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Fine Mapping of the McLeod Locus (XK) to a 150–380-kb Region in Xp21

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Summary

McLeod syndrome, characterized by acanthocytosis and the absence of a red-blood-cell Kell antigen (Kx), is a multisystem disorder involving a late-onset myopathy, splenomegaly, and neurological defects. The locus for this syndrome has been mapped, by deletion analysis, to a region between the loci for Duchenne muscular dystrophy (DMD) and chronic granulomatous disease (CGD). In this study, we describe a new marker, 3BH/R 0.3 (DXS 709), isolated by cloning the deletion breakpoint of a DMD patient. A long-range restriction map of Xp21, encompassing the gene loci for McLeod and CGD, was constructed, and multiple CpG islands were found clustered in a 700-kb region. Using the new marker, we have limited the McLeod syndrome critical region to 150–380-kb. Within this interval, two CpG-rich islands which may represent candidate sites for the McLeod gene were identified.

Introduction

McLeod syndrome is a rare, X-linked recessive disorder involving the Kell blood group system. Red blood cells in McLeod syndrome are characterized by acanthocytic morphology, reduced in-vivo survival, weak Kell antigens, and absence of a ubiquitous antigen called "Kx" (Wimer et al. 1977). Immunoprecipitation studies have established that the Kx antigen is a marker of ~37 kDa on the red-blood-cell membrane (Redman et al. 1988), while Kell antigens reside on a 93-kDa glycoprotein (Redman et al. 1986). The relationship between Kell and Kx is not well understood. The absence of Kx is unique to McLeod syndrome and is associated with a marked reduction of Kell antigens.

The biochemical defect responsible for McLeod syndrome is currently unknown, and no deficiencies or abnormalities were found in many factors known to affect cell shape (Symmans et al. 1979; Tang et al. 1981). The most prominent deficiency is the absence of Kx, but its function remains uncertain. In the absence of a defined biochemical defect, the alternative approach to elucidate the nature of the McLeod gene product is to clone the gene on the basis of its known subchromosomal location.

The first clue to the localization of McLeod locus (XK) to Xp21 was based on observations of patients with contiguous-gene syndromes. Patient BB, who exhibited McLeod syndrome in association with Duchenne muscular dystrophy (DMD), chronic granulomatous disease (CGD), and retinitis pigmentosa form 3 (RP3), had a cytogenetically detectable lesion in Xp21 (Francke et al. 1985). Microdeletions in the same region were also found in patient SB, who exhibited McLeod syndrome, CGD, and RP3 phenotypes (de Saint-Basile et al. 1988), and patient OM, who had McLeod syndrome and CGD (Frey et al. 1988). Deletion analysis of these and other patients with combinations of the four disorders has established the order of the gene loci in Xp21 to be Xpter–DMD–XK–CGD–RP3–Xcen (van Ommen et al. 1986; Bertelson

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et al. 1988). The region containing the McLeod locus was further defined by comparing deletions observed in a DMD patient (JD) and in a pair of first cousins with McLeod syndrome. Although the patients do not share a common phenotype, a region of deletion overlap encompassing the cloned loci of p469(DXS196)/p634 and p378-40-1(DXS307)/p145-12(DXS141) was identified (fig. 1*A*). On the basis of this observation, the McLeod locus was assigned to a region flanked by p378-40-1/p145-12 (the relative order of these two clones has not been established) and the gene for CGD (Bertelson et al. 1988). Physical mapping using pulsed field gel electrophoresis (PFGE) with DNA fractionated up to 1,600 kb has not been able to link p145-12 and p378-40-1 to CGD, although p378-40-1 could be linked to p145-12 (data not shown). To isolate new marker(s) that map closer to the McLeod locus, our approach was to clone the deletion breakpoint in patient JD. The deletion in this patient spans a region of ~6,000 kb (Wilcox et al. 1986) and includes the previous markers p145-12/p378-40-1 (fig. 1*A*).

In the present study, we report the isolation of two probes, 3BH/R 0.8 and 3BH/R 0.3 (DXS 709), from the proximal side of the deletion breakpoint in patient JD. PFGE analysis of the region containing the McLeod and CGD gene loci reveals the presence of multiple CpG islands. The physical limits of the McLeod syndrome critical region as defined by flanking markers 3BH/R 0.3 and CGD is estimated to be 150–380 kb. Within this region, two CpG-rich islands were identified.

Patients and Methods

Patients

All male patients used in the present study have been reported previously. JD had a cytogenetically detectable lesion in Xp21 but exhibited only the DMD phenotype (Wilcox et al. 1986). BB had McLeod syndrome in association with three other X-linked disorders: DMD, CGD, and RP3 as described (Francke et al. 1985). S/H are first cousins with McLeod syndrome and a microdeletion in Xp21 (Bertelson et al. 1988). FD exhibited the McLeod phenotype but no apparent rearrangements in his X chromosome (Carter et al. 1990). Control DNAs include leukocytes from a normal male individual and a lymphoblastoid cell line containing four X chromosomes GM1416B (NIGMS, Human Genetic Cell Repository, Camden, NJ).

DNA Probes

The probes used in the identification of the distal deletion breakpoint in JD were (1) DMD cDNA 5b-7 (Koenig et al. 1987), (2) P20 (DXS269) (Wapenaar et al. 1988), and (3) cosmid cPT4 (Blonden et al. 1989). Probes used in the construction of the long-range restriction map were (1) CGD-cDNA (CYBB) (Royer-Pokora et al. 1986) and (2) 3BH/R 0.3 (DXS 709) (proximal sequence of a junction fragment isolated from JD).

DNA Isolation, Digestion, Fractionation, Transfer, and Hybridization

Genomic DNA in solution was isolated from lymphoblastoid cell lines and peripheral blood leukocytes by standard methods (Herrmann and Frischauf 1987). Digestion, fractionation of DNA by electrophoresis, and Southern blot transfers were according to methods described elsewhere (Monaco et al. 1985). Purified DNA fragments and whole cosmids were labeled by the random priming method of Feinberg and Vogelstein (1983). Radioactively labeled cosmids were preannealed with 100 μg of sonicated human DNA before hybridization. Prehybridization and hybridization were performed at 42°C in a buffer containing 50% formamide (Monaco et al. 1985). Filters were washed to a final stringency of 0.1 × SSC, 0.1% SDS at 65°C for 30 min before exposure to Kodak XAR-5 film.

Construction of a Deletion-Junction Library

A phage library was constructed in EMBL 3, as described elsewhere (Frischauf et al. 1983; Frischauf 1987), by using partially *Mbo*I-digested DNA of patient JD, which had an average size of 12–18 kb. Phages were packaged in vitro using Gigapack Gold (Stratagene) and were transduced into *Escherichia coli* strain P2PLK (Stratagene) to an efficiency of 2.17×10^7 pfu/μg DNA. A library of $\sim 1.7 \times 10^6$ phages was plated and transferred onto Hybond N^+ (Amersham) for screening.

