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ORIGINAL PAPER

A new candidate region for the positional cloning of the *XLP* gene

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X-linked lymphoproliferative disease (XLP) is an inherited immunodeficiency characterised by selective susceptibility to Epstein-Barr virus and frequent association with malignant lymphomas chiefly located in the ileocecal region, liver, kidney and CNS. Taking advantage of a large bacterial clone contig, we obtained a genomic sequence of 197620 bp encompassing a deletion (XLP-D) of 116 kb in an XLP family, whose breakpoints were identified. The study of potential exons from this region in 40 unrelated XLP patients did not reveal any mutation. To define the critical region for XLP and investigate the role of the XLP-D deletion, detailed haplotypes in a region of approximately 20 cM were reconstructed in a total of 87 individuals from 7 families with recurrence of XLP. Two recombination events in a North American family and a new microdeletion (XLP-G) in an Italian family indicate that the *XLP* gene maps in the interval between DXS1001 and DXS8057, approximately 800 kb centromeric to the previously reported familial microdeletion XLP-D.

Keywords: immunodeficiency; intestinal lymphomas; Epstein-Barr virus; familial microdeletions

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Introduction

X-linked lymphoproliferative disease (XLP, originally called Duncan disease; MIM 308240) is a rare, inherited

immunodeficiency characterised by selective susceptibility to Epstein-Barr Virus (EBV). Whilst more than 98% of individuals from the general population experience an EBV infection with little or no clinical manifestation, affected individuals, when exposed to EBV infection, develop fatal or chronic infectious mononucleosis, acquired agammaglobulinaemia (or hypogammaglobulinaemia), aplastic anaemia and malignant lymphoma.[1]

The *XLP* gene was initially mapped by linkage analysis in the Xq24–q25 region at 4% recombination from the DXS42 locus.[2] Further linkage studies established that the *XLP* gene is telomeric with respect to markers DXS42 and DXS37, at about 1–2% recombination from DXS42 (Z = 17.5) and at 3% (Z = 11.8) recombination from the DXS37 locus. A third marker, DXS12, was found to be closely linked to the disease locus and no recombination (Z = 7.5) was demonstrated.[3,4] A cytogenetically visible deletion in Xq25 was observed in an XLP family by Wyandt *et al*.[5] Subsequently three constitutional deletions were reported in unrelated XLP patients. In the first one the markers DXS6, DXS739 and DXS100 are missing; in the second one the markers DXS739 and DXS100 are deleted; whilst in the third one only the marker DXS739 is missing.[6,7]

Recently a novel constitutional deletion of no more than 120 kb in an XLP patient (XLP-D), nested within the three cytogenetically visible deletions, was reported by two of our groups.[8,9] A large scale sequence analysis of the bacterial clones from this region carried out in the present work did not lead to the identification of the *XLP* gene. In order to establish whether this critical region really represented a candidate interval for the *XLP* gene we performed linkage analysis on seven unrelated pedigrees. To this end highly polymorphic markers spanning the critical region and covering a genetic distance of approximately 20 cM were used. Haplotype reconstruction using these markers allowed us to localise the *XLP* gene in an interval between DXS8081 and DXS8057, about 800 kb proximal to the previously reported XLP-D deletion. In addition, the genotyping of an Italian family using DXS8057 led to the identification of a novel constitutional deletion (XLP-G) present in one affected member as well as in two carrier females. This genetic and physical mapping defines the location of the *XLP* gene in the interval between DXS1001 and DXS8057, a new candidate region for the positional cloning of the *XLP* gene.

Materials and Methods

Patients and Families

Patient XLP-D has been previously described.[8,9] Genomic DNA extracted from paraffinated blocks of different tissues of his maternal uncle, who died of fatal infectious mononucleosis, was used for the amplification of EST 74809, xSTS916.283N, DF83, xSTS46709N, DXS7365, DXS7364, 4670.29 (see results).

In each of the seven families genotyped in the present work (three italian, three North-American and one Swiss), at least two maternally related male members had features of XLP (life threatening infectious mononucleosis, malignant lymphoma and acquired hypogammaglobulinaemia).

