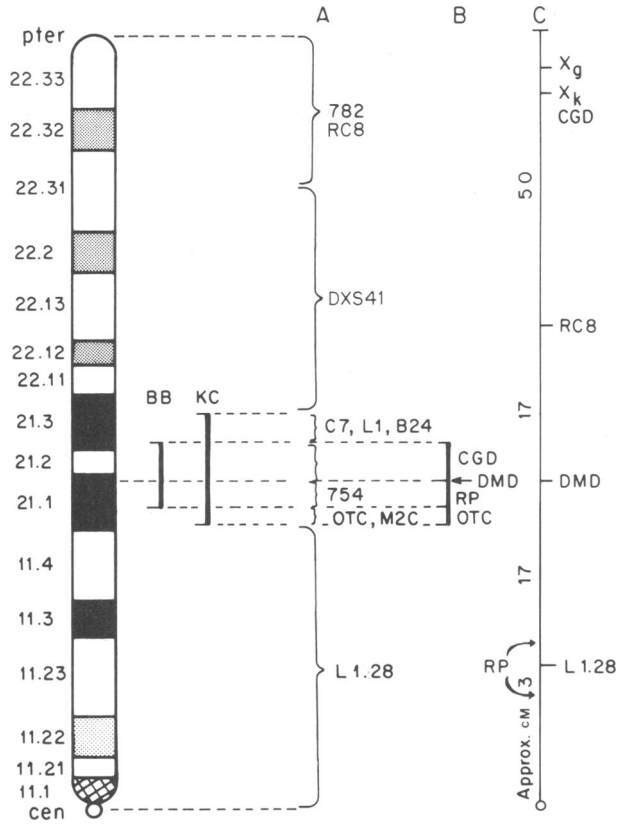

FIG. 6.—Standard (ISCN 1981) [33] ideogram of the human X chromosome short arm. *Brackets* indicate the deletions in patients KC and BB. *A*, Cytological localizations of DNA sequences as derived from data reported in this and in the accompanying paper [14]. *B*, Tentative localizations of loci for chronic granulomatous disease (CGD), Duchenne muscular dystrophy (DMD), and retinitis pigmentosa (RP) based on studies of our two deletion patients (this paper and [12–14]). *C*, Linkage map of Xp with approximate distances as reported in the literature, see text for references.

phenotype. Cytogenetic analysis suggested that he may have a small interstitial deletion of part of band Xp21 on the short arm of the X chromosome. Because he exhibited a mild degree of mental retardation, we did not expect him to be nullisomic for a cytologically detectable piece of the X chromosome, but we rather suspected to find a balanced insertion. Such an insertion involving two breakpoints within band Xp21 could have inactivated some or all of the involved loci, analogous to events postulated for the females with apparently balanced Xp21/autosome translocations who manifest symptoms of DMD [35–37]. However, molecular analyses were necessary to demonstrate that the patient, indeed, did have a deletion. A unique DNA sequence, identified by hybridization to probe 754, was absent from patient BB's lymphoblastoid cell line, while other sequences located also in band Xp21 on either side of 754 were still present; this constitutes evidence for an interstitial deletion. Probe 754

detects a *Pst*I restriction fragment length polymorphism with two alleles. In the course of linkage studies, the sequence has been detected in DNA from at least 200 normal individuals and DMD patients [28]. The linkage data suggest a genetic distance of about 3 cM (lod score 6.2) with 95% confidence limits of 1–12 cM ([28] and P. L. Pearson et al., unpublished results, 1984).

The extent of the deletion can be inferred from molecular hybridization studies summarized in figure 6 [12, 14]. A slightly larger deletion has been identified in a female patient (KC) that was associated with random X-inactivation [13]. This deletion, designated del(X)(Xpter-p21.3::p21.1-Xqter) as well, has removed most of band Xp21. As documented [14], it spans across the breakpoints in the t(X;9)(p21;p22) and the t(X;11)(p21;q13) translocations in DMD females. Since probes B24, L1, and C7 (distal) and M2C and OTC (proximal) are flanking patient BB's deletion on both sides, but map inside patient KC's deletion, the latter must include the former entirely. Further, since 754 maps proximal to *DMD* but inside patient BB's deletion, the deletion very likely extends proximally offwards of the *DMD* locus. It is therefore unlikely that the proximal breakpoint of patient BB's deletion is related to DMD sequences. Since no sequence has as yet been identified that maps within patient BB's deletion but distal to the DMD translocation breakpoint, we cannot be certain how far patient BB's distal breakpoint is from the DMD breakpoint.