PFGE

High-molecular-weight DNA in agarose blocks was prepared and digested with rare-cutting restriction enzymes according to a method described elsewhere (Herrmann et al. 1987). PFGE was performed in a contour-clamped homogenous electric field gel system (CHEF-DRII; Biorad). Phage λ multimers and chromosomes of *Saccharomyces cerevisiae* (strain YNN295; Biorad) were used as size markers. To opti-

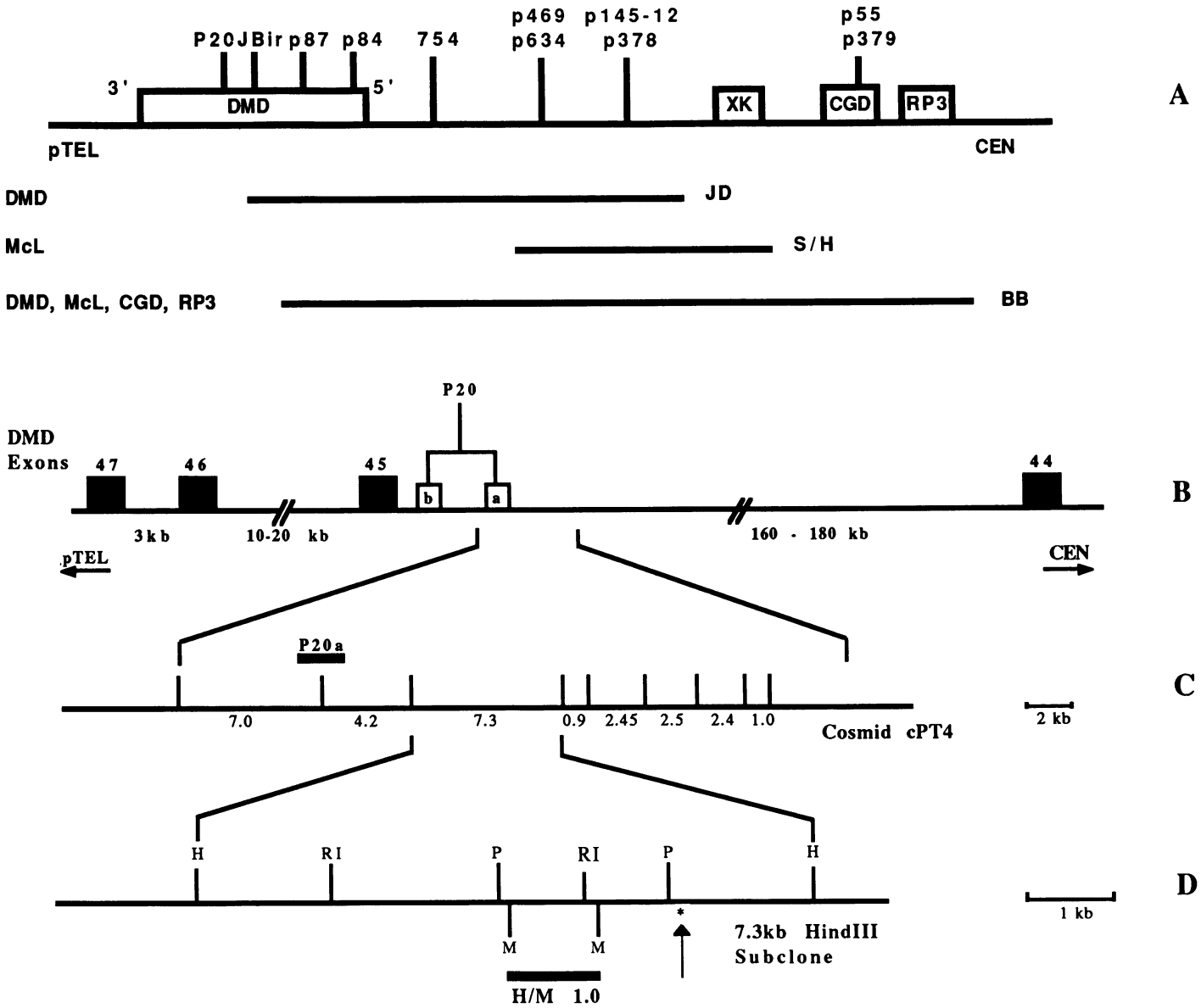

Figure 1 Schematic representation of Xp21 and stepwise magnification of region containing distal deletion endpoint in patient JD. *A,* Map of Xp21, indicating relative positions of genes and cloned loci. In cases where the order has not been ascertained, loci are assigned to the same site (e.g., p145-12 and p378-40-1). Below the map, the location and extent of deletions and the clinical manifestations in patients BB, S/H, and JD are indicated. *B,* Organization of region containing DMD exons 44–47, intron sizes, and location of probe P20. *C, Hin*dIII restriction map of cosmid cPT4. *D,* Restriction map of 7.3-kb *Hin*dIII fragment derived from cosmid cPT4, indicating sites for *Eco*RI (RI), *Pst*I (P), and *Msp*I (M). The vertical arrow points to an approximate location of the distal deletion endpoint in patient JD, and the position of an unique 1.0-kb *Msp*I fragment (H/M 1.0) is indicated below the map.

mize sizing of fragments, DNA was separated into two windows of resolution: a lower range of 50–700 kb and an upper range of 200–1,600 kb. Specific electrophoretic conditions were as described in the figure legends.

Results

Identification of the Distal Breakpoint in Patient JD

The order and sizes of most of the exon-containing genomic *Hin*dIII and *Pst*I fragments detected with DMD-cDNA subclones has been established (Koenig et al. 1987; Den Dunnen et al. 1989). Thus deletion breakpoints within the DMD gene can be readily assigned by hybridizing cDNA subclones to digests of genomic DNA isolated from patients. The distal deletion endpoint in patient JD has been previously mapped within the DMD gene, between the cloned genomic loci J66 and JBir (Monaco et al. 1987; Bertelson et al. 1988). Hybridization of cDNA subclone 5b-7 to *Hin*dIII and *Pst*I genomic DNA digests of JD detected three normal size fragments that correspond to exons 45–47, in contrast to the detection of exons 30–47 in the control lane (fig. 2*A*). As no altered size fragments were observed on Southern blot analysis, the result indicated that JD was deleted for all exons 5′ of exon 45 and that the distal breakpoint must have occurred within the intron separating exon 45 from exon 44. The size of this intron has been estimated to be 160–180 kb (Den Dunnen et al. 1989), which renders cDNA 5b-7 unsuitable as a distal probe to screen for the junction fragment.

Probe P20 (DXS269) consists of two genomic segments located 32 kb apart and was derived from the ends of a deleted cosmid. The distal segment of this probe, P20(b), maps 10.5 kb centromeric to exon 45 (fig. 1*B*). Hybridization of P20 to DNA digested with frequently cutting enzymes such as *Eco*RI, *Hin*dIII, and *Pst*I did not detect any abnormal size fragments in patient JD (data not shown), which suggested that the breakpoint mapped farther proximal to P20(a). With less frequently cutting enzymes such as *Eco*RV, *Kpn*I, and *Bgl*II, altered size bands were observed in JD compared with the control DNA (fig. 2*B*). Despite this finding, the distance from P20(a) to the distal breakpoint in JD was still in the range of 12–20 kb.

