

High-resolution mapping of the X-linked lymphoproliferative syndrome region by FISH on combed DNA

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Abstract. X-linked lymphoproliferative syndrome is an inherited immunodeficiency for which the responsible gene is currently unknown. Several megabase-sized deleted regions mapping to Xq25 have been identified in XLP patients, and more recently a 130-kb deletion has been reported (Lamartine et al., 1996; Lanyi et al., 1996). To establish a physical map of this deleted region and to identify the XLP gene, two cosmid contigs were established (Lamartine et al., 1996). However, the physical map of this region is still uncompleted and controversial and three points remain unsolved: (1) the centromeric-telomeric orientation of the whole region, (2) the relative orien-

tation of the two contigs, and (3) the size of the gap between the two contigs. To provide a definitive answer to these questions, high-resolution mapping by fluorescence in situ hybridization on combed DNA and molecular approaches were combined to establish the physical map of the XLP region over 600 kb. Our results identified a gap of 150 kb between the two contigs, established the relative orientation of one contig to the other, and determine the centromeric-telomeric orientation of the whole region. Our results show that the order of the marker over this region is: cen...1D10T7-DF83-DXS982...tel.

X-linked lymphoproliferative syndrome (XLP; MIM 308240), originally called Duncan disease (Purtilo et al., 1995) is an inherited immunodeficiency characterized by a selective susceptibility to Epstein-Barr virus (EBV) and for which the responsible gene is currently unknown. EBV infection of af-

ected boys results in severe and often fatal infectious mononucleosis, with survivors developing hypo- or agammaglobulinemia or lymphoma. EBV is a causative agent of infectious mononucleosis and has been directly implicated in the development of Burkitt's lymphoma and nasopharyngeal carcinoma (Rickinson and Kieff, 1996). EBV is also strongly associated with lymphoproliferative syndromes in individuals with genetic or acquired immunodeficiency, Hodgkin's lymphoma, lymphoepithelioma carcinomas of the stomach, gastric adenocarcinomas, and other malignancies (Rickinson and Kieff, 1996).

Although the XLP syndrome is rare in the general population, isolation of the XLP gene should provide insights into the normal control of EBV-induced lymphoproliferation and other EBV associated diseases. Early genetic linkage analysis assigned the XLP gene to the Xq24→q26 region between DXS37 (centromeric border) and DXS100 (telomeric border) (Skare et al., 1989). Overlapping deleted regions varying in size from 2 to 6 Mb (see XLP-739, Fig. 1A) and including the markers DXS6,

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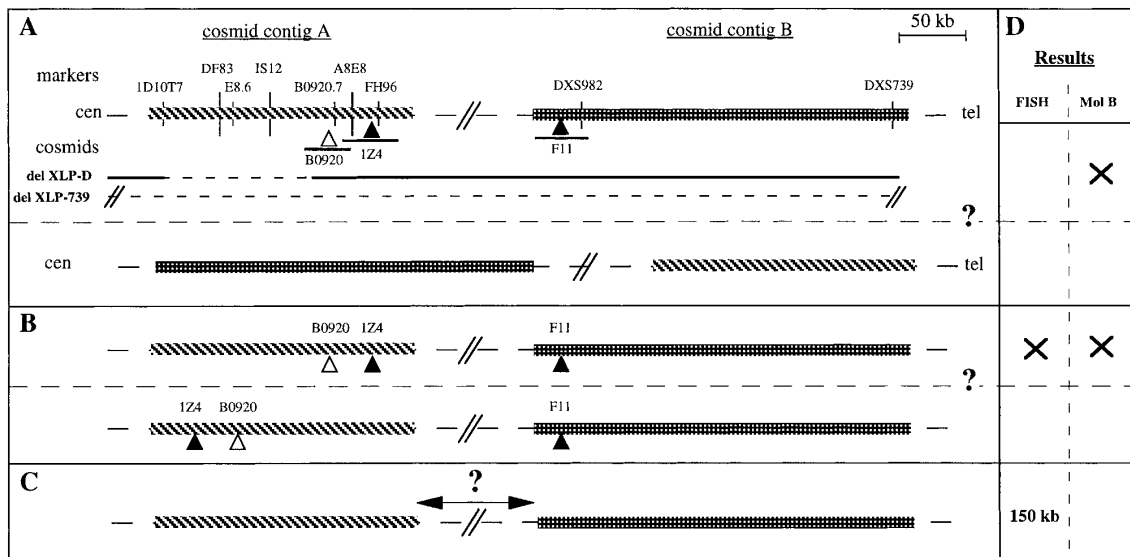


