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Identification of Novel RFLPs in the Vicinity of CpG Islands in Xq28: Application to the Analysis of the Pattern of X Chromosome Inactivation

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Summary

Probes for CpG islands were cloned from the distal long arm of the human X chromosome; three of them were found to be polymorphic. A *Hin*dIII RFLP was identified by the probe 2-25 (DXS606), and it was mapped to the Xq27-Xq28 boundary. Probes 2-19 (DXS605) and 2-55 (DXS707), which identify *Eco*RI and *Msp*I polymorphisms, respectively, have been mapped to the distal part of Xq28, in the G6PD-RCP/GCP gene region. Probe 2-19 has been further localized about 16 kb from the 3′ end of the G6PD gene. The new RFLPs may be useful for the precise mapping of the many disease genes localized in this part of the human X chromosome. Probe 2-19 is highly informative, and it has been studied in greater detail. Using the methylation-sensitive rare-cutter enzyme *Eag*I in conjunction with the polymorphic *Eco*RI site, we were able to demonstrate that the RFLP may be used both to study randomness of X chromosome inactivation and for carrier detection in X-linked syndromes where nonrandom X inactivation occurs. It is conceivable that the combined use of 2-19 and of the probes described so far (pSPT-PGK and M27β) will make analysis of X inactivation feasible in virtually every female.

Introduction

We have recently isolated probes for genes of the distal long arm of the human X chromosome, from Xq24 to the telomere. From a genomic library of *Eag*I-*Eco*RI fragments, we have identified clones which are likely candidates as probes for CpG islands (Maestrini et al. 1990). They contain additional sites for rare-cutter restriction enzymes such as *Bss*HII and *Sac*II and correspond to demethylated regions of DNA. In addition, for most of them the *Hpa*II methylation pattern obtained from digestion of male and female DNAs was compatible with the presence of a methylated CpG island on the inactive X chromosome. Since all CpG islands isolated have been found to contain the 5′ end of a gene (Bird 1987), the *Eag*I-*Eco*RI fragments having the characteristics of a CpG island may represent probes for genes of the human X chromosome. In four cases, partial nucleotide sequencing of the clones has demonstrated correspondence to already known genes: LAMP2 (Manoni et al. 1991), HPRT, G6PD, and GdX (D. Toniolo, unpublished observations).

Many inherited diseases have been mapped to the distal long arm of the human X chromosome: it therefore would be of interest to determine whether any of the genes identified by our CpG-island probes corresponds to a known disease. The isolation and sequencing of cDNAs may give some insight into the role of the corresponding genes. However, the identification of polymorphic loci in the vicinity of each CpG island may also be very useful for defining more precisely their localization with respect to a known disease. The precise mapping of a gene is especially important, since the molecular defect of most genetic diseases is

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not known but could be identified from the cloning and sequencing of the corresponding gene.

X chromosome–specific RFLPs may also enable one to distinguish between the active and the inactive X chromosome. X chromosome inactivation is normally a random process, involving the maternally and paternally derived X chromosome (Lyon 1972). However, nonrandom X inactivation has been observed in certain mature cell populations in female carriers of X-linked disorders, and it has been attributed to selection against cell precursors expressing the defective gene. In X-linked immunodeficiencies, selective inactivation of the X chromosome carrying the mutant gene results in heterozygous females who are phenotypically normal (reviewed in Conley and Puck 1988). The assessment of the X inactivation pattern has thus been used for determination of the carrier status for X-linked severe combined immunodeficiency (X-SCID) (Conley et al. 1988; Goodship et al. 1988), X-linked agammaglobulinemia (XLA) (Fearon et al. 1987), and Wiskott-Aldrich syndrome (WAS) (Fearon et al. 1988; Greer et al. 1989). CpG islands probes from the PGK gene (Keith et al. 1986) and the HPRT (Wolf et al. 1984) gene enable one to distinguish between the methylation pattern of the CpG island on the active X chromosome and that of the CpG island on the inactive X chromosome, and RFLP heterozygosity has been used successfully. More recently, the VNTR probe M27β (DXS255) has also been shown to recognize a different methylation pattern on the active versus the inactive X chromosome (Boyd and Fraser 1990).

In the present paper we report the finding of three RFLPs—2-25 (DXS606), 2-19 (DXS605), and 2-55 (DXS707)—identified by CpG island probes. One of them, 2-19, is highly informative. We show that this RFLP can be used to study the randomness of X chromosome inactivation.