The K001 family (Figure 1) is the original Duncan pedigree from David Purtilo's XLP Registry (Omaha, NE, USA). In this six-generation pedigree at least 19 males died of XLP.[1,10]

The patient from the GL01 family (Figure 2) was admitted to the Gaslini Institute (Genova) at the age of 6 years for fever, hepatosplenomegaly and persistent high levels of transaminase enzymes after an EBV infection. The liver and bone marrow biopsies suggested the presence of a lymphoproliferative disease. The proband's mother had a first cousin who died of a lymphosarcoma. A suppressive steroid therapy supported by infusion of immunoglobulins every week was started soon after the diagnosis and followed up at monthly intervals by infusions of immunoglobulins during the past 4 years. At present the clinical condition of the patient is stable.

Amplification and Sequencing of the Junction Fragment of the XLP-D Deletion

In order to amplify the junction fragment, the following primers were used as standard PCR: 6846U, CAGCACACTGATGGGTCTTGACT; 15700L, GGACCAGTTTGATACCTTGTGGAT. Sequencing of the junction fragment was carried out using the purified PCR product and the same primers (see Results).

Exon Trapping and Sequencing of the XLP-D region

Each of the cosmids[8] spanning the critical region were digested with *Bam* HI-*Bgl* II, cloned in the pSPL3 vector. After Cos cells transfection, a mini library of the RT-PCR products was constructed for each cosmid as described by Church *et al*.[11]

The cosmids covering the XLP-D deletion were subcloned on the basis of a detailed restriction map[8] (and unpublished data, Yin L, 1998) and sequenced using Dye Primer cycle sequencing chemistry with an Applied Biosystems 377A automated DNA sequencer. Plasmid and cosmid end sequencing was performed using Dye Terminator sequencing chemistry with Taq polymerase together with T3/T7/Sp6 primers.[12] Primer walking using Dye Terminator sequencing chemistry was used to complete the sequence of each subclone in addition to confirming sequence overlaps of adjacent subclones. All sequences thus generated were assembled using Applied Biosystems programs FACTURA version 1.2.0 and AUTOASSEMBLER version 1.3.0.

The prediction of exons was performed on unmasked sequence using GRAIL 1, 1A, 1.3, 2[13] and the Gene-Finder options FEXH, HEXON, FGENEH[14] through URL http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf. Only loci predicted at least three times by different programs were