Fig. 1. (A, upper section) Schematic representation of the XLP region physical map encompassing the XLP-D deletion in Xq25. Cosmid contigs A (192 kb) and B (280 kb) constructed from the YAC 916D1 (Lamartine et al., 1996) are represented to scale with the position of the main markers they contained. The exact position of three cosmid probes is noted, as well as the deleted region of the XLP-D deletion (del XLP-D, dashed line = 130 kb) (Lamartine et al., 1996) and the XLP-739 deletion (del XLP-739, dashed line = 2 Mb) (Sylla et al., 1993). Three points remained unsolved concerning this map: (A) The centromeric-telomeric orientation of the whole region, (B) the relative orientation of the cosmid contigs A and B in relation to each other, and (C) the size of the gap between the two contigs. (D) Summary of the results we obtained by either FISH and molecular combing or molecular biology (Mol B).

DXS739, and DXS100 have been identified in XLP patients (Skare et al., 1993; Sylla et al., 1993). Recently, we have reported a new constitutional deletion (XLP-D, Fig. 1A) of approximately 130 kb in size affecting an XLP patient (Lamartine et al., 1996). The same deletion was also described by Lanyi et al. (1996). This interstitial constitutional deletion overlaps the previously reported deletions. To establish a physical map of the deleted region and to identify the XLP gene, a cosmid minilibrary was constructed from a single mega-YAC (YAC 916D1) encompassing this small deletion and used to establish two cosmid contigs, A and B (Lamartine et al., 1996, Fig. 1A). The contig of 192 kb (contig A, Fig. 1A) spans the entire small XLP deletion of 130 kb, whereas the second contig of 280 kb (contig B, Fig. 1A) maps to a region that is not deleted in this patient.

Since no potential exons have been found by analyzing the contig A sequence (Yin et al., manuscript submitted for publication), we think that a part of the gene responsible for XLP disease or its regulatory elements might be confined outside the region covered by this contig. Therefore, we decided to define more precisely the physical map of a larger genomic region that is deleted in other XLP patients (Lamartine et al., 1996), such as the 2-Mb deletion XLP-739 (Fig. 1A, Sylla et al., 1993), by using an additional contig (contig B, Fig. 1A). Previous reports positioned DXS982 centromeric to DXS739 and DF83 centromeric to DXS982 (Wu et al., 1993; Lamartine et al., 1996). However, more recently Lanyi et al. (1996) proposed the reverse orientation, positioning DXS982 centromeric to DF83. The orientation of the ends of the contig A itself relative to the

contig B, as well as the physical distance between the two contigs, has not been established. Therefore, three points remained unsolved concerning the XLP region: (1) the centromeric-telomeric orientation of the whole region (Fig. 1A), (2) the relative orientation of contig A to contig B (Fig. 1B), and (3) the size of the gap between the two contigs (Fig. 1C).

Since the XLP gene could either be confined to the 192-kb contig covering the small deletion or span over several hundred kilobases outside this interval, it was important to determine the precise physical map of the whole region. To resolve these issues, we have adopted a recently developed strategy based on high-resolution mapping by fluorescence in situ hybridization (FISH) called molecular combing (Bensimon et al., 1994). Briefly, this technique involves the mechanical stretching of naked DNA molecules, bound by their unmodified extremities to chemically modified glass surfaces. The resulting combed fibers have been found to be uniformly stretched in one direction only, thereby providing distances in kilobases to be calculated from distances measured in micrometers under the microscope (2 kb/ μm). Molecular combing combined with FISH has been shown to be a relatively fast and efficient method for genome mapping and the analysis of genomic rearrangements on cloned DNA (Weier et al., 1995; Monier et al., manuscript submitted for publication) as well as on genomic DNA (Michalet et al., 1997) and is the only DNA stretching method available that can provide a reproducible degree of stretching (reviewed by Monier et al., 1997).

