Pulse-field linkage of the P3, G6pd and Cf-8 genes on the mouse X chromosome: demonstration of synteny at the physical level

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ABSTRACT

Utilising pulse-field gel electrophoresis physical linkage between three mouse X-linked genes has been demonstrated. The three genes, *P3*, *G6pd* and *Cf-8* all lie within 400Kb of DNA. This physical linkage mirrors the situation on the human X chromosome, respresenting the first demonstration of mouse/human synteny at the physical level. A detailed physical map encompassing 1.6 Mbp of this region is presented. A number of the rare cutter restriction enzyme sites within this map are partially blocked on the inactive X chromosome, presumably due to the methylation of CpG rich islands. Pulsed field gel electrophoresis therefore provides a useful tool for the study of X-inactivation over large regions of the X chromosome.

INTRODUCTION

New technologies in mammalian molecular genetics, in conjunction with extensive classical genetic information, have led to considerable progress towards the molecular analysis of the mouse X chromosome. Attention has been focused on a number of loci, many of which are putative homologues of human X-linked disease loci, and in addition on the comparative evolution of human and mouse X chromosomes and the mechanisms of X chromosome inactivation.

Interspecific crosses between *Mus musculus domesticus* and *Mus spretus* have been utilized to position anonymous DNA clones and known X-linked genes with a high degree of accuracy (1,2,33). The resultant molecular map now contains some 50 localised markers, providing an average probe density of one every 2 cM (approximately 3,000 Kbp). It is now possible to contemplate linking up a number of these genetically localised markers in a physical map using pulsed field gel electrophoresis (PFGE).

Considerable information concerning the syntenic relationship of the human and mouse X chromosomes has been derived from these studies. The PLP gene has been localised to the mouse and human X chromosomes. In mouse the neurological *jp* mutation has been shown to correspond to an alteration of the PLP gene (4). Sequences from the human DMD gene were localised to the *mdx* locus (5,6,7,34). A number of other human X-linked genes have been localised on the mouse X chromosomes; *CGD* (8), *P3*, (3), *F8*, *F9* and *CBP* (3,9). These, together with other known and putative genetic homologies, have made it possible to define five major conserved segments, suggesting a minimum number of five breakpoints to account for the divergence of human and mouse X chromosomes (3,8,10). More detailed studies may reveal additional breakpoints and it is likely that a number of smaller chromosomal rearrangements will have occurred within syntenic blocks.

Little is known about the molecular mechanism of X chromosome inactivation. The *Xce* locus has been strongly implicated in the control of this process (11). It is also known

that CpG rich islands which are found in association with a wide variety of genes (12,13) are hypermethylated on the inactive X chromosome relative to the active X chromosome. Genes found in the pseudoautosomal region of the human chromosome escape X inactivation and apparently remain hypomethylated within their CpG rich islands (14,15,16). It is thought that methylation on the inactive X chromosome plays a role in maintaining the inactive state, but is not the primary event causing the inactivation process to occur (17).

The advent of PFGE has provided an essential tool for the molecular analysis of complex genomes. Used in conjunction with restriction enzymes which cut infrequently in the mammalian genome (rare cutters) it is possible to construct long range restriction maps of the mammalian genome (18). The high CpG content, and methylation sensitivity of the recognition sequences for rare cutter restriction enzymes means that target sites often occur in the hypomethylated CpG rich islands associated with many mammalian genes (19). PFGE maps therefore provide an indication of the position of uncharacterised genes, as well as providing a means of determining the physical distance separating genetically linked DNA markers.

On the human X chromosome PFGE has been used to establish physical maps around the DMD locus (20), the pseudoautosomal region (15,16) and in the Xq27−Xqter interval (21,22). In this paper we describe the detailed physical mapping of a region of the mouse X chromosome which is syntenic with part of the human Xq27−Xqter interval, and the detailed comparative distributions of 3 genes−P3, G6PD and F8. Furthermore, the resultant physical map was used to examine differences in the methylation status of the active and inactive X chromosomes within this region.

MATERIALS AND METHODS

DNA probes

Probes G28A and G28B were derived from a mouse genomic clone (λG28) isolated using the human pGD3 probe that contains the P3 gene (35). Probe sp64VIII contains a full length human factor VIII cDNA insert (gift of Genetics Institute). Probe pGD-T-5B contains a full length human G6PD cDNA insert (gift of L. Luzatto). All probes were labelled by the random priming method (23).