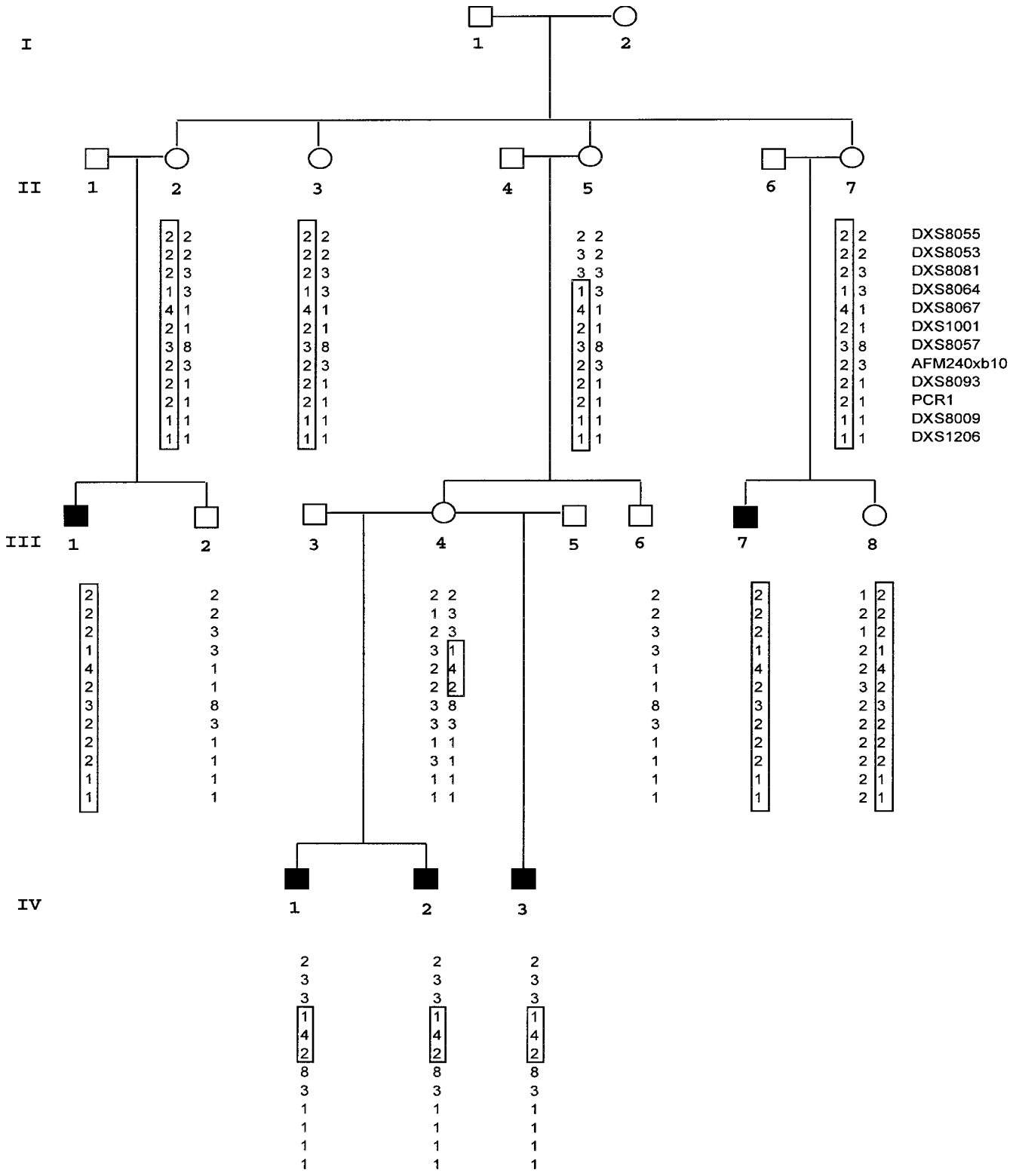

Figure 1 *Haplotype reconstruction in the K001 family. Only markers encompassed by DXS8055 and DXS1206 are shown from centromere (top) to telomere (bottom). The at-risk haplotypes are boxed. The five affected individuals (filled symbols) share alleles only at the DXS8064, DXS8067 and DXS1001 loci. The recombination events demonstrated in this pedigree localise the* XLP *gene between markers DXS8081 and DXS8057, approximately 800 kb centromeric to the XLP-D deletion (see text).*