Using FISH on combed DNA, the orientation of contig A with respect to contig B, as well as the length of the gap between

them, was determined. The relative contig orientation was independently confirmed by a molecular approach, using small YACs mapping to this region. This approach was also used to determine the centromeric-telomeric orientation of the whole region. The combination of these in situ and molecular approaches allowed the construction of a complete physical map of the XLP region over a distance of 600 kb.

Materials and methods

DNA clones

YAC 916D1 contains a human genomic fragment of 1.5 Mb and was isolated by PCR screening of pooled DNA of the CEPH mega-YAC library using oligomers from X2-183 (DXS982). YAC yWXD592 contains a human genomic fragment of 0.8 Mb. This clone was localized within the large XLP deletions previously described (Wyandt et al., 1989). YACs yWXD318, 195, and 642 contain human genomic fragments of 0.3, 0.2, and 0.2 Mb, respectively, corresponding to the human Xq25 region.

Cosmid clones 1Z4, B0920, and F11 contain genomic fragments of 41.5, 35, and 38 kb, respectively, in size. Cosmids B0920 and 1Z4 were isolated from a YAC 916D1 minilibrary (Lamartine et al., 1996), and their inserts share an overlapping region of 8.2 kb (Yin et al., manuscript submitted for publication). Both cosmid clones B0920 and 1Z4 are included in a cosmid contig of 192 kb (contig A, Fig. 1A) spanning the XLP-D deletion of 130 kb, whereas cosmid clone F11 is included in another contig of 280 kb (contig B, Fig. 1A) mapping to the Xq25 region (Lamartine et al., 1996).

Southern-blot analysis of YAC clones

YAC clones yWXD318, 195, and 642 were obtained from the Center for Genetics in Medicine (St. Louis, MO). Yeast cell culture and DNA isolation were performed as described by Green and Olson (1990). DNA separation and blotting were performed according to standard protocols (Sambrook et al., 1989). The filters were hybridized in Church solution (0.5 M phosphate buffer [pH 7.2], 7% SDS, and 1% bovine serum albumin) at 65 °C and then washed in SSC/SDS buffer as previously described (Sambrook et al., 1989). DNA probes were ³²P-labeled by random priming (Amersham) and used at a specific activity of 1 × 10⁶ cpm/ml of hybridization solution. Markers used in this analysis has been previously described (Lamartine et al., 1996).

Metaphase chromosome preparations

Metaphase chromosome spreads from stimulated human male lymphocytes were obtained by standard techniques, viz., Colcemid treatment, hypotonic shock, and methanol-acetic acid fixation. Prior to hybridization, preparations were denatured for 2 min in an 80 °C oven.

Preparation of target DNA and molecular combing

Yeast DNA containing YAC 916D1 and YAC yWXD592 were prepared from 50-ml yeast cultures (Evans, 1996). Yeast cells were embedded in 1% low-melting agarose blocks (1 µg DNA/100 µl of block) using a standard pulse-field gel electrophoresis (PFGE) block preparation protocol (Davies, 1988). Before the YAC clones were used for combing, their sizes were checked by PFGE.

YAC DNA was then combed by dynamic molecular combing (DMC) as described in Michalet et al. (1997). Briefly, yeast DNA embedded in gel was stained with YOYO-1 (Molecular Probes) at a ratio of five base pairs to one YOYO-1 dye molecule. YAC molecules were then extracted in solution by digesting the gel fragment with β-agarase I (Biolabs) and subsequently diluted with 50 mM MES at pH 5.5 (1 µg DNA/4 ml MES). Finally, the combing process was performed, first by anchoring extremities of DNA molecules in solution to glass cover slips that had been pretreated with vinyl silane (Allemand et al., 1998) and then by pulling the cover slips out of the DNA solution at a constant speed.