When attempting to correlate the clinical findings with the cytogenetic and molecular results, one has to consider different possible explanations:

(1) The deletion has removed the DNA sequences at the *DMD, RP, CGD,* and *Xk* loci; lack of the genes produces the clinical phenotypes, and, consequently, all these loci map within the deletion.

(2) The deletion has altered the chromatin configuration in the central part of Xp21 that interferes with proper regulation and expression of these genes. The responsible DNA sequences may not have been physically removed and, thus, may not map within the deleted segment, but in the vicinity.

(3) The deletion of part of band Xp21 has removed genetic information that is required for the normal expression of the wild-type alleles at some or all of the loci. In this case, the disease loci could be situated outside of the deletion. For example, an X-linked recessive mutation has been reported that causes both muscle dystrophy and neutrophil dysfunction. The basic defect appears to involve ultrastructural abnormalities in mitochondria [38].

(4) One or more of the disorders present in patient BB may have been due to an inherited or de novo mutation independent of the deletion. This possibility could not be evaluated further since no family members were available for study.

Muscular Dystrophy

The diagnosis of *DMD* in this patient was based on clinical findings and progression of the disease, on serum CPK levels, muscle histology, and EMG. Independent evidence from several females with DMD and X/autosome translocations [35–37] and from family linkage studies with flanking RFLP markers

[26] supports the notion that the *DMD* locus resides within band Xp21. Thus, it is reasonable to assume that the deletion in patient BB has removed or inactivated the normal allele at the *DMD* locus. The gene for a milder form of X-linked muscular dystrophy (Becker type, BMD) has also been mapped to the X short arm, by linkage with *DXS7,* the locus identified by probe L1.28 [39]. Since the recombination distances between *DMD* and *DXS7* (17 cM with 95% confidence interval 7–33 cM) and between *BMD* and *DXS7* (19 cM with 95% confidence limits 10–31 cM) were similar, and based on data with other probes that *BMD* is distal to *DXS7,* the possibility has been raised that BMD and DMD could be allelic mutations at the same locus [39]. If that were the case, the milder BMD phenotype could be due to a partial defect associated with residual function of the gene. The progression of the muscular dystrophy in patient BB was of the most severe type that is seen in classical DMD. His clinical course would thus be consistent with deletion (or complete inactivation) of the gene. Screening of X-specific clone banks for sequences missing in this patient's DNA should be a useful approach for developing DNA markers near the DMD locus and for isolating the gene itself.

Retinitis Pigmentosa

The diagnosis of retinitis pigmentosa (RP) was based on bilateral progressive retinal dystrophy, peripheral field constriction, and severely abnormal rod and cone function. The RP phenotype may be inherited in autosomal dominant, autosomal recessive, or X-linked recessive fashion with the X-linked recessive type being present in 6%–20% of all cases [40, 41]. While the early onset and rapid progression in patient BB are consistent with the X-linked type [40–42], the lack of family information does not allow one to rule out autosomal inheritance. However, the possibility that his RP resulted directly from the Xp21 deletion has to be considered seriously in view of the recently reported linkage between X-linked RP (*XLRP*) and *DXS7* detected by probe L1.28. In five informative families, a maximal lod score of 7.89 was obtained at a distance of 3 cM with 95% confidence limits of 0–15 [43]. In a single large pedigree, Nussbaum et al. [44] obtained a greater recombination distance between *XLRP* and *DXS7* of 12.5 cM (95% confidence limits 3–35 cM). Assuming that our patient's RP was caused by deletion of this locus, the following gene order is suggested by our data: centromere-*DXS7-OTC-RP-DMD*. Both *DXS7* and *OTC* are outside and proximal to patient BB's deletion, and *DXS7* and *DMD* are 17 cM apart. *OTC* has to be distal to *DXS7* since the two are separated by the breakpoint in the female deletion patient KC [12, 14]. A high-frequency polymorphism is detected with *Msp*I at the *OTC* locus [45]. It is clear that linkage studies with both probes (L1.28 and OTC) need to be done in families in which *XLRP* or *DMD* are segregating in order to confirm the suggested gene order and to determine precisely the genetic distances.