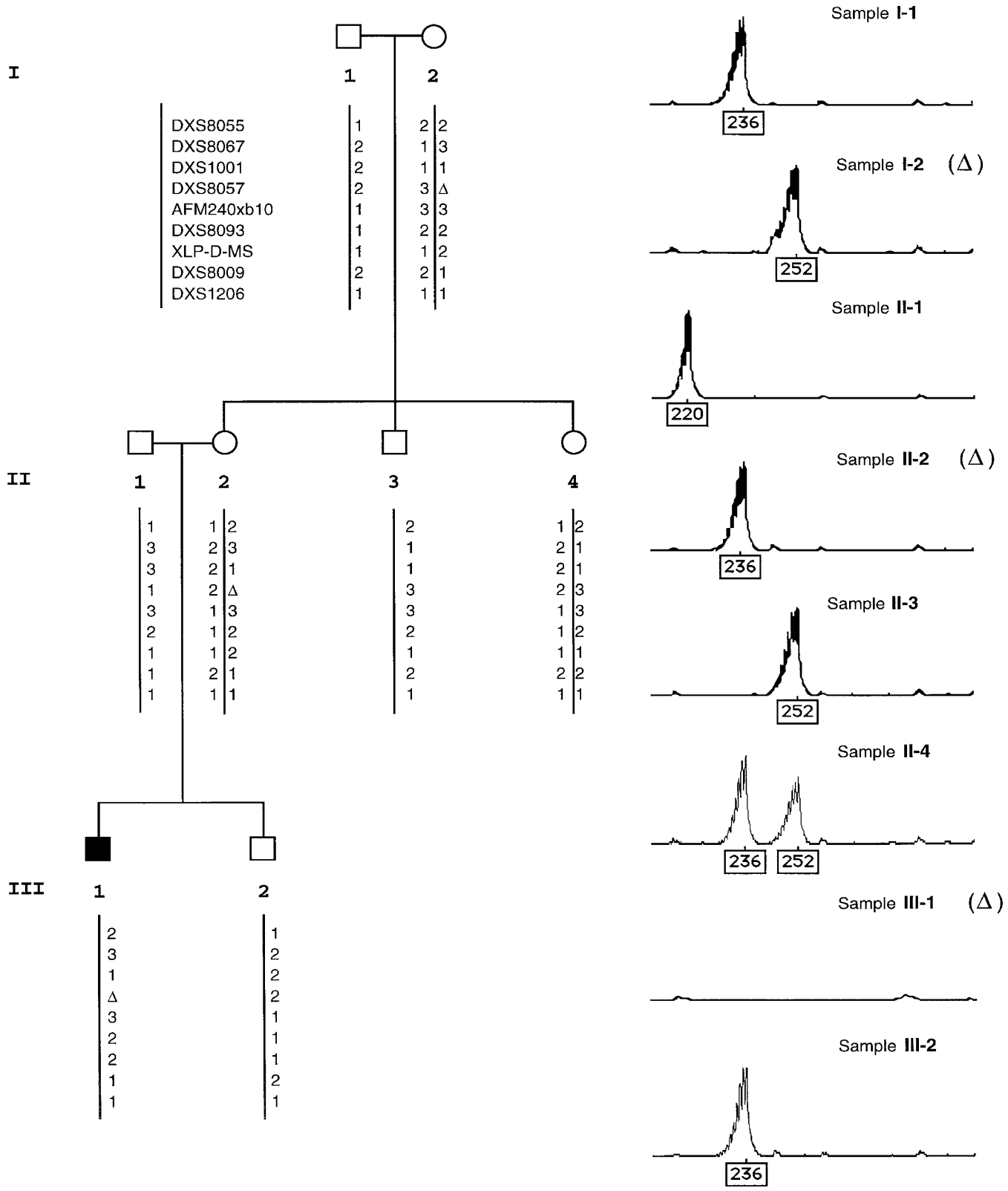

Figure 2 *Haplotype reconstruction in the GL01 family. Only markers encompassed by DXS8055 and DXS1206 are shown. A null allele at the DXS8057 locus (indicated by △) was demonstrated in III-1, II-2 and was probably also present in I-1. On the right part of this figure the 3 alleles of DXS8057 are reported in bp: 220 bp corresponds to allele 1, 236 bp to allele 2 and 252 bp to allele 3.*

considered to represent best candidate exons. The entire sequence was also analysed using BLASTN[15] versus dbEST, dbESTNEW, EMBL, EMNEW, VERTRNA (vertebrate RNA in EMBL, non-EST) and CpG island databases, and BLASTX versus SWISSPROT, SPTREMBL and WORM-PEP databases. Data from all the sequence analysis were collected and graphically displayed using an ACEDB database.[16]

Analysis of Mutations and RT-PCR on the four best Candidate Exons

The primers used to amplify the four best candidate exons (A, B, C and D, see results) were designed from the sequence which can be accessed through GenBank (AF001905) at the following nucleotide positions: AF9622–9646, AR 9833–9811; BF 25867–25889, BR 26100–26080; CF 143118–143142, CR 143308–143286; DF 169672–169696, DR 169838–169814. PCR products were analysed on the ABI377 automatic sequencer. Total RNA was extracted from cell pellet using TRIzol®Reagent from GIBCOBRL following the protocol recommended by the manufacturer. Approximately 1 μg of total RNA was reverse transcripted using the RNA PCR Core Kit from Perkin Elmer. PCR amplification was carried out using the same kit with the addition of Taq Start Antibody (CLONTECH), mixed 1:1 with the Taq polymerase 5 min prior to the reaction. The primer sequences (located inside the predicted exons) for potential exon A, B, C and D corresponded to the following nucleotide positions: AF 9702–9725, AR 9769–9746; BF 25965–25988, BR 26032–26009; CF 143174–143195, CR 143233–143212; DF 169730–169755, DR 169801–169776.