The quality of each treated cover slip batch was checked carefully with a control DNA of known size (lambda phage) to ensure that the degree of stretching of the combed DNA molecules remained at a value of 2 kb/µm. No further internal control was then needed to check the degree of DNA stretching.

Probe labeling and hybridization

Probes were labeled by nick translation or by random priming with biotin or digoxigenin. Biotinylated YAC (400 ng) and cosmid clones (100 ng) were hybridized separately on human male metaphase chromosome spreads with a 50-fold excess of human Cot-1 DNA (GIBCO BRL). Probes were detected with avidin-FITC (Vector Laboratories) (Lichter et al., 1988), and the preparations were counterstained with DAPI (200 ng/ml) in fluorescence antifade buffer containing 90% glycerol, 0.1 M Tris-HCl (pH 8.0), and 2.3% DABCO (1,4-diazobicyclo-2,2,2-octane).

Three two-color hybridization sets were performed on combed YAC DNA: 1Z4-biotin and B0920-digoxigenin probes (100 ng each) on combed YAC 916D1 (hybridization set No. 1), 1Z4-biotin and F11-digoxigenin probes (100 ng each) on combed YAC yWXD592 (hybridization set No. 2), and B0920-biotin and F11-digoxigenin probes (100 ng each) on combed YAC yWXD592 (hybridization set No. 3). A refined protocol for FISH on combed DNA has been described by Monier et al. (1998). Briefly, slides were submitted to a blocking step before hybridization with 4 × SSC, 3% BSA for 30 min at 37 °C, washed in 2 × SSC, and dehydrated in ethanol. Target DNA was denatured in a solution of 70% formamide, 2 × SSC (pH 7) at 73 °C for 2 min, whereas probes were denatured in 50% formamide, 2 × SSC at 75 °C for 5 min. Probes were hybridized overnight with a 50-fold excess of competitor DNA (human Cot-1 DNA). Following hybridization, the preparations were washed three times (5 min each) in 50% formamide, 2 × SSC (pH 7) at room temperature and then three times (5 min each) in 2 × SSC alone, again at room temperature. A blocking step with 4 × SSC, 3% BSA, and 0.1% Tween20 was then carried out for 30 min at room temperature. Detection was performed by using FITC and TRITC with a five-layer system, as described by Michalet et al. (1997). Preparations were mounted in antifade buffer without counterstaining.