Pulsed Field Gel Analysis

Thymocytes were isolated from the thymus of 3 week old male and female C57Bl/10 mice. DNA was prepared in agarose blocks using established methods (24). Restriction enzyme digests (including Hind III digests) were performed on agarose blocks for 4−20 h under standard conditions. Partial digestions with Bssh II were achieved using a combination of variable time intervals and variable salt concentrations, (details in relevant figure legend). Concatamerised λ DNA and yeast chromosomes (*S.cerevisiae*, strain YP148 and *S. pombe*) were used as molecular weight standards (25). Uncertainty concerning the precise size of some yeast chromosomes, together with variable migration of mouse DNA as a function of sample concentration (20) means that fragment sizes represent an estimate and may show inaccuracies of 10−15%. Generally, agarose blocks contained approximately 10 μg of mouse DNA.

Initial PFGE experiments were performed on the LKB pulsaphor OFAGE apparatus. Conventional runs, resolving 50−1000 Kbp were performed on 1% agarose gels in 0.5×TBE buffer at 13°C. A voltage gradient of 6V/cm was used for 40 h. Switching intervals are indicated in the relevant figure legends. Very high molecular weight separations (1−7 Mbp) were performed on 0.65% agarose gels at 13°C for a total of 14 days at

1.1V/cm. Switching intervals are indicated in the relevant figure legend. Subsequent PFGE experiments were performed on a CFGE apparatus (26). Conditions were essentially the same as with OFAGE, except the voltage gradient was 4V/cm. Switching intervals are indicated in the figure legends.

Southern Analysis

Gels were stained with ethidium bromide and often destained for 1−2 hours in running buffer prior to photography. PFGE gels were exposed to U.V. light for 2−5 min. in order to nick the DNA prior to blotting. Gels were then transferred to Gene Screen nylon membranes, after standard denaturing and neutralising procedures. The DNA was fixed to nylon membranes by baking (2 h, 80°C) followed by brief exposure to U.V. light.

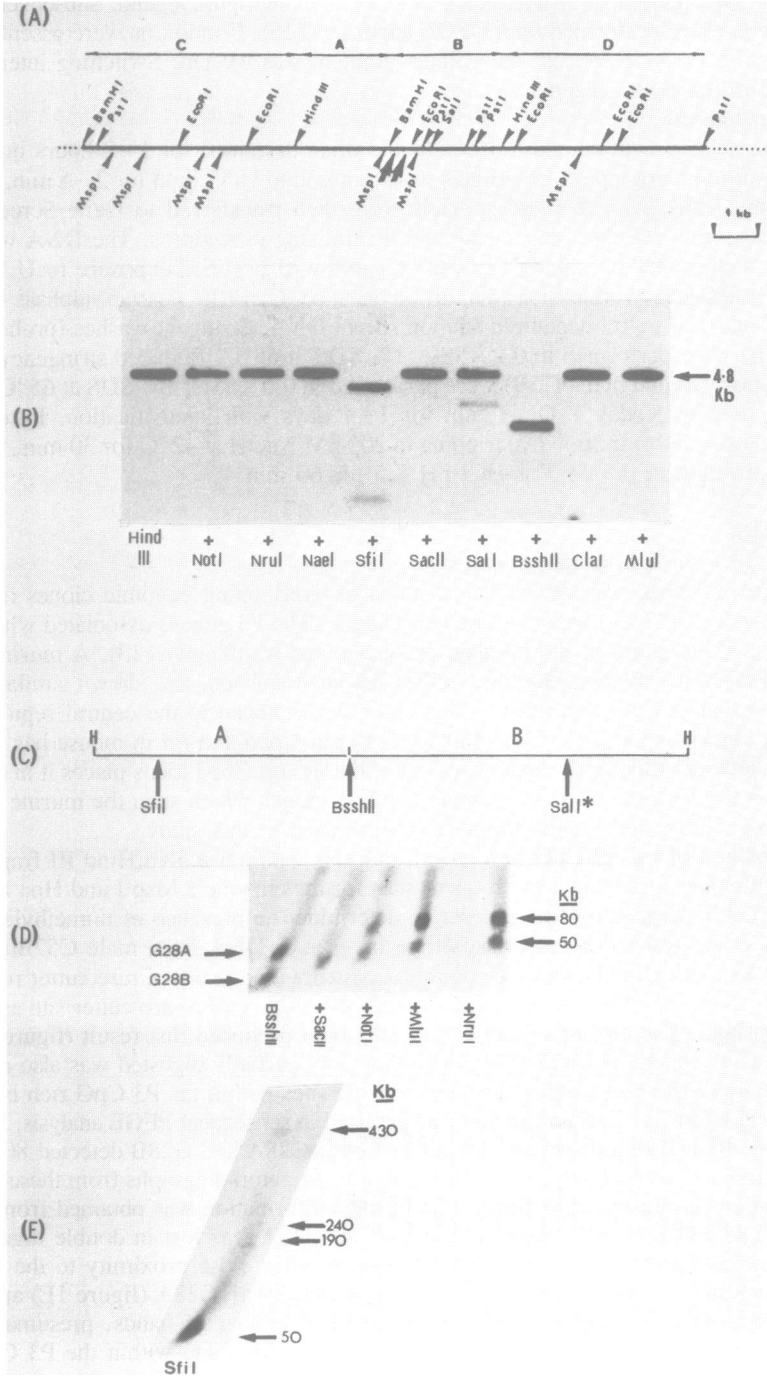
Pre-hybridisation/hybridisation was carried out at 65°C in 10% dextran sulphate, 5×SSC, 1% SDS and 100 μg/ml denatured salmon sperm DNA. Stringent washes (probes G28A and G28B) were performed in 0.2×SSC, 1% SDS at 65°C. Reduced stringency washes (probes sp64VIII and pGD-T-5B) were performed in 0.5×SSC, 1% SDS at 65°C. Filters were exposed to Kodak X-Omat film for 1−7 days with intensification. Filters were prepared for re-hybridisation by stripping in 200mM NaOH at 42°C for 30 min, followed by neutralization in 0.5 M Tris-HCl pH 7.5 for 60 min.