Haplotype Reconstruction in Seven Families

The following microsatellite markers were used to genotype the seven XLP pedigrees: cen – DXS8088 – DXS1220 – DXS8055 – DX8053 – DXS8081 – DXS8064– DXS8067 – DXS1001 – DXS8057 – AFM240xb10 – DXS8093 – XLP-D-MS – DXS8009 –DXS1206 – DXS8078 – DXS1046 – DXS1047 – DXS8041 – tel. The order as well as physical distances for markers from AFM240xb10 to DXS8078 have been reported in the 4.5 Mb physical contig developed by Porta *et al*.[17] The physical order of markers from DXS8088 to DXS8057 and from DXS8078 to DXS8041 was determined on the basis of the PAC contigs available at the Sanger Centre (http://www.sanger.ac.uk/HGP/ChrX/). Finally, the physical distance between the marker DXS8057 and AFM240xb10 was estimated to be approximately 500 kb on the basis of the Sanger contig (Coffey A *et al*, 1998, unpublished data).

All PCR reactions were performed under standard conditions. Primers used to amplify the marker AFM240xb10 were reported in the GDB database. XLP-D-MS is a microsatellite marker identified by us from the XLP-D deletion (bp 36843–36882), whose primer sequences and the PCR condition were reported in the GDB database (accession number 6674083). Primers and annealing temperatures used to amplify the remaining microsatellites were reported in the Généthon genetic map,[18] except for the following markers, for which primers have been redesigned based on the sequences of these loci reported in the GDB database: DXS8064, forward GCCTGACCAACATGGTGAAC, reverse GGCTGCCAACTCTTTTCTCC; DXS8093, forward GGCTATTTGCACACCCGGAG, reverse GAGGTGCC-GAGAGCAAGAG; DXS8057, forward GCTCTGTA-GAAGGGGTAATATGC, reverse CAGCCTCT-GAAAGTGTTGGG. Genomic DNAs were typed using primers labelled with TET fluorescent amidite and alleles were visualised by an automated sequencer ABI mod 373A and using the Genescan software.

Physical Mapping and FISH

The PAC clone dJ1104I16 was identified by PCR screening of the PAC library RPCI-5 using primers at the DXS8057 locus. PAC clones dJ231B20, dJ1052M9, dA101O7, dA173I14, dJ221H19, dJ369O24, dJ618F1, dJ693G4, dJ876G16 and dJ1104I16 were provided by the Sanger Centre. In addition, BAC clones 258H9, 99N24, 63G16, 281I22 were selected by Dr. J. Sumegi (1998, unpublished data).

FISH experiments were performed with PAC clones dJ231B20, dJ1052M9, dA101O7, dA173I14, dJ221H19, dJ369O24, dJ618F1, dJ693G4, dJ876G16 and dJ1104I16 on metaphase chromosomes obtained from lymphoblastoid cell line of the GL01 patient, according to a protocol described by Pinkel *et al*[19] with minor modifications.

The following STSs, 281i22-T7, 63g16-Sp6, 258h9-T7, 281i22-Sp6, 63g16-T7, 99n24-T7 and 258h9-SP6, were obtained through end sequencing of BAC clones 281I22, 63G16, 99N24, and 258H9 (Sumegi J *et al*, 1998, unpublished data). On the other hand, STSs 1052m9-T7 and 231b20-T7 were constructed with the end sequences of the PAC clones from the Sanger Centre (dJ1052M9 and dJ231B20). All primer sequences for these STS are available upon request.

Results

Molecular Characterisation of the XLP-D Deletion

The total length of the sequencing contig encompassing the XLP-D deletion and consisting of 197620 bp can be accessed (AF001905). The sequence of the junction fragment was obtained from a purified 0.4 Kb PCR product (see Materials and Methods). A fragment of 116924 bp was missing in patient XLP-D between bases 31089 and 148013 of the wild type sequence. In addition 26 bp between nucleotides 31033 and 31058 were also missing in patient XLP-D, possibly as result of the same event which led to the deletion of 116924 bp. Since this 26 bp deletion is not present in 60 X chromosomes from 40 normal control individuals (20 males and 20 females), it is unlikely that it represents a polymorphism.