Material and Methods

Probes

Probes 2-19 (DXS605, 2-25 (DXS606), and 2-55 (DXS707) were isolated from an *Eag*I-*Eco*RI human genomic library according to a method described elsewhere (Maestrini et al. 1990). They are all cloned in Bluescript. Probe 2-19 is a 6-kb DNA fragment, and probe 2-55 is a 2.5-kb DNA fragment. Probe 2-25 was originally a 9-kb DNA fragment; however, it is highly unstable, and several derivatives with insert size ranging from 2 to 4 kb have been isolated. In each derivative clone a region has been identified which is deleted to different extents. This region, however, does not appear to be variable in the human genome. Analysis of the randomness of X inactivation was carried out with pSPT-PGK and M27β DNA probes, as described elsewhere (de Saint-Basile et al., submitted).

Cell Lines, DNAs, and Hybridization

The TC hybrids were isolated from a human-hamster cell hybrid containing only the human X chromosome (HY.84P11), according to a method described elsewhere (Suthers et al. 1990). In brief, the HY.84P11 cells were treated with FUDR and caffeine to induce both the expression of the FRAXA and breakage of the X chromosome. Clones resistant to 6-thioguanine and diamide (Rosenstraus and Chasin 1975; D'Urso et al. 1983) and that have lost the human HPRT and have retained the human G6PD gene were isolated.

DNA methylation was studied on genomic DNA prepared from leukocytes of normal individuals and from the cell types indicated for each carrier female, according to a method described elsewhere (Toniolo et al. 1984). Southern blotting and hybridizations were as described by Maestrini et al. (1990).

Results

Identification of Three Polymorphic DNA Sites

In the course of the characterization of the *Eag*I-*Eco*RI recombinant clones from our library, RFLPs have been identified by the probes 2-25 (DXS606), 2-19 (DXS605), and 2-55 (DXS707): 2-25 identifies a *Hin*dIII RFLP with 7.5-kb and 5-kb alleles; 2-19 is an *Eco*RI RFLP with two alleles, one of 6.5 kb and one of 8.3 kb; 2-55 is an *Msp*I RFLP with 2.3-kb and 1.4-kb alleles. The allelic frequencies of the polymorphisms are shown in table 1.

The *Hin*dIII polymorphism was studied in 66 unrelated X chromosomes; low heterozygosity was demonstrated. In addition, the 5-kb allele may not be present in every population, since, in our preliminary screening, it was found only in people originally from southern Italy. The *Msp*I polymorphism was studied in 83 unrelated X chromosomes; the 2.3-kb allele is also not very common, and it too may be more frequently found in southern Italy. The *Eco*RI polymorphism identified by 2-19 is more informative and equally distributed in all populations analyzed.

Table 1

Allele Frequency

Locus	Probe	Alleles (kb)	Enzyme	Allele Frequency	No. of Chromosomes Analyzed
DXS605.....	p2-19	6.5/8.3	*Eco*RI	.61/.39	69
DXS606.....	p2-25	7.5/5[a]	*Hin*dIII	.89/.11	66
DXS707.....	p2-55	2.3/1.4	*Msp*I	.30/.70	83

[a] Found only among people from southern Italy.

Physical Mapping

Probe 2-25 was mapped to Xq27, and probes 2-19 and 2-55 were mapped to Xq28 (Maestrini et al. 1990). To better define their localization they were hybridized to a small panel of Chinese hamster-human hybrids with breakpoints in Xq27-Xq28 and were isolated as described in Material and Methods. The X chromosome DNA present in each hybrid was studied by hybridization to the probes indicated in figure 1. This analysis has shown that two of the hybrids (TC2.9 and TC4.9) possess only the region of the X chromosome corresponding to the G6PD and GCP/RCP genes. Other hybrids (TC2.7 and TC4.8) also retain DNA hybridizing to the probes DX13 (DXS15) and St14 (DXS52). One hybrid (TC1.2) has, in addition, DNA hybridizing to probes in the Xq27.3-Xq28 region. With the exception of TC4.9, all hybrids also carry most of the short arm of the X chromosome (not shown). In this panel of hybrids probe 2-25, which hybridizes to TC1.2, TC2.7, and TC4.8, maps to the Xq27-Xq28 boundary.