Sequences proximal to P20(a) have been cloned in cosmid cPT4 and characterized according to *Hin*dIII fragments (Blonden et al. 1989) as shown in figure 1*C*. Southern analysis of whole-cosmid hybridization to *Hin*dIII digests of JD's DNA was carried out and indicated that a 7.3-kb *Hin*dIII fragment present in control DNA has been rearranged to a different size fragment of 13 kb in patient JD (fig. 2*C*). To localize precisely the site of the breakpoint, the 7.3-kb *Hin*dIII fragment was subcloned into a plasmid vector (Bluescript, Stratagene) and was mapped with several restriction enzymes (fig. 1*D*). The presence of two *Pst*I sites within the fragment subdivided it into three smaller segments, each of which was hybridized sequentially to the digest of JD's DNA. When the most proximal segment (1.7-kb *Hin*dIII/*Pst*I) was used, JD was found to be deleted for all enzymes tested; from the middle portion, a unique 1.0-kb *Msp*I fragment (designated as H/M1.0) detected altered size bands in JD, for all tested enzymes except *Pst*I (fig. 2*D*). This result suggested that the distal deletion endpoint in JD was within the 1.7-kb *Hin*dIII/*Pst*I segment, located just proximal to the *Pst*I site. This placed H/M1.0 ~1.0 kb telomeric to the distal deletion endpoint in JD (fig. 1*D*).

Cloning of the Junction Fragment from JD

Screening a ninefold haploid genome equivalent (4.5-fold for the X chromosome) bacteriophage library of patient JD with H/M1.0 identified three positive phage clones. These were mapped with several restriction enzymes (fig. 3*A*). A total of 27.0 kb around the deletion breakpoint was isolated in three overlapping phages. From phage 3B, two *Hin*dIII/*Eco*RI fragments, one of 0.8 kb and one of 0.3 kb (designated as 3BH/R 0.8 and 3BH/R 0.3, respectively) were isolated and mapped 10 kb and 12 kb, respectively, from the centromeric side of the deletion breakpoint (fig. 3*A*). Verification that these probes were derived from the centromeric region of the deletion breakpoint in JD was obtained by hybridization to the DNA of patients S/H and BB. Both sequences were deleted in S/H and BB, as shown in figure 3*B* and *C*. Although 3BH/R 0.3 maps just 2.0 kb proximal to 3BH/R 0.8, hybridization of the former probe to genomic Southern blots of conventional and pulsed-field gels consistently detected an additional, less intense cross-hybridizing band (figs. 3*C*, 4*B*, 5*B*, and 6*B*). As this band was not observed in patients BB, JD, and S/H (fig. 3*C*), the origin of the cross-hybridizing sequence was inferred to be X chromosome specific and to map to Xp21.

Physical Mapping Around the McLeod/CGD Loci by PFGE

The samples used for mapping were DNA isolated from whole blood from a normal male individual and

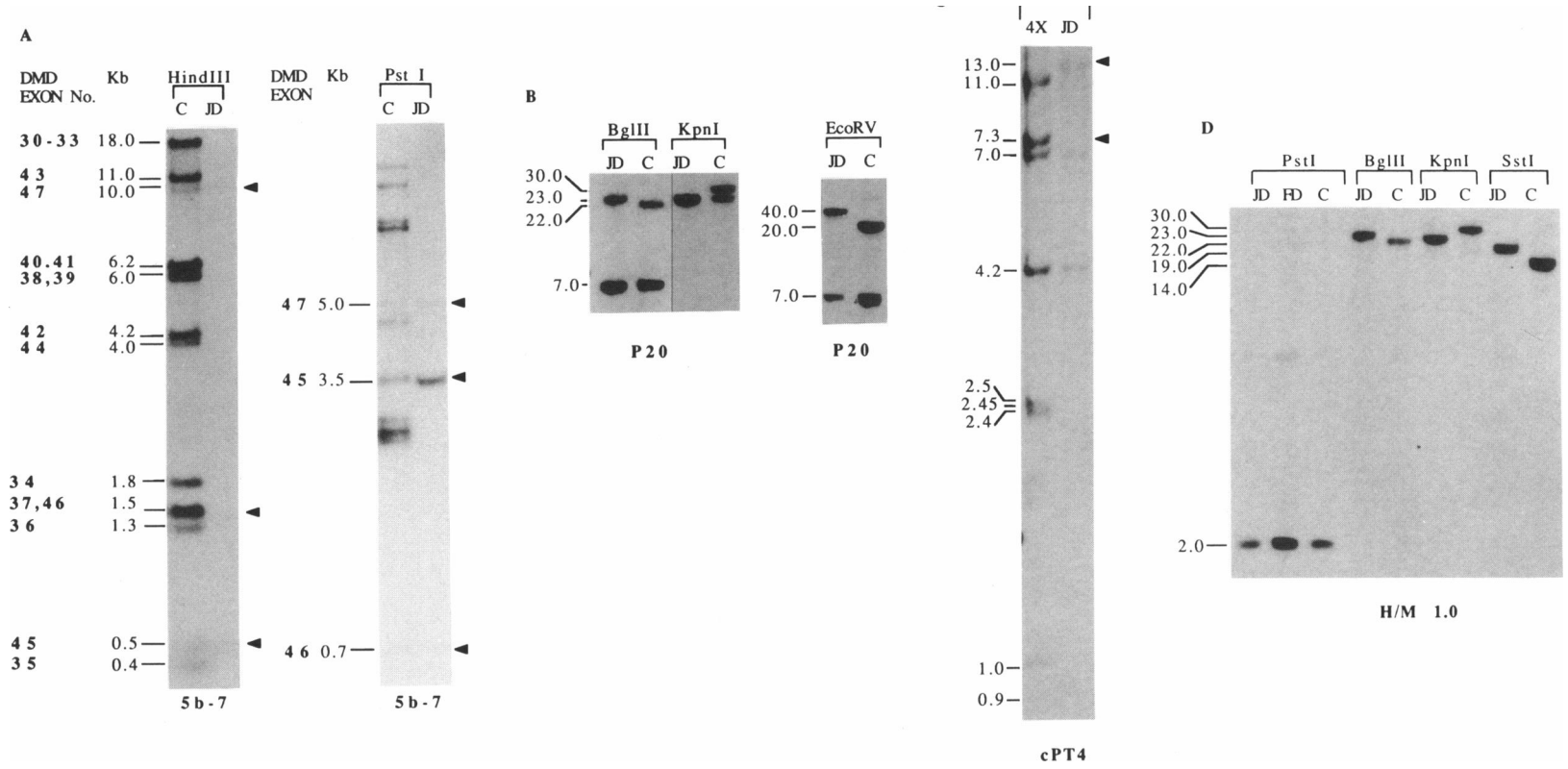

Figure 2 Localization of distal deletion endpoint in patient JD. *A*, Hybridization of *Hin*dIII- and *Pst*I-digested genomic DNA from normal male (lanes C) and patient JD to DMD cDNA 5b-7. DMD exons containing genomic *Hin*dIII and *Pst*I fragments are indicated in boldface, and arrowheads indicate the presence of only exons 45–47 in JD. *B*, Detection of deletion junction fragments in JD, on Southern blot analysis with probe P20. Sizes of large fragments were determined by comparison to a high-molecular-weight marker (BRL and Gibco) on a 0.3% agarose gel. *C*, Mapping JD deletion breakpoint by whole-cosmid hybridization to *Hin*dIII-digested genomic DNA. The lower arrowhead indicates the deleted 7.3-kb fragment in JD, while the upper arrowhead shows the presence of a 13-kb junction fragment. *D*, Southern blot analysis with H/M 1.0, which detected altered size fragments in JD for all tested enzymes except *Pst*I.