RESULTS

Mapping of the P3 CpG rich island

The human P3 gene was detected in a series of overlapping genomic clones (spanning approximately 100 Kb) containing the G6PD gene. The P3 gene is associated with a CpG rich island. The nature of the P3 gene product remains unknown (13). A murine clone, homologous to the human P3 gene (λ G28) has been isolated, and shown similarly to be associated with a CpG rich island. This clone was mapped to the central region of the mouse X chromosome (35). Close linkage between *G6pd* and *P3* in mouse has not been formally proved, although genetic mapping of the mouse G6pd locus places it in the same vicinity as the P3 gene (28). Two single copy subclones which span the murine P3 CpG rich island (G28A and G28B, figure 1A) were used in this study.

The position of the P3 CpG rich island, that lies within a 4.3Kb Hind III fragment of the λ G28 clone, is indicated by frequent sites for the enzyme's Msp I and Hpa II (figure 1A, doublet of large arrows). In order to determine the presence of unmethylated sites for rare cutting restriction enzymes within this island, DNA from male C57Bl/10 mice was digested with Hind III and then double digested with a series of rare cutter restriction enzymes. Cleavage of this fragment indicated the presence of a rare cutter site associated with the island. The enzymes Bssh II and Sfi I both produced this result (figure 1B). In addition a site for the enzyme Sal I which was only partially digested was also detected. The position of the rare cutter restriction enzyme sites within the P3 CpG rich island are indicated in figure 1C. This information was utilized in subsequent PFGE analysis. Southern analysis of Bssh II digests revealed that the probes G28A and G28B detected 80 Kb and 50 Kb fragments respectively. Superimposition of the autoradiographs from these separate hybridisations is illustrated in figure 1D. Further information was obtained from double digests. The 80 kb Bssh II fragment detected by G28A was lost in double digests with Sac II, indicating the presence of a Sac II site in fairly close proximity to the P3 CpG island. Digests with the enzyme Sfi I, when hybridised with G28A (figure 1E) and G28B (not shown) gave a complex result consisting of a number of bands, presumably as a consequence of partial digestion of certain sites. The Sfi I site within the P3 CpG rich