Genomic DNA extracted from paraffin embedded tissues of the maternal uncle of patient XLP-D who died of fatal infectious mononucleosis was found deleted for XLP-D-MS (bp 36843–36882), xSTS916.283N (bp 50458–50618), DF83 (bp 63537–63889), xSTS46709N (bp 109236–109706), DXS7365 (bp 111492–111640), which are deleted in XLP-D, whilst the markers EST 74809 (bp 30111–30460), DXS7364 (bp 156847–156948) and

4670.29 (bp 174094–174176) are not deleted in either the XLP-D patient or his uncle.

The only significant, potentially coding BLASTN homology found was to HSXQD,[20] with 100% identity over more than 1 kb between 63518–64656 in XLP-D (78–1216 in HSXQD). However, this sequence was masked by RepeatMasker2 as L1 element. No significant homologies were found as a result of BLASTX searches. The potential exon analysis of the 197620 bp sequence carried out as described in Materials and Methods led to the identification of four candidates: A (9702–9769 bp), B (25963–26032 bp) and D (169806–169730 bp) were identified by three independent sequence analysis methods, and potential exon C (143174–143236 bp) was identified twice by these methods and once by exon trapping. Sequencing in 40 XLP patients for these four best candidate exons did not detect any apparent mutation. RT-PCR assay did not identify any significant expression in monocytes, lymphoblastoid cell lines, the neuroblastoma cell line SK-NBE-5Y and the teratocarcinoma cell line BA129 (data not shown). The screening of human spleen cDNA library with potential exon C as probe did not yield any positive clone.

Haplotype Analysis in the 20 cM Region of Xq25

The seven available families were typed using the markers reported under Materials and Methods which cover a region of approximately 20 cM. The results from haplotype reconstruction for five of the seven families analysed are not shown, since no recombination nor microdeletion events were apparent. The seven families show different at-risk haplotypes. Figure 1 reports the haplotype reconstruction in the K001 family for the regions encompassed by DXS8055 and DXS1206. Since two recombination events occurring through two subsequent generations were found, this pedigree was typed with four additional markers, DXS8088, DXS1220, DXS8053 and DXS8064, in order to define the recombination breakpoints. In the second generation of the K001 pedigree, three out of four sisters II-2, II-3, and II-7 have the same at-risk haplotype observed in the affected male III-1. The fourth, II-5, shows a first recombination event between markers DXS8081 and DXS8064. In the third generation, the affected males III-1 and III-7 as well as the carrier female III-8 share almost all the at-risk haplotype. A further recombination in the at-risk haplotype was observed between markers DXS1001 and DXS8057 in the III-4 female. Her three sons (IV-1, IV-2, and IV-3) share therefore with these second cousins (III-1 and III-7) alleles only at the DXS8064, DXS8067 and DXS1001 loci. These observations indicate that the *XLP* gene in this pedigree must be between markers DXS8081 and DXS8057.

Characterisation of a new Microdeletion in an XLP Patient

Figure 2 shows the results of the genotyping on the GL01 family. No recombination event was found between the disease gene and any of the markers used. Patient III-1 carried a null allele at the DXS8057 locus in the grand-maternal haplotype, whilst his unaffected brother III-2 inherited the intact grand-paternal haplotype. The same genomic alteration was shown also in their mother II-2, who inherited only the paternal allele 2, suggesting a genomic deletion of DXS8057. DNA from 40 unrelated XLP patients were amplified using primers at the DXS8057 locus. No additional microdeletion was demonstrated. In addition, genomic DNA from 24 normal males was tested for the same marker. No deletion was found. The absence of deletion in DXS8057 in a total of 64 males indicate that the null allele of this marker is not a deletion polymorphism.