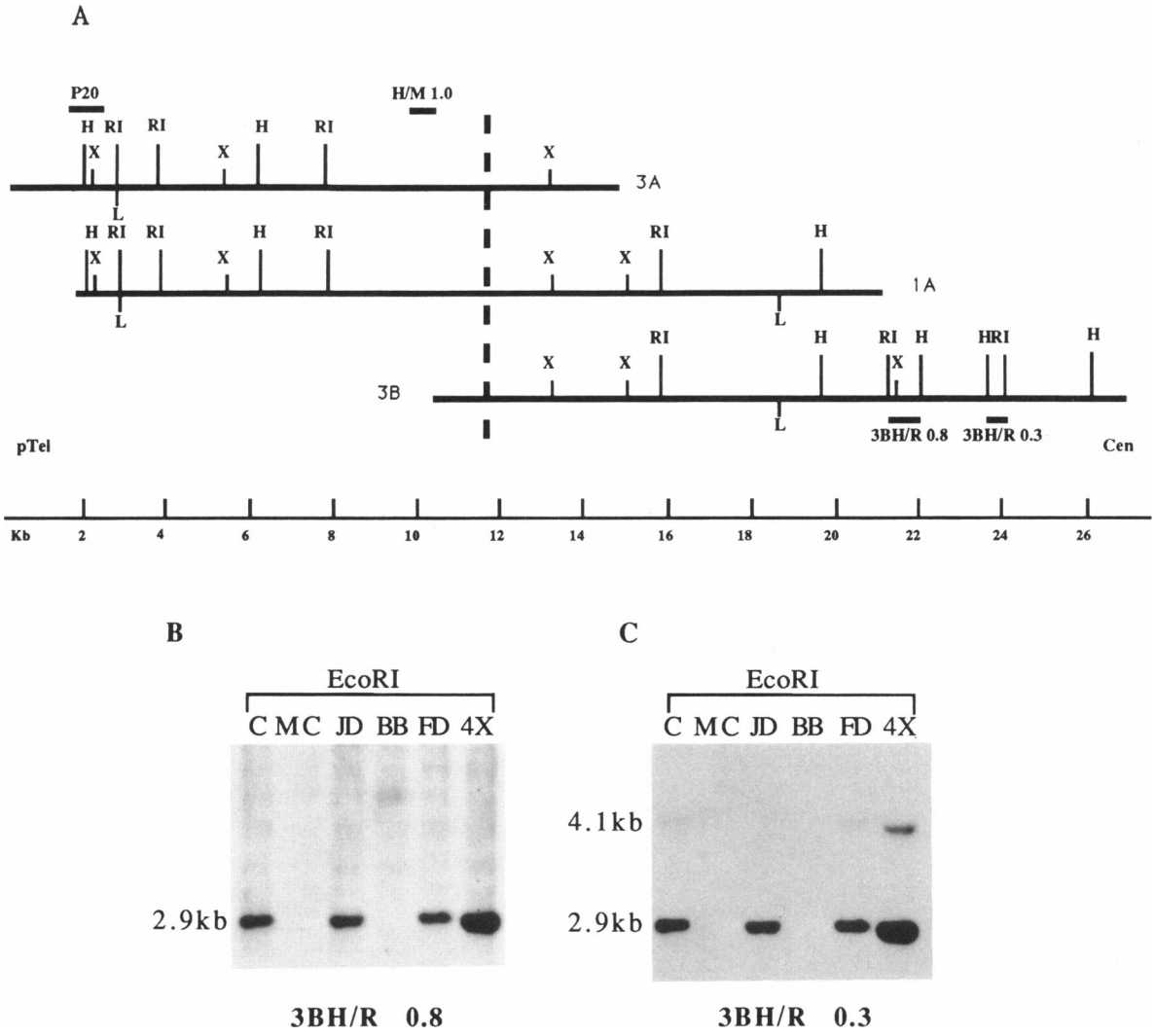

Figure 3 Characterization of region around deletion breakpoint. *A*, Restriction map of three overlapping phages containing junction fragment of patient JD. The restriction enzymes sites are *Hin*dIII (H), *Eco*RI (RI), *Xba*I (X), and *Sal*I (L), and the site of the breakpoint is indicated by the vertical broken line. Two sequences designated as 3BH/R 0.8 and 3BH/R 0.3 were isolated from the proximal side of the breakpoint. *B*, and *C*, Sequential hybridization of 3BH/R 0.3 to the same Southern blot containing *Eco*RI-digested genomic DNA of control samples (lanes C) and 4X and patients MC (one of the McLeod cousins), JD, BB, and FD. Note that the cross-hybridizing sequence is detected only with 3BH/R 0.3.

patient FD, while DNA from patient JD was obtained from a lymphoblastoid cell line. PFGE analysis using probe p145-12 detected the same bands as did probe p378-40-1, for all enzymes tested (data not shown). However, neither markers could be linked to CGD on gels fractionated up to 1,600 kb. The sizes of fragments detected by 3BH/R 0.3 and CGD-cDNA are summarized in table 1. On the basis of these observations, a long-range restriction map encompassing the McLeod and CGD gene loci, both for the normal X chromosome as well as the deleted X chromosome of patient JD, was constructed, as shown in figure 7. In view of the number of CpG islands detected, these sites have been designated I–V to facilitate discussion.

PFGE Analysis of DNA from a Normal Male

The physical map was constructed initially from a series of partial *Sfi*I digestion products detected by CGD-cDNA and 3BH/R 0.3. Fragment sizes of 220 kb, 580 kb, 760 kb, and 1,120 kb were detected by