Probes 2-19 and 2-55, which also hybridize to TC2.9 and TC4.9, map to the G6PD-GCP/RCP region. Since this is not a very large DNA region (Poustka et al., in press), we have tried to localize the probes by hybridization to Southern blots of high-molecular-weight DNA fractionated by pulsed field gel electrophoresis (PFGE). Probe 2-19 was found to hybridize to the same 400-kb *Mlu*I and 300-kb *Not*I fragments as did the G6PD gene in a lymphoblastoid cell line derived from a normal male (not shown). Hybridization to cloned DNA (cosmids and lambda phages) of the G6PD region showed that 2-19 hybridizes to a cosmid, pTV1, containing the 3′ region of the G6PD gene, to its 3′ flanking DNA, and to the 5′ DNA flanking the adjacent P3 gene (Martini et al. 1986; Alcalay and Toniolo 1988). Probes 2-19's precise location in the map of the region is shown in figure 2.

X Chromosome Inactivation

Restriction-enzyme analysis of genomic DNA of homozygous and heterozygous females has shown that the polymorphic *Eco*RI site corresponds to the *Eco*RI cloning site of 2-19 (not shown). Restriction fragments derived from 2-19 (fig. 3*A*) were used to study methylation of male and female genomic DNA. When 2-19ES was used as a probe in *Eco*RI + *Hpa*II digestions, bands common to male and female DNA—as well as higher-molecular-weight bands, in females, that may correspond to methylation of the DNA on the inactive X chromosome—were detectable (not shown). However, in heterozygous females, we were unable to see any fragment that could be related to the RFLP. When 2-19EP was used, only very small *Hpa*II fragments were detectable, and no methylation

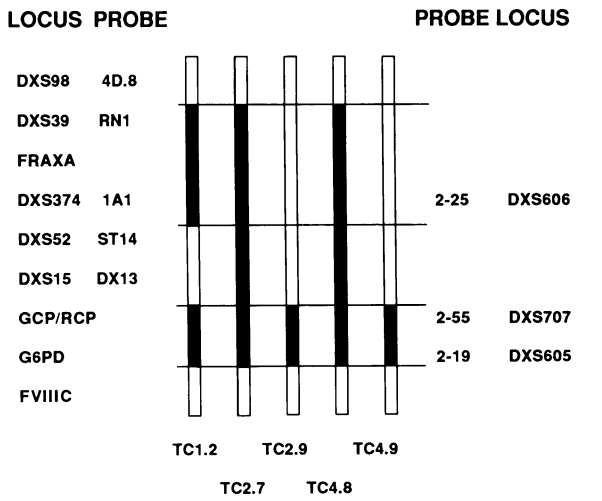

Figure 1 Schematic representation of the hybridization data obtained with the hybrid panel. Blackened regions denote presence of hybridization signal. The order of loci is based mainly on the PFGE map of Poustka et al. (in press).

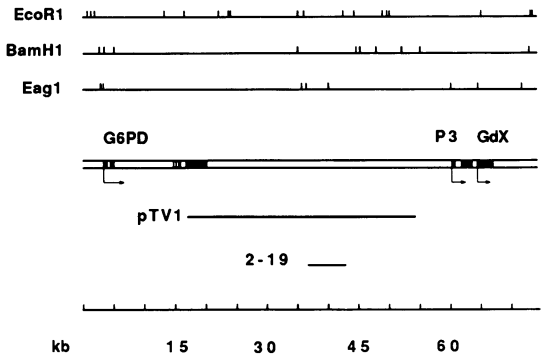

Figure 2 Localization of 2-19 on the human X chromosome. The G6PD, P3, and GdX genes are positioned under the restriction map for *Eco*RI, *Bam*HI, and *Eag*I. Blackened regions are exons. Below is the cosmid pTV1 (Martini et al. 1986) and p2-19.

differences between male and female DNA were detected. It is therefore likely that the CpG island is separated from the polymorphic *Eco*RI site by at least one *Hpa*II site unmethylated on both active and inactive chromosome.