Microscopy and digital imaging

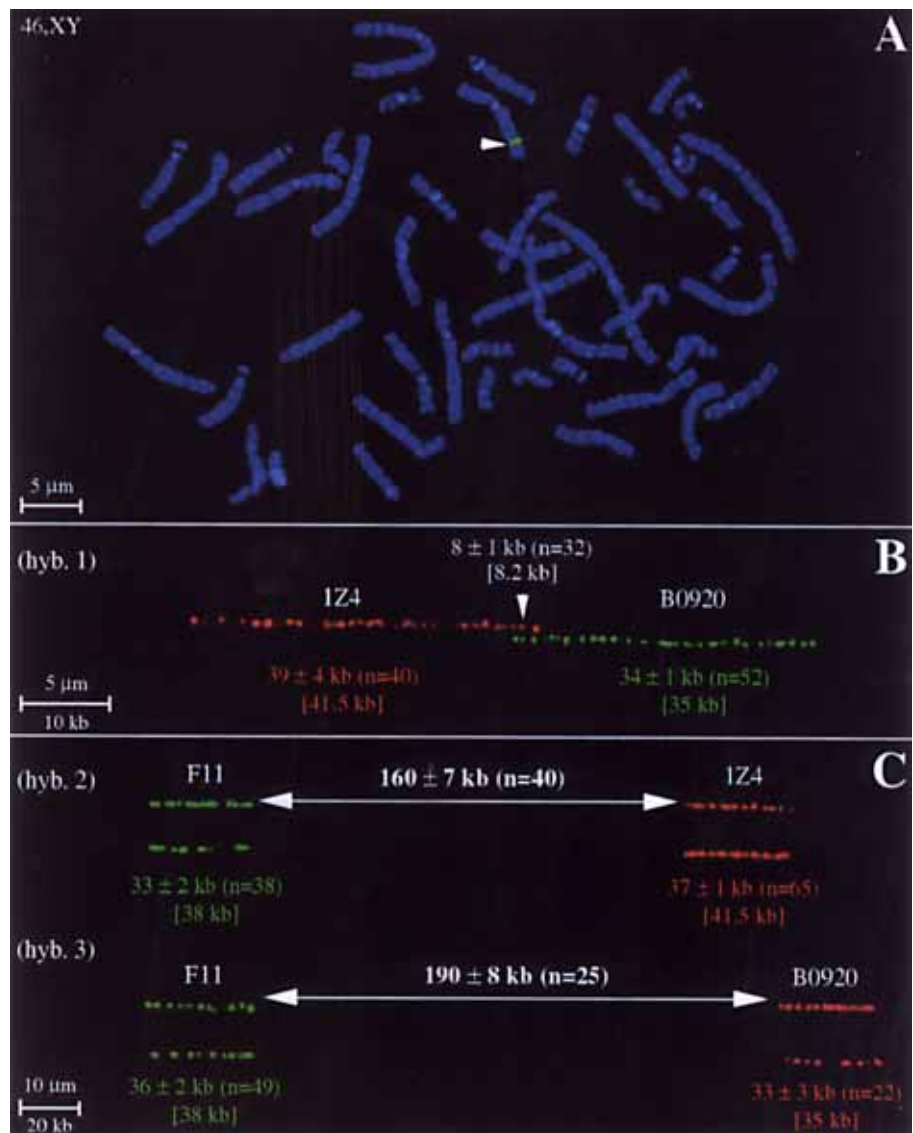
Preparations were observed using an epifluorescence microscope equipped with a 100-W mercury lamp and filter set 17 (Zeiss BP 485/20, dichroic filter 510, and BP 515-565) for FITC and rhodol green fluorescence and filter set 15 (Zeiss BP 546/12, dichroic filter 580, and LP 590) for TRITC fluorescence. Digital 16-bit images were collected with a cooled black-and-white charge-coupled device (CCD) camera mounted on the microscope. Each fluorochrome was imaged separately by switching red- and green-fluorescence-specific filters either manually or automatically. To correct for the shift generated by switching the filters, a third image was acquired with a double bandpass filter (Zeiss filter set 24), allowing the simultaneous collection of red and green fluorescence emissions. Hybridization signals were imaged using either a 100× or a 63× oil-immersion objective. Pixel sizes varied from 0.1 to 0.38 µm depending upon the acquisition conditions used.

Image analysis and distance measurements

FISH images obtained with conventional epifluorescence microscopes are always blurred by glare and out-of-focus light emissions. So far, in our laboratory, a procedure called iterative deconvolution, based on a modeling of the optical system in the microscope, was developed and used for the processing of 16-bit images acquired with a cooled CCD camera mounted on a fluorescence microscope (Monier et al., 1996). This procedure is included in a software program, called CytoFISH, developed by Y. Usson, which improves the resolution of FISH images by an estimated 30% (unpublished results). Iterative deconvolution was therefore applied to raw images of combed DNA.

After a reduction of the 16-bit color depth to 8 bits, red and green images were merged. The shift between red and green images generated by the manual switching of red- and green-fluorescence-specific filters was corrected by adjusting the two red and green images according to the double green and red image. The size of a hybridization signal was measured in micrometers with software we developed by placing a cursor at the beginning and at the end of the signal on the magnified image. The gap between the two signals was determined by measuring the distance between the two facing ends of a couple of red and green hybridization signals. Only pairs of perfectly aligned red and green signals were considered to ensure that both signals belonged to the same molecule. Sequence and gap sizes were estimated on at least 25 molecules by subsequently converting distances from micrometers into kilobases, using a conversion factor of 2 kb/µm.