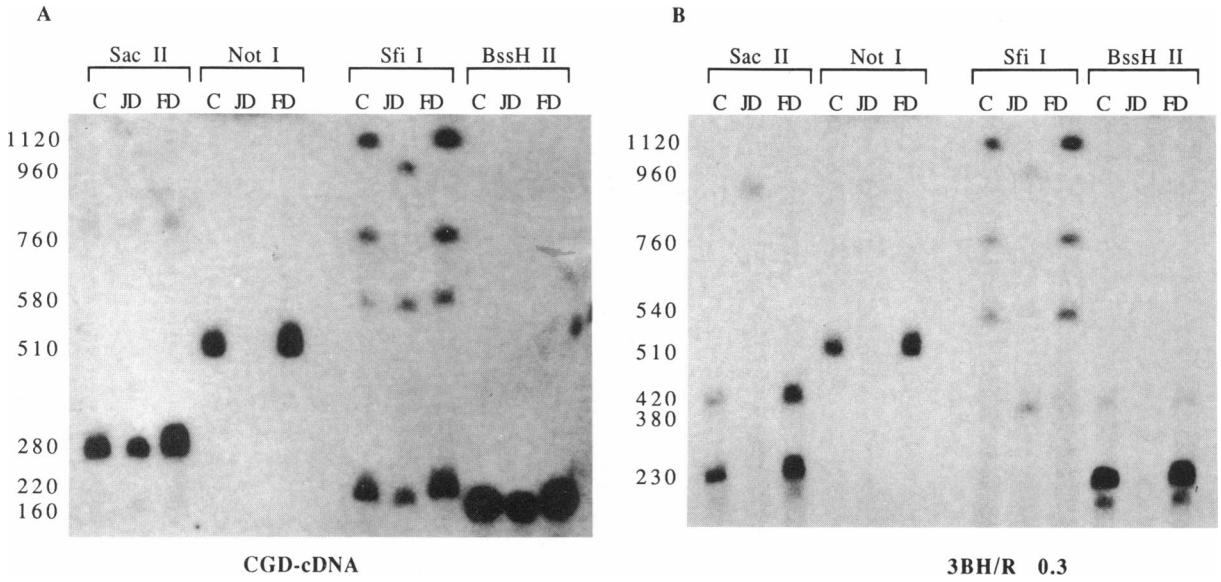

Figure 4 Physical linkage of 3BH/R 0.3 and CGD-cDNA to common *Not*I fragment. CGD-cDNA and 3BH/R .03 were hybridized sequentially to a PFGE Southern blot containing genomic DNA from a normal male (lanes C) and from patients JD and FD. The DNA was digested to completion with the restriction enzymes indicated and was fractionated through a 0.8% agarose gel in 0.5 × TBE at 170 V on a Biorad CHEF-DRII apparatus. The pulse times used were initially 60 s for 21 h followed by 100 s for 15 h and 150 s for 8 h. Chromosomes prepared from *Saccharomyces cerevisiae* and lambda oligomers were used as size markers.

CGD-cDNA, as shown in figure 4*A*. The order of *Sfi*I sites was established from the hybridization pattern of the distal marker 3BH/R 0.3 which identified common fragments of 1,120 kb and 760 kb (as detected by CGD) but that also identified a 540-kb band as the smallest hybridizing fragment (fig. 4*B*), indicating that 3BH/R 0.3 resides on a 540-kb *Sfi*I fragment adjacent and distal to the 220-kb *Sfi*I fragment on which CGD lies. The two probes were therefore linked on a 760-kb *Sfi*I partial digestion fragment. Further evidence of physical linkage was obtained when both probes hybridized to a common 510-kb *Not*I fragment (fig. 4*A* and *B*). Coincidental sizes and comigration of bands were excluded from the analysis of double-digest patterns. For example, in double digests of *Sfi*I/*Not*I, partial digest bands of 160 kb and 510 kb were detected by CGD-cDNA, while fragment sizes of 350 kb and 510 kb were identified by 3BH/R 0.3; the sum of the two smallest hybridizing fragments is equivalent to the 510-kb band observed in the single *Not*I digest. This result also localized the position of the *Not*I sites with respect to the partial *Sfi*I site at IV (fig. 7).

Identification of Multiple CpG Islands within and Flanking the McLeod Locus

The assignment of CpG islands I–V, shown in figure 7, was based on fragment sizes observed from a series of single and double digests described as follows: in single-enzyme digests with *Bss*HII and *Eag*I, CGD-cDNA detected identical size bands of ~165 kb (fig. 5*A*) which, on double digestion with *Sfi*I, reduced it to a size similar to that of *Sfi*I/*Not*I digest (figs. 5*A* and 6*A*). This result indicated that both *Bss*HII and *Eag*I sites were located adjacent to the *Not*I site. Similarly, hybridization of CGD-cDNA to *Sfi*I/*Sac*II double digests resulted in a series of partials with the strongest hybridizing band at 160 kb (fig. 5*A*). In summary, site V contained a CpG island that was recognized by at least four different rare-cutting enzymes (fig. 7). In contrast to the fragments detected by CGD-cDNA, hybridization of 3BH/R 0.3 to single-enzyme digests with *Bss*HII and *Sac*II detected identical size fragments of 230 kb and a cross-hybridizing band of 190 kb (fig. 5*B*).

Detection of a Cross-Hybridizating Fragment by 3BH/R 0.3

In *Mlu*I digests, 3BH/R 0.3 identified a single 420-kb fragment, while CGD-cDNA hybridized to sequences in the limiting mobility (>1,000 kb) (fig. 5). When double digests of *Mlu*I in combination with either *Eag*I, *Not*I, *Bss*HII, or *Sac*II were performed, CGD-cDNA identified a 165-kb fragment, while 3BH/R 0.3 detected a 230-kb band in all double digests (fig. 5). These findings suggested that both mark-