Since several different rare-cutter enzyme sites were found in the 2-19 region, we tested the use of methylation-sensitive rare-cutter restriction enzymes to study the methylation pattern of the 2-19 CpG island. Double and single digestions were performed with *Eco*RI and *Eag*I and with *Bss*HII and *Nru*I. *Eag*I did demonstrate reproducible differences between male and female DNA. Male leukocyte DNA in *Eag*I + *Eco*RI digestions probed with 2-19EP showed only one band corresponding to methylation of site Ea3 and to demethylation of site Ea2 (fig. 3*B*, lanes 1–6). All homozygous females showed two bands: one that was the same size as that in male DNA and one that corresponded to methylation of site Ea2 in addition to Ea3. (Fig. 3*B*, lanes 7 and 8). Site Ea3 was always found methylated, in male and in female DNA. Heterozygous normal females (fig. 3*B*, lanes 9–13) showed four bands. The same results were obtained from DNA of leukocytes of 6 males, 6 homozygous females, and 10 heterozygous females.

To conclusively demonstrate that the pattern in heterozygous females correlates with X inactivation, we analyzed nine heterozygous females from pedigrees affected with XLA or WAS. Five females were obligate carriers, and four belonged to pedigrees with sporadic occurrence of the disease (table 2). In all but one (subject AM) nonrandom X inactivation in the relevant cell type was confirmed with pSPT-PGK and/or M27β probes. Nonrandom X chromosome inactivation was also demonstrated with probe 2-19. In eight cases (fig. 3*C*) in the *Eco*RI + *Eag*I digestions, only two bands were detectable, those corresponding to complete demethylation of one of the two alleles and to methylation of the other allele. The only exception was AM, whose DNA showed four bands.

Discussion

In the present paper we have reported the characterization of three RFLPs of the distal long arm of the human X chromosome, in Xq28. The *Hin*dIII RFLP identified by 2-25 was mapped to the proximal part of Xq28. An *Msp*I RFLP identified by probe 2-55 was mapped to distal Xq28, in the G6PD-RCP/GCP gene region. They may be useful markers in the genetic map of the human X chromosome, as they are localized in a DNA region where many inherited diseases have been mapped, but few polymorphic DNA sites are available for linkage studies. The third RFLP identified by 2-19 also maps to the distal part of Xq28. By sequential hybridizations to a small hybrid panel with breakpoints in Xq28, to PFGE filters, and to a large cloned region of DNA flanking the G6PD gene, we were able to localize 2-19 to a region 16 kb downstream from the 3′ end of the G6PD gene. It is worth mentioning that this is the first common polymorphic DNA site found in this part of the X chromosome, where DNA variations are apparently very rare (D'Urso et al. 1988) despite the high number of mutations in the G6PD coding region, which are responsible for the numerous G6PD variants described in many parts of the world (Beutler 1991).

Probe 2-19 corresponds to a CpG island: several rare-cutter enzyme sites map in the region, and the methylation pattern of the *Hpa*II sites is typical of a CpG island on the X chromosome (Toniolo et al. 1984). Accordingly, a cDNA corresponding to 2-19 has also been isolated (not shown). Thus a fourth gene is apparently located within the 80 kb of DNA where the G6PD and two housekeeping genes of unknown function P3 and GdX have been identified (Alcalay and Toniolo 1988). This is an extremely high concentration of genes, and it will be interesting to determine both how frequent such clustering of genes is along the chromosomes and whether it has some functional significance.

Unlike methylation of most mammalian DNA, which is often variable, the pattern of DNA methylation of CpG islands on the X chromosome is highly