Chronic Granulomatous Disease

Chronic granulomatous disease (CGD) is genetically heterogeneous with X-linked recessive and autosomal recessive forms. Several different associated

biochemical defects have been identified [46]. The heme containing cytochrome b_{-245} was absent in all of 19 men with X-linked recessive CGD but was present in all of eight individuals (one male, seven females) with presumably autosomal recessive CGD [47]. In view of the accumulating evidence that cytochrome b is involved in the oxidative system of neutrophils, it is possible that this cytoplasmic cytochrome b is the primary product of the defective gene and that cytochrome b deficiency represents the molecular lesion responsible for X-linked CGD [48]. Heterozygous females for the X-linked form of CGD have neutrophils, of which approximately 50% fail to reduce NBT [16], and their total neutrophil population has intermediate concentrations of cytochrome b_{-245}, demonstrating that this gene is inactivated [47]. Since patient BB's neutrophils lacked cytochrome b_{-245}, it is most likely that he had the X-linked recessive form of CGD. Because his mother and other family members were unavailable for testing, we were unable to confirm the genetic type present in patient BB. The hypothesis that a gene for X-linked CGD is located within patient BB's deletion is supported by our recent finding that the female patient KC with a deletion of most of Xp21 is heterozygous for CGD. In the endotoxin-coated NBT slide test [16], 33% of her neutrophils failed to reduce NBT [13], a finding consistent with random X-inactivation in her granulocytes and supporting localization of a *CGD* gene within band Xp21. The co-occurrence of CGD and DMD has been reported in a single male whose mother was a CGD carrier [49].

McLeod Phenotype

The McLeod phenotype, characterized by weakened or absent antigenicity in the Kell blood group system, has been identified in several boys with X-linked CGD [50, 51]. However, the majority of male patients with CGD do not have the McLeod phenotype [50]. Individuals with the McLeod phenotype often have mild hemolytic anemia and acanthocytosis, as was present in patient BB. Their red blood cells lack K_x antigen, the product of the X_k gene [50]. It is believed that K_x represents the precursor molecule of the Kell antigen [52]. A specific allele at the X_k locus has been postulated to be responsible for the association of CGD with the McLeod phenotype [50, 52].

On the Map of Xp

The X_k locus is closely linked to the *Xg* blood group locus [52, 53]. This is consistent with other family linkage data that also suggest close linkage of *CGD* (McLeod type not specified) and *Xg* with a maximal lod score of 3.85 at 0 cM (95% confidence interval 0–17 cM) [54]. However, *Xg* is located in the most distal band on Xp [10] and escapes X-inactivation in females [55], while *CGD* as well as X_k [50] are clearly inactivated. In a very recent report, males with inherited deletions of Xp22.32→pter did not suffer from CGD [56].

Furthermore, previous family studies have shown no linkage between *Xg* and *DMD* [57] or between *Xg* and *XLRP* [58]. The inferred distance between *Xg* and *DMD*, calculated from data regarding marker loci in between, is at least

55 cM [59], or possibly greater in the absence of measurable linkage between *Xg* and *DXS9* (RC8) [60].

How can we attempt to explain the huge discrepancies between the cytological and the linkage map data for the X chromosome short arm, summarized in figure 6? First, the linear distances and recombination distances on the X chromosome short arm would be disproportionate if recombination occurred predominantly in the middle of the arm, and, specifically, between the *CGD* and *DMD* loci. Second, the reported linkage studies may not have been with representative CGD or RP families but may have included biased samples, for example, families with unrecognized X-chromosome rearrangements. Third, there could be more than one locus on the X chromosome that when mutated give rise to similar phenotypes. Such may be the case for *XLRP* [44]. Fourth, since it seems a priori unlikely that patient BB's small deletion should contain three disease loci that are widely dispersed on the Xp linkage map, could there be a single defect, for example, involving a cell membrane component, that indirectly causes the different disease phenotypes? Cytochrome b, in order to carry out its function in the oxidative pathway of neutrophils, has to be translocated to the cellular membrane [48]. K_x, the product of X_k, is a membrane protein, the backbone of or precursor to the Kell red cell antigen [50]. Patients with DMD do not have the McLeod phenotype, but they have occasionally been reported to have abnormal red cell morphology [61]. Similarly, elevated serum CPK levels can be seen in individuals with McLeod red cell phenotype [62], and histologic changes of active myopathy have recently been documented in two such men who had no clinical evidence of muscular dystrophy [63]. There is independent evidence that the DMD mutation may involve a membrane defect [64].

All these instances of phenotypic overlap and co-occurrences of different disease manifestations suggest uncertainty as to the number and basic functions of the genes involved in these disorders. Molecular genetic markers that are precisely mapped cytologically will have to be applied in family linkage studies in order to dissect heterogeneous disease entities and to distinguish and map individual gene loci.

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