Fig. 2. (A) Test of DNA clone specificity. Hybridization of YAC yWXD592 on a 46,XY human metaphase chromosome spread shows a single hybridization signal on the distal part of Xq (arrowhead), thus demonstrating that the YAC yWXD592 is not chimeric. The DAPI-G banding is improved by a procedure for image restoration (Monier et al., 1996). **(B, C)** Quantitative data obtained by FISH mapping on combed YAC molecules with cosmid probes from the XLP region. **(B)** Simultaneous hybridization of cosmid probes B0920 (green) and 1Z4 (red) on combed molecules of the YAC 916D1. Note that the red signal is shifted upward on purpose to allow better visualization of the extent of the overlap. However, the green and red signals are on the same fiber. Cosmid probes appear as extended signals in the form of strings of fluorescent beads. A small overlap is visible between the B0920 and 1Z4 signals (arrowhead). Below each signal and upward the overlap is indicated the mean size in kilobases (mean \pm SD) of the corresponding sequences estimated by (n) signal length measurements. Distances in kilobases were converted from distances in micrometers using a conversion factor of 2 kb/ μ m. For comparison, the corresponding expected sizes in kilobases defined by restriction mapping are given in brackets. **(C)** Hybridization of cosmid probes F11 and 1Z4 (hyb. 2) and F11 and B0920 (hyb. 3) on combed molecules of the YAC yWXD592. Note that between the cosmid probes, the combed YAC molecules are not visible since they have not been counterstained. The B0920 signal (red, hyb. 3) is located farther from the F11 signal (green, Hyb. 2) than the 1Z4 signal (red, Hyb. 2). Thus one can deduce that the 1Z4 probe is located between B0920 and F11. This observation is confirmed by quantitative data: above each gap and below each signal the corresponding size in kilobases (mean \pm SD; n = number of measurements). The sizes, in kilobases, defined by restriction mapping are given in brackets. The positions of the 1Z4 signal (hyb. 2) relative to the B0920 signal (hyb. 3) was confirmed in the complementary hybridization presented in **B**.



Results

Relative orientation of the contig A according to the contig B: gap length estimation

Three cosmid probes, B0920, 1Z4, and F11, were hybridized on combed YACs yWXD592 and 916D1, containing 800 kb and 1.5 Mb of human genomic DNA, respectively. The integrity of the YAC inserts was tested by PCR for the presence of several markers and the size of the inserts determined by PFGE. The specificity of the cosmid and YAC probes were assessed by in situ hybridization on metaphase chromosomes. All five probes gave an intense signal in the Xq25 band (Fig. 2A), indicating that the clones were not chimeric and were therefore suitable for FISH studies on combed DNA.

As the YAC 916D1 was the source from which the cosmid minilibrary was established, this YAC was used to confirm the

relative positions of the two overlapping cosmid probes, B0920 and 1Z4, which belong to the cosmid contig A (Fig. 1). When hybridized simultaneously on combed YAC 916D1, the B0920 (green) and 1Z4 (red) probes appeared as two contiguous signals in the form of strings of fluorescent beads, showing an overlap over a short region (hyb. 1, Fig. 2B). Note that in Fig. 2B the red signal is shifted upward on purpose to improve visualization of the extent of the overlap. The sizes of the hybridization signals were in good agreement with the lengths of the probes defined by restriction mapping (Fig. 2B). An 8-kb overlap was estimated from measurements performed on 32 molecules (standard deviation [SD] = 1). This value was concordant with the restriction maps of the two cosmid inserts from which an overlap of 8.2 kb was determined.

To establish the relative orientation of the two cosmid contigs A and B, two cosmid probes belonging to contigs A and B

Table 1. Presence of specific markers in YAC clones mapping in the Xq25 region

Marker	1D10T7	DF83	E8.6	IS12	B0920.7	A8E8	FH96	X2-183 (DXS982)
YAC yWXD592 (0.8 Mb)	+	+	+	+	+	+	+	+
YAC yWXD318 (0.3 Mb)						+	+	+
YAC yWXD195 (0.2 Mb)					+	+	+	
YAC yWXD642 (0.2 Mb)								+