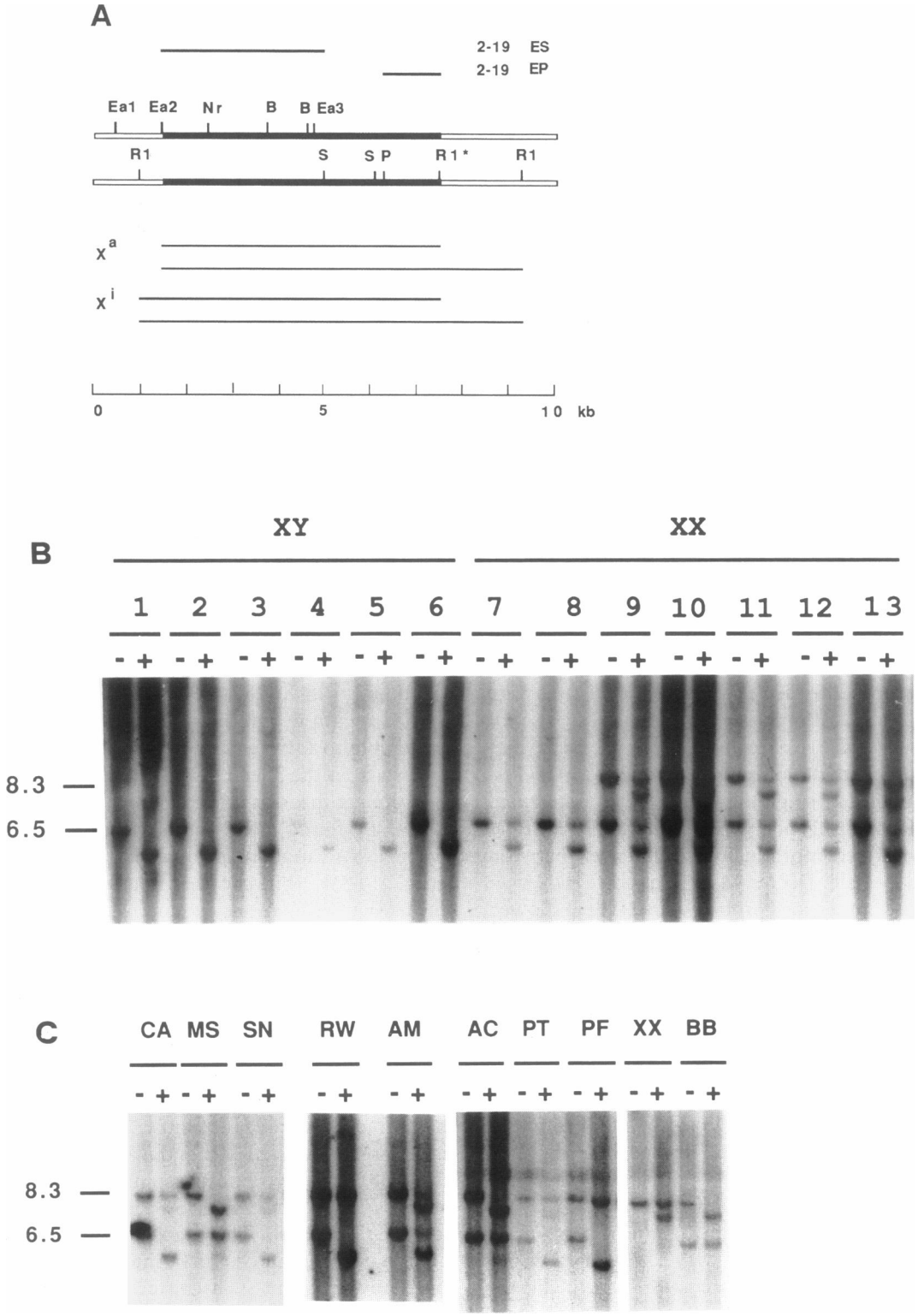

Figure 3 *A*, Restriction map of the 2-19 genomic region, derived from pTV1 (blackened region is 2–19). Above are the probes used in the hybridizations. Below is a schematic representation of the bands obtained in the *Eco*RI + *Eag*I digestion of the active (X^a) or inactive (X^i) chromosome. Restriction enzymes are indicated: Ea = *Eag*I; RI = *Eco*RI; Nr = *Nru*I; B = *Bss*HII; S = *Sac*I; P = *Pst*I. *B*, Southern blot of leukocyte DNA, from normal males (XY) and females (XX), digested with *Eco*RI (–) or *Eco*RI + *Eag*I (+) hybridized to 2-19EP. *C*, Southern blot as in A, except that DNAs are from the cell types and the heterozygous females described in table 2.

Table 2

Pattern of X Inactivation in Females from Pedigrees with X-linked Immunodeficiencies