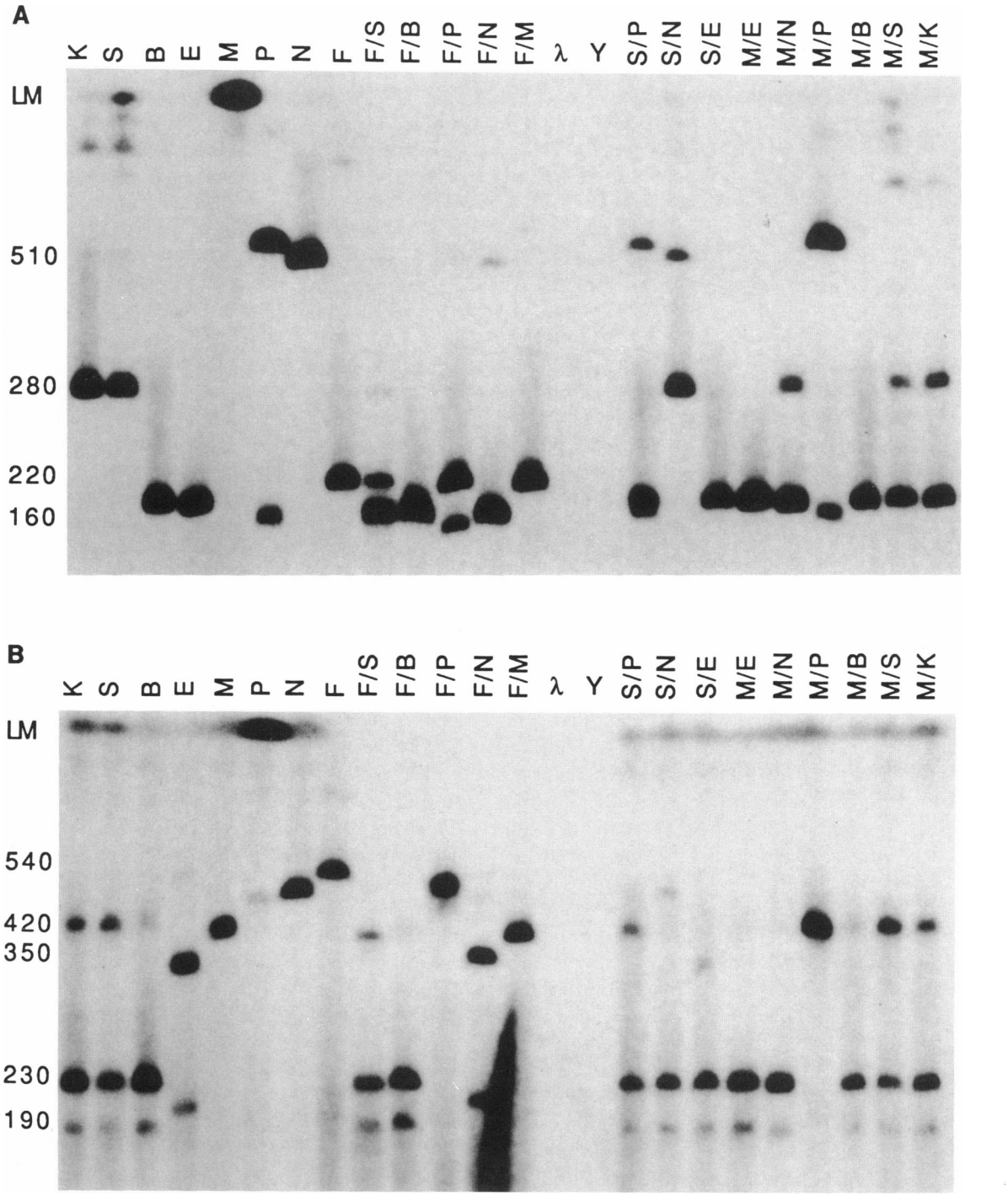

Figure 5 PFGE analysis of DNA from normal male. Hybridization is of CGD-cDNA (*A*) and 3BH/R 0.3 (*B*) to a Southern blot containing genomic DNA from a normal male. The DNA was digested to completion with the restriction enzymes indicated and was fractionated through a 0.8% agarose gel in 0.5 × TBE at 170 V. The pulse times used were 25 s for 17 h followed by 60 s for 16 h and 120 s for 5 h. Restriction enzymes used were *Ksp*I (K), *Sac*II (S), *Bss*HII (B), *Eag*I (E), *Mlu*I (M), *Fsp*I (P), *Not*I (N), and *Sfi*I (F). LM = hybridization in limiting-mobility region (i.e., >1,000 kb).

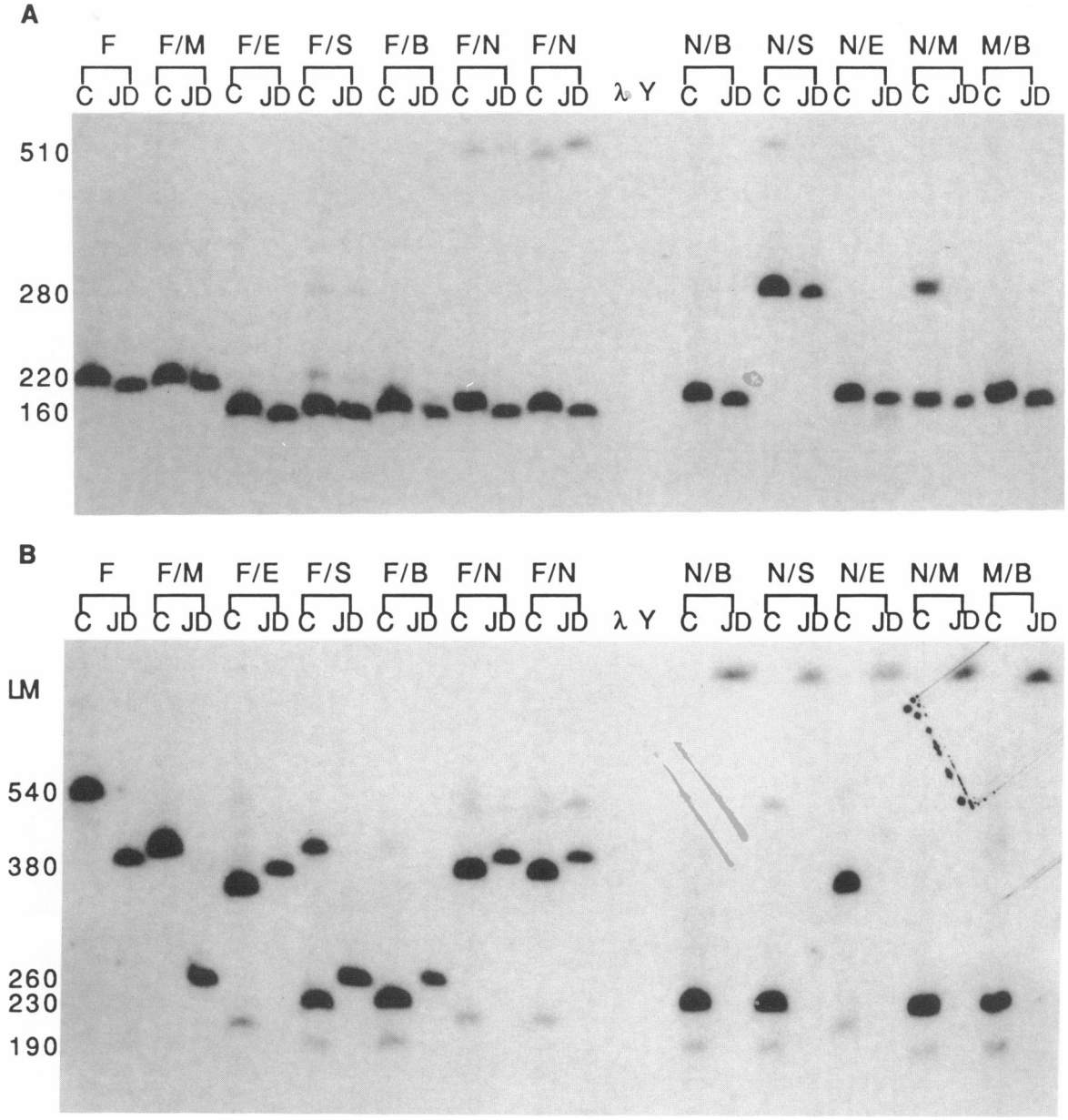

Figure 6 PFGE analysis of normal X-chromosome and deleted X chromosome of patient JD. *A*, Hybridization of CGD-cDNA to filter containing DNA from normal male (lanes C) and patient JD. DNA was digested and fractionated as described in fig. 5. Migration differences in identical enzyme digests between DNA of control and of JD were consistently observed and could be a consequence of unequal DNA concentration. Of the seven restriction enzymes used, only one methylation difference between DNA from leukocytes and the lymphoblastoid cell line of JD was identified in the *NotI*/*MluI* digests. The *MluI* site in leukocytes was found to be partially methylated but to be unmethylated in the lymphoblastoid cell line of JD. *B*, Hybridization to same blot with 3BH/R 0.3, which detected altered size fragments in JD for all enzymes tested. Size markers were lambda oligomers and chromosomes from *Saccharomyces cerevisiae*. Restriction enzymes are abbreviated as in fig. 5.