were hybridized together. Since YACs containing small inserts are less sensitive to fragmentation during combing, the small YAC yWXD 592 was used. One experiment involved cosmid probes F11 (green) and 1Z4 (red) (hyb. 2, Fig. 2C) and the other involved F11 (green) and B0920 (red) (hyb. 3, Fig. 2C). As in the previous experiment, the size of the 1Z4, B0920, and F11 signals correlated well with the expected insert sizes of the probes as defined by restriction mapping (Fig. 2C). As shown in Fig. 2C, the 1Z4 signal appeared closer to the F11 signal (hyb. 2, Fig. 2C) than to the B0920 signal (hyb. 3, Fig. 2C). These data led to the conclusion that 1Z4 is located between B0920 and F11. The relative orientation of contig A according to contig B can therefore be deduced from this result (Fig. 1D, middle) and corresponds to the representation on the upper part of Fig. 1B.

In hybridization 2, the length of the gap separating the F11 and 1Z4 signals was 160 kb ($SD = 7$, $n = 25$), whereas in hybridization 3, F11 and B0920 were separated by a gap of 190 kb ($SD = 8$, $n = 40$), (Fig. 2C). As the probe lengths estimated from hybridization 3 correlated better with their expected size, the hybridization 3 result was therefore used to determine the size of the gap between the two cosmid contigs. In hybridization 3, a distance of 190 kb was found between the two facing ends of F11 and B0920. Since 39 kb separates the closest extremity of contig A facing contig B, the size of the gap between contigs A and B was estimated to be 151 kb ($190 \text{ kb} - 39 \text{ kb} = 151 \text{ kb}$) (Fig. 1D, lower section).

Centromeric-telomeric orientation

Until recently, the orientation of the whole region was thought to be the one proposed by Wu et al. (1993) and Lamartine et al. (1996). However, Lanyi et al. (1996) proposed the reverse orientation, positioning DXS982 centromeric to DF83. To settle this controversy, the centromeric-telomeric orientation was confirmed by Southern blot analysis on YAC DNA. Small YACs, yWXD 318, yWXD 195, and yWXD 642, mapping to this region, with sizes varying from 200 to 300 kb, were hybridized with several marker probes from the contigs. The data presented in Table 1 indicate that B0920.7, a subfragment of the cosmid B0920, maps centromeric to DXS982 (X2-183), which is included in cosmid F11. Since 1D10T7, one of the end markers of the contig A, is not present in the YAC yWXD 318, 1D10 was shown to be centromeric to B0920, which, in turn, was found to be centromeric to F11. These results are concordant with the combing results (Fig. 1D, middle section) and indicate that the order of markers over this region is: cen...1D10T7-DF83-DXS982-DXS739...tel (Fig. 1D, upper section).

Discussion

By combining FISH on combed DNA and conventional mapping procedures, we have shown that (1) contig A is centromeric to contig B (Fig. 1D, upper section), (2) the B0920 probe is centromeric to the 1Z4 probe in the cosmid contig A (Fig. 1D, upper and middle sections), and (3) the two contigs are 150 kb distant (Fig. 1D, lower section). The distance of 345 kb between DXS982 and DF83 that we could deduce from combing measurements agrees with the distance of 300 kb estimated by restriction mapping (Lanyi et al., 1996). However, in their paper, Lanyi et al. (1996) determined the reverse orientation of contig A and B, since they found DXS982 to be closer to the centromere than DF83. The orientation of the markers in contig B that we deduce from our data (viz., DXS982 centromeric to DXS739) are concordant with the results obtained by Wu et al. (1993), using an *in situ* approach.

This report confirms the power of FISH coupled with molecular combing for high-resolution mapping and shows that this technique can be used to order cosmid contigs over a 600-kb region, even in regions with numerous repeated sequences (the centromeric contig contains 67% repeated sequences, based on sequencing data [Yin et al., manuscript submitted for publication]) and duplications of several unique sequences (Lamartine et al., 1996; Porta et al., 1997; D. Schlessinger, personal communication). The establishment of the exact order of the contigs in the XLP locus will contribute to a better characterization of this region and should help to identify the gene involved in XLP disease. In addition, this extended map of 600 kb should also be of interest for analysis of chromatin organization in this region in the interphase nucleus.

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