Subject	Carrier Status	Cell Type	X Inactivation[a] with				Reference (sample)
			2-19	PGK	HPRT	M27β	
SN.....	Carrier of XLA	B lymphocyte	Nonrandom	Nonrandom	NI	NT	Guioli et al. 1989 (family N)
RW.....	Carrier of XLA	B lymphocyte	Nonrandom	NI	NI	Nonrandom	Guioli et al. 1989 (family P)
MS.....	Mother of a sporadic case of WAS	White blood cell	Nonrandom	Nonrandom	NT	Nonrandom	de Saint-Basile et al., submitted (family XXV)
CA.....	Mother of a sporadic case of WAS	B lymphocyte	Nonrandom	NT	NT	Nonrandom	L. D. Notarangelo, unpublished data
AM.....	Carrier of WAS	T lymphocyte	ND°	Nonrandom°	NT	Nonrandom°	L. D. Notarangelo, unpublished data
AC.....	Mother of a sporadic case of WAS	B lymphocyte	Nonrandom	Nonrandom	NT	Nonrandom	de Saint-Basile et al., submitted (family XXIII)
PT	Carrier of WAS	T lymphocyte	Nonrandom	NI	NT	Nonrandom	de Saint-Basile et al., submitted (family IX)
PF.....	At risk for WAS	B lymphocyte	Nonrandom	NI	NT	Nonrandom	de Saint-Basile et al., submitted (family IX)
BB.....	Mother of a sporadic case of WAS	B lymphocyte	Nonrandom	NI	Nonrandom	Nonrandom	L. D. Notarangelo, unpublished data

[a] NI = noninformative; ND = not determined; and NT = not tested.

[b] Bands corresponding to inactivation of the normal X chromosome were also present. The ratios were 92:8 for pSPT-PGK and 90:10 for M27β. For discussion of the results with 2-19, see text.

conserved among different individuals and tissues. For this reason X-specific polymorphic probes corresponding to CpG islands (i.e., PGK and HPRT) have represented an extremely useful and very reliable tool for analyzing the pattern of X chromosome inactivation and for performing carrier detection in several X-linked diseases. However, use of the probes is impaired by the low heterozygosity. As many as 50% of the females are noninformative for both pSPT-PGK and HPRT probes, thus limiting the feasibility of carrier detection. More recently, an X chromosome–specific DNA probe (M27β) which detects both a VNTR sequence and a different methylation pattern on the active vs the inactive X chromosome has been isolated. While heterozygosity at this locus is extremely high (about 90% in the literature and 88% in our own experience), the methylation pattern is more complex than initially described (Fraser et al. 1989; Boyd and Fraser, in press). The active X is always methylated. The methylation pattern on the inactive X is variable at this locus, although unmethylation is preferentially observed. This phenomenon may at times affect interpretation of the results. The Xq28 probe 2-19, reported in the present paper, detects a simple, CpG island–associated methylation pattern when a rare-cutter restriction enzyme, *Eag*I, is used. Both the heterozygosity at this locus and the simple pattern of methylation make this probe of interest for the study of X inactivation and for carrier detection. It is thus conceivable that the combined use of the probes described so far makes analysis of randomness of X inactivation feasible in virtually every female.

We have applied the novel probe 2-19 to the analysis of the pattern of X chromosome inactivation in nine heterozygous females belonging to pedigrees with XLA or WAS. Several studies have demonstrated that obligate carriers of XLA or WAS exhibit a nonrandom pattern of X inactivation in the cell lineages affected with the disease. In eight of nine cases a complete concordance was observed between the patterns detected by probe 2-19 and by pSPT-PGK, M27β, or HPRT. A single female, AM, showed four bands on digestion with *Eag*I + *Eco*RI and hybridization to 2-19 (fig. 3*C*), a pattern characteristic of random X chromosome inactivation. Two bands corresponding to demethylation of the *Eag*I site Ea2 on both chromosomes were more prominent. This female is an obligate carrier of WAS: she is the mother of two affected males and belongs to a pedigree with three generations affected (not shown). Analysis of X chromosome inactivation in this female by means of the pSPT-PGK and M27β probes gave a pattern of incomplete nonrandom X inactivation (by densitometry, the ratios between the active X's and the inactive X's bands were 92:8 and 90:10, respectively). Although, as a rule, carriers of WAS display a nonrandom pattern of X inactivation, some cases have been reported in which random X inactivation has been demonstrated (Gealy et al. 1980; Fearon et al. 1988). These data have suggested that the WAS mutation may not be completely deleterious to differentiation and proliferation of hematopoietic precursors expressing the mutated X chromosome as the active X. Our data are in keeping with these observations. Furthermore, the unusual pattern detected by 2-19 in this subject suggests that variations in the methylation pattern may occasionally be present also at this locus; caution should therefore be used in the analysis of methylation for diagnostic purposes. That these variations are not common occurrences, however, is indicated by the reproducible pattern found in the normal population (6 males and 16 females analyzed in the present study).

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