The XLP-G deletion was confirmed and characterised by FISH as well as by STS analysis (Figure 3). PAC clones dJ1104I16, dJ876G16, dJ693G4, dJ231B20, positive for DXS8057, detected a clear hybridisation signal in lymphoblastoid cell lines from normal controls, but not in patient III-1. The dJ618F1 clone gave a weak hybridisation signal only in 28% of the metaphases analysed, suggesting that the telomeric breakpoint of the XLP-G deletion is contained in this PAC (Figure 3). Furthermore, PAC clones dJ36O24, dJ221H19 and dA173I14, telomeric to and partially overlapping with dJ618F1, were all present in the patient III-1. On the other hand, the centromeric clones dA101O7 and dJ1052M9 were absent in the XLP-G deletion by FISH analysis suggesting that the centromeric boundary of the deletion locates centromerically to these two PAC clones (Figure 3).

These data were further confirmed by STS analysis. The most centromeric 28li22-T7 STS, contained in BAC clone 281I22, is present in patient III-1, whilst STS 1052m9-T7, telomeric to the previous one and contained in clones 281I22, dJ1052M9 and dA101O7, is deleted in patient III-1. The telomeric breakpoint of the deletion is defined by STS 99n24-T7 and marker DXS7144. In particular, STS 99n24-T7 contained in clones 258H9, 99N24, dA173I14, dJ221H19, dJ369O24, dJ618F1, dJ693G4 and dJ876G16 is deleted in patient