Table 1

Summary of Fragment Sizes Observed

Probe Enzyme	Fragment Size in[a] (kb)			
	3BH/R 0.3		CGD-cDNA	
	Control	JD	Control	JD
*Sfi*I	540	~380	220	220
	760	600	580	600
	1,120	960	760	960
			1,120	
*Not*I	510	LM	510	LM
*Eag*I	350	LM	165	165
	210[b]			
*Bss*HII	230	LM	165	165
	190[b]			
*Sac*II	230	~900	280	280
	420[c]		510[c]	~800[c]
	700[c]		700[c]	LM
	LM		LM	
	190[b]			
*Mlu*I	420	~900	LM	LM
*Fsp*I	530	ND	150	ND
			530	
*Sfi*I/*Not*I	350	380	160	160
	510[c]	540[c]	510[c]	540[c]
	540[c]			
	210[b]			
*Sfi*I/*Eag*I	350	375	160	160
	510[c]		165[c]	165[c]
	210[b]			
*Sfi*I/*Mlu*I	420	260	220[c]	220[c]
			225[c]	225[c]
*Sfi*I/*Bss*HII	230	260	160	160
	190[b]		165[c]	165[c]
			350[c]	
*Sfi*I/*Fsp*I	530	ND	150	ND
			220	
*Sfi*I/*Sac*II	230	260	160	160
	420[c]	380[c]	220[c]	220[c]
	510[c]		280[c]	280[c]
	190[b]		340[c]	340[c]
*Not*I/*Eag*I	350	LM	165	165
	210[b]			
*Not*I/*Mlu*I	230	LM	165	165
	190[b]		280	
*Not*I/*Bss*HII	230	LM	165	165
	190[b]			
*Not*I/*Sac*II	230	LM	280	280
	510		510	LM
	190[b]			
*Eag*I/*Mlu*I	230	LM	165	165
	190[b]			
*Eag*I/*Bss*HII	230	LM	165	165
	190[b]			
*Eag*I/*Sac*II	230	LM	165	165
	350[c]			
	190[b]			
*Mlu*I/*Bss*HII	230	~900	165	165
	190[b]			
*Mlu*I/*Sac*II	230	~900	165	165
	420[c]		280[c]	280[c]
	190[b]		690[c]	LM
			800[c]	
			LM	
*Mlu*I/*Fsp*I	420	ND	150	ND
			530	
*Sac*II/*Bss*HII	230	~900	165	165
	190[b]			

Note.—Fragment sizes observed in patient FD (not shown) are identical to those in control DNA.

[a] Data are approximate values of restriction-fragment sizes detected by PFGE of DNA from control and patient JD. LM = hybridization in the region of limiting mobility (>1,000 kb); and ND = not determined.

[b] Cross-hybridizing bands.

[c] Weak hybridizing bands resulting from partial digestion.

ers were localized between separate and distinct CpG islands. The detection of an identical size fragment on double digestion has enabled the assignment of four different rare cutters to a "single" site (within the limits of resolution permitted by PFGE) designated as islands II and V and shown in figure 7.

In addition to the strongly hybridizing band of 230 kb, 3BH/R 0.3 also detected a fainter cross-hybridizing fragment of 190 kb (fig. *5B*) in the above series of double digests. No cross-hybridizing fragments were observed in single-enzyme digests of *Mlu*I, *Not*I, and *Sfi*I, while a 210-kb cross-hybridizing fragment was observed in digests with *Eag*I, *Eag*I/*Sfi*I, *Eag*I/*Not*I, and *Not*I/*Sfi*I. The remaining combinations of restriction-enzyme digests produced an identical 190-kb cross-hybridizing fragment (figs. 4–6). On the basis of these observations, the region containing sequences homologous to 3BH/R 0.3 appeared to correspond to the 190-kb region flanked by CpG islands I and II. This was consistent with the finding that enzyme di-

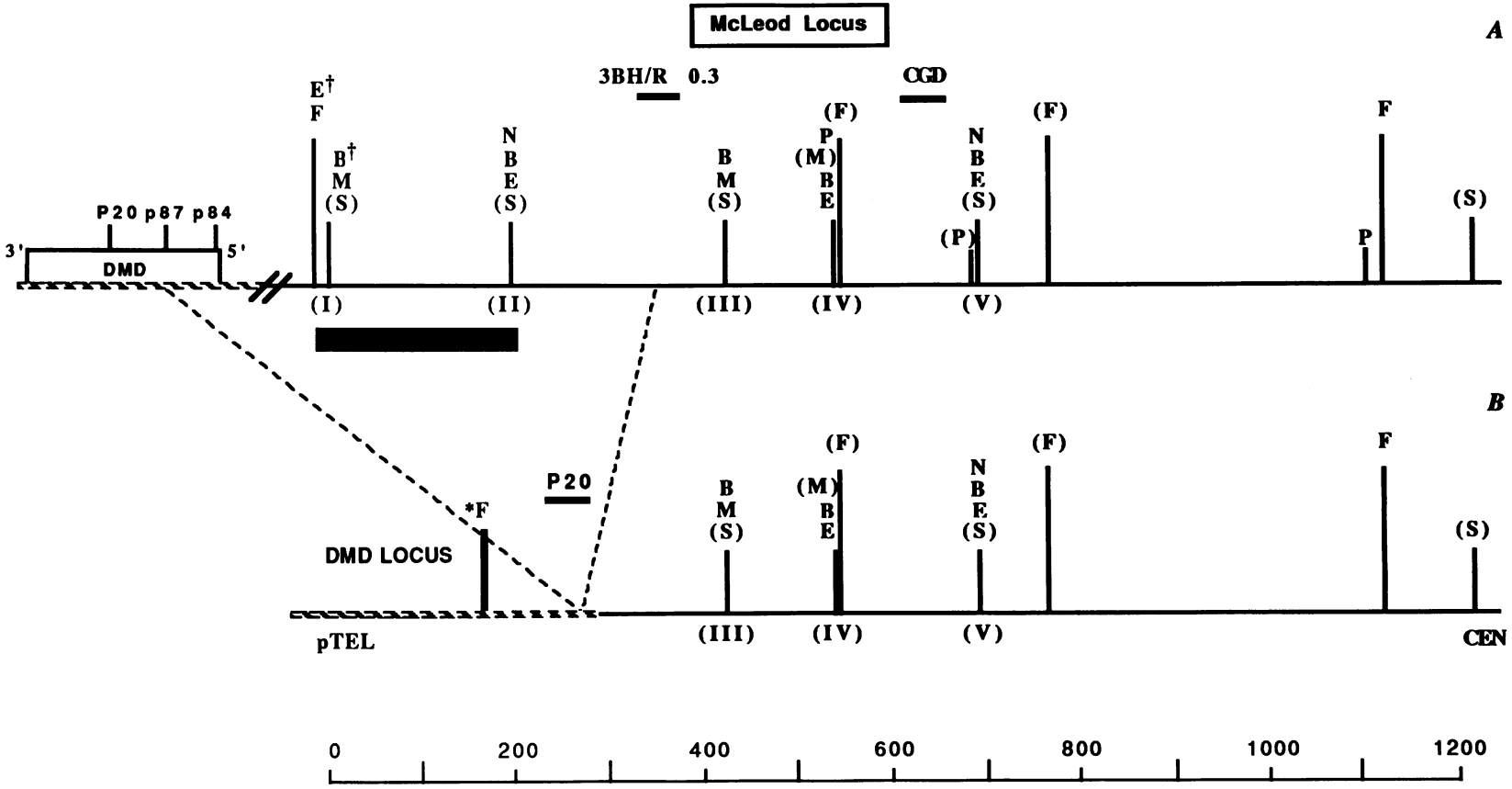

Figure 7 Comparative physical maps of region around McLeod locus in normal and in deleted X chromosome of patient JD. *A*, Restriction map encompassing McLeod and CGD loci that was constructed using DNA isolated from blood of normal male individual and digestion with all combinations of restriction enzymes *Sfi*I (F), *Mlu*I (M), *Bss*HII (B), *Eag*I (E), *Not*I (N), *Sac*II (S), and *Fsp*I (P). CpG islands identified by the presence of rare-cutting enzyme sites have been designated I–V. Comparative mapping against the deleted X chromosome of JD has enabled the assignment of the new marker, 3BH/R 0.3, to a region located 30–150 kb distal to island III. The minimal regions to which CGD, P20, and the McLeod locus could be assigned are indicated above the map. The blackened box below islands I and II highlights the region of cross-hybridization detected by 3BH/R 0.3; the presence of *Bss*HII and *Eag*I sites at island I (indicated by [†]) was inferred from the size of the cross-hybridizing band observed. *B*, Restriction map of deleted X chromosome in patient JD. The broken lines indicate a region of ~6,000 kb that was deleted in JD, and the horizontal hatched line denote sequences from the DMD locus. The *Sfi*I site which originates from the DMD locus is indicated by an asterisk (*). Partially cleaved restriction-enzyme sites are shown in parentheses.

gests which produced fragments that contained the 190-kb region (between islands I and II) and the 3BH/R 0.3 region (between islands II and III) did not give rise to any cross-hybridizing fragment. In the series of single and double digests with *Eag*I, a cross-hybridizing fragment of 210 kb was detected, indicating the presence of an *Eag*I site located 20 kb distal to CpG island I (fig. 7).