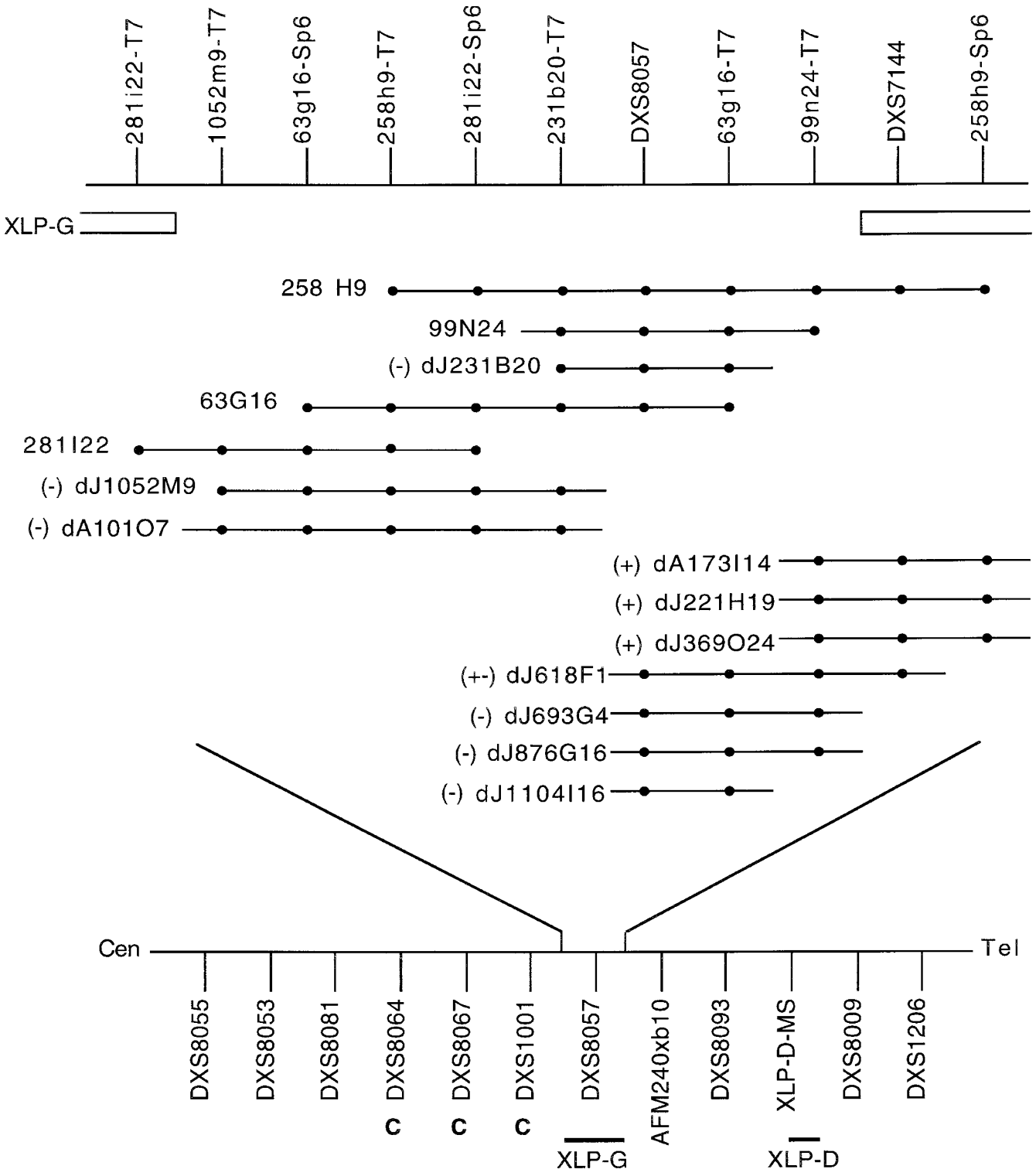

Figure 3 *Physical and genetic mapping of the new candidate XLP deletion. The results of FISH analysis are reported on the left of the PAC clones used as probes to analyse metaphases from a lymphoblastoid cell line of patient GL01. +: clear hybridisation signal, +–: weak hybridisation signal, indicating partial retention of the corresponding genomic region, –: no hybridisation. The centromeric and telomeric breakpoints of the XLP-G deletion indicated in this figure were defined by amplification of the STSs reported at the top (see results). At the bottom of the figure, the locations of the XLP-G and XLP-D deletions are shown, and the markers concordant in affected individuals of pedigree K001 are marked C.*

III-1, whilst marker DXS7144 contained in clones 258H9, dA173I14, dJ221H19, dJ369O24 and dJ618F1 is present in III-1. In conclusion, the two breakpoints of the XLP-G deletion fall in the centromeric 281I22 and the telomeric dJ618F1 clones, respectively. On the basis of the sizes of the BAC and PAC clones contained in the contig of Figure 3, the XLP-G deletion is estimated to span approximately 250–300 kb of genomic DNA.

Discussion

After defining the breakpoints of the XLP-D deletion, the entire DNA segment absent in this patient (116924 bp) and the two flanking sequences (31089 bp centromerically and 49606 bp telomerically) were analysed and four potential exons were identified on the basis of their prediction by at least three different prediction methods (see Results). None of them matched the existing ESTs, nor had any significant homology with known proteins. No apparent point mutation was found in genomic DNA from 40 XLP patients, nor was any significant expression detected for the four putative exons in four different cell lines: monocyte, lymphoblastoid, neuroblastoma, SK-NBE-5Y and teratocarcinoma BA129. The possibility of a sporadic association between the XLP clinical phenotype and the deletion occurring in patient XLP-D was therefore taken into account, in spite of the familial recurrence of the XLP-D deletion (see Results). As alternative hypotheses, we considered that this deletion (a) might contain only a very small portion of the *XLP* gene; (b) might be located outside the *XLP* gene, influencing its expression by long-range positional effect.[21]

Following the latter hypothesis we focused our efforts on further genetic mapping of the XLP region. We therefore genotyped seven pedigrees for some of the polymorphic markers available over a wide interval in the Xq25 region.

The recombinations found in kindred K001 define a new location of the XLP gene between centromeric marker DXS8081 and the telomeric marker DXS8057. On the other hand, the XLP-G deletion in family GL01 which involves only the DXS8057 but not the proximal marker DXS1001 nor the distal marker XLP-D-MS suggests that the *XLP* gene maps distal to DXS1001 and centromeric to XLP-D deletion. The genetic data obtained from K001 and GL01 pedigrees are thus concordant, pointing to a second candidate region for the *XLP* gene delimited by DXS1001 and DXS8057 (Figure 3), which spans at least 2 Mb. The physical distance between the telomeric boundary of this second candidate region and the centromeric breakpoint of the XLP-D deletion is approximately 800 kb.

The discrepancy arising from the observation of two candidate regions may be explained by the existence of a second non-apparent mutation in one of the two pedigrees showing a microdeletion (XLP-D and XLP-G) or by the presence of genetic heterogeneity (two closely linked genes for XLP on the X chromosome). While the latter hypothesis is based on several examples of X-linked genes, the former cannot be excluded until a candidate *XLP* gene is cloned and point mutations can be identified in all XLP patients.

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