*Ksp*I (Boehringer Mannhein), an isoschizomer of *Sac*II that is not sensitive to the presence of 5′-methylcytosine, was used alongside *Sac*II to examine the methylation status of the DNA used. In both enzyme digests, an identical series of partial digestion products were observed (fig. 5), indicating that this restriction site is refractory to digestion, by virtue of sequence environment rather than by methylation.

PFGE Analysis of DNA from Patient JD

Hybridization of CGD-cDNA to patient JD's DNA digested with *Sac*II or *Bss*HII did not reveal any aberrant size fragments, as shown in figure 4*A*. In *Not*I digests, however, both markers hybridized in the limiting mobility (>1,000 kb), as opposed to the 510-kb fragment seen in the control lane (fig. 4), which suggested that the CpG island at site II has been deleted in patient JD. Confirmation of this was obtained by hybridization of 3BH/R 0.3 to a series of double digests using *Not*I and the enzymes present at CpG island III. As shown in figure 6*B*, 3BH/R 0.3 hybridized to a much larger fragment as a result of the deletion at that site.

It is interesting that in *Sfi*I digests 3BH/R 0.3 identified novel fragments of 380 kb, 600 kb, and 960 kb (fig. 4*B*). This result indicated the presence of a "new" *Sfi*I site which maps 380 kb distal to CpG island IV. However, as sequences distal to P20 were not disrupted by the deletion, the *Sfi*I site observed was inferred to be the DMD gene region that elsewhere has been designated as site "F" (Den Dunnen et al. 1987). This was confirmed by hybridization with probe P20 which detected fragments whose size was identical to that of 3BH/R 0.3 in JD, for all enzymes tested (data not shown). Probe P20 has been mapped 80–200 kb proximal to this *Sfi*I (F) site (Wapenaar et al. 1988). These data, taken together with the known distance from P20 to 3BH/R 0.3 (fig. 3), were used to limit the site of 3BH/R 0.3 to a region located 30–150 kb distal to the CpG island III.

Hybridization of 3BH/R 0.3 to double digests of *Sfi*I with rare-cutting enzymes with sites at island III, detected an identical size fragment of 260 kb in JD (fig. 6*B*). As there are currently no markers that mapped between islands III and IV, these data were the only evidence that unequivocally confirmed the presence of a CpG island at that site.

Discussion

The McLeod locus was initially assigned, by deletion analysis, to a region flanked by the cloned loci of p145-12/p378-40-1 and the gene for CGD (Bertelson et al. 1988). Contrary to a previous estimate (Bertelson et al. 1988), the physical distance between these flanking markers was found to be >1,600 kb, as neither marker could be physically linked to CGD on PFGE analysis.

Cloning the junction fragment of patient JD has led to the isolation of two new distal markers with which to study the McLeod locus. The new markers, designated 3BH/R 0.8 and 3BH/R 0.3, are located, respectively, 10 kb and 12 kb proximal to the deletion breakpoint in JD. Because of the lack of a cell line or fresh blood sample from patients S/H, the proximal deletion endpoints in these patients could not be mapped by PFGE; consequently the critical region containing the McLeod locus was redefined by 3BH/R 0.3 and CGD.

When 3BH/R 0.3 and CGD were used as flanking markers, a physical map of 1,100 kb encompassing the gene loci for McLeod and CGD was constructed using DNA from a normal male individual. The restriction enzymes used were methylation sensitive and contained CpG dinucleotides in their recognition sequence. Extensive studies have found CpG islands at the 5′ end of all housekeeping genes and of several tissue-specific genes (Bird 1986; Lindsay and Bird 1987). Localization of CpG islands can therefore provide a powerful means with which to identify expressed sequences. From the physical map (fig. 7), five CpG islands (sites I–V) were found clustered within a 700-kb region. Each of these islands contained sequences that are recognized, on average, by four rare-cutting restriction enzymes, making them potential candidates for genes such as McLeod and CGD which are located in this Xp21 region.

Physical mapping of the rearranged X chromosome of patient JD has identified an *Sfi*I (F) site in the DMD gene. This restriction site has been particularly valuable in verifying the rare-cutter sites present at CpG island III and has enabled the localization of 3BH/R

0.3 to a region 30–150 kb distal to island III. However, the exact position of the CGD gene between islands IV and V is unknown. If the uncertainty concerning the precise location of both markers is taken into account and if it is assumed that the genomic size of the CGD gene is 25–30 kb (Orkin 1989), then the size of the region containing the McLeod locus can be estimated, from the map, to be 150–380 kb. Within this region, there are two independent CpG islands located 120 kb apart which represent potential sites for the McLeod gene.

It is interesting to note that, although 3BH/R 0.3 maps just 2.0 kb proximal to 3BH/R 0.8 (fig. 3), only the former detected a cross-hybridizing band on Southern blot analysis of both conventional and pulsed-field gels. The presence of a cross-hybridizing sequence rather than partial digestion product is supported by the fact that it is consistently observed for all enzymes tested and is absent in deletion patients BB, JD, and S/H. Analysis using PFGE has localized the region of cross-hybridization to a 190-kb interval defined by CpG islands I and II. In view of both its specific localization within Xp21 and copy number, the nature of this cross-hybridizing sequence is currently being investigated.

DNA analysis of McLeod patient FD, on conventional electrophoresis and PFGE, did not detect any abnormal size fragments (fig. 3). This suggests that a point mutation, a minor deletion, or a non-X-linked form of McLeod syndrome is responsible for the phenotype.

Chromosome walking in yeast artificial chromosomes (YAC) and cosmids has been initiated from the cloned loci of 3BH/R 0.3 and CGD, to isolate overlapping cosmid and YAC clones that span the McLeod syndrome critical region. These clones will be analyzed for (1) the presence of CpG islands identified in genomic DNA, (2) conserved sequences, (3) expressed sequences in RNA and cDNA, and (4) sequence differences between normal and McLeod patients. However, despite the usefulness of CpG islands as landmarks and starting positions from which to search for expressed sequences, it has been observed that not all genes are associated with CpG islands (Bird 1986; Lindsay and Bird 1987). Hence, to isolate all cDNAs from the McLeod syndrome critical region, whole YAC and cosmids that mapped within that interval will be labeled to high specific activity and will be used as probes to screen cDNA libraries according to a method described elsewhere (Elvin et al. 1990).

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