(A)
C
A
B
D
BamHI
PstI
EcoRI
HindIII
SalI
MspI
1 kb
(B)
4·8
Kb
Hind III
NotI
NruI
NaeI
SfiI
SacII
SalI
BsshII
ClaI
MluI
(C)
H
A
B
H
SfiI
BsshII
SalI*
(D)
Kb
80
50
G28A
G28B
BsshII
+SacII
+NotI
+MluI
+NruI
(E)
Kb
430
240
190
50
SfiI

island apparently is cut to completion (figure 1B), so it was assumed that the partial bands detected with G28B represented consecutive Sfi I sites in the G28B direction. The same bands were detected with the G28A probe, but it was difficult to determine whether any additional bands were also present. Only a small fragment of the G28A probe lies on the left side of the internal Sfi I site (figure 1C) and it is likely that any bands detected by this portion of the probe would be relatively weak.

The enzymes Mlu I, Nru I and Not I produced fragments in the limiting mobility region of conventional pulsed field gels. In order to resolve these fragments, pulsed field gels resolving fragments in the range 1 – 7 Mbp were carried out, (see Materials and Methods). A 1.4 Mbp fragment was detected in Mlu I digests (figure 2). Nru I produced two fragments of 1.4 and 1.6 Mbp fragment (data not shown). No clear bands were seen with the enzyme Not I. With this data it was therefore not possible to determine the position of the P3 island within these large fragments. The data obtained with the G28A and G28B probes is summarised in table 1 and in figure 5.

Linkage of the P3 and Cf-8 genes

Close genetic linkage between the G28 clone and the mouse homologue of the coagulation Factor VIII gene (Cf-8) was recently demonstrated (9,33). We obtained a human factor VIII complete cDNA clone (sp64 VIII) for use in PFGE analysis. Initially, physical linkage of the mouse Cf-8 and P3 genes was indicated by the common hybridisation of G28A and sp64 VIII to the 1.4 Mbp MluI fragment (figure 2B). Single and double digests with the other rare cutter enzymes provided a detailed map encompassing approximately 250 Kbp around the Cf-8 gene (table 1 and figure 5). Double digests demonstrated that sites for MluI and NruI are present within this map. The result suggested that the Cf-8 gene lies at one end of the large MluI and NruI fragments. The NruI site closest to the Cf-8 gene was apparently cut to completion, suggesting that the 1.4 and 1.6 Mbp NruI fragments detected with P3 probes occurs through partial digestion of an NruI site 1.4 Mbp distant from the Cf-8 gene. The data obtained in these experiments, however, was not sufficient to allow the precise positioning of the P3 gene within the long range map.

Incorporation of G6pd within the map

A human G6PD cDNA clone (pGD-T-5B) was utilized in further experiments. This clone detected the 1.4 Mbp MluI fragment, in common with both Cf-8 and P3 probes (figure 2B). Analysis of Bssh II digests revealed that pGD-T-5B detects an 80 Kbp fragment, in common with G28A, and also a 240 Kbp fragment in common with Cf-8 (figure 3A,B,C). This result suggested that the G6pd exons span the 80 Kbp and 240 Kbp Bssh II fragments, and that the probable gene order was Cf-8-G6pd-P3. This supposition was confirmed as follows. A series of samples were digested to completion with MluI and then partially digested with Bssh II. Because the Cf-8 gene lies at one end of the MluI fragment, the ladder of bands produced by partial Bssh II digests represent sequential Bssh II sites within

Figure 1. Mapping of the P3 CpG rich island

Restriction map of the genomic clone G28(A) showing the position of the MspI/HpaII cluster (large arrows), and the position of the Hind III sites flanking the CpG rich island. Arrowed lines (top) indicate the subclones G28A (Hind III/Bam H1) and G28B (Bam H1/Hind III) used in this study. Rare cutter sites within the 4.8 Kbp Hind III fragment were detected using Hind III/rare cutter double digests and probing with G28A (B) and G28B (not shown). A map of the CpG island was produced (C) and provided the basis for subsequent PFGE analysis. (D) illustrates one such analysis, where the subclones G28A and G28B detected different sized Bssh II fragments (autoradiographs from both hybridizations superimposed). (E) illustrates the hybridisation pattern observed with the G28A probe on SfiI digests. Southern analysis is described in materials and methods. PFGE was performed on the OFAGE apparatus, with a switching interval of 60 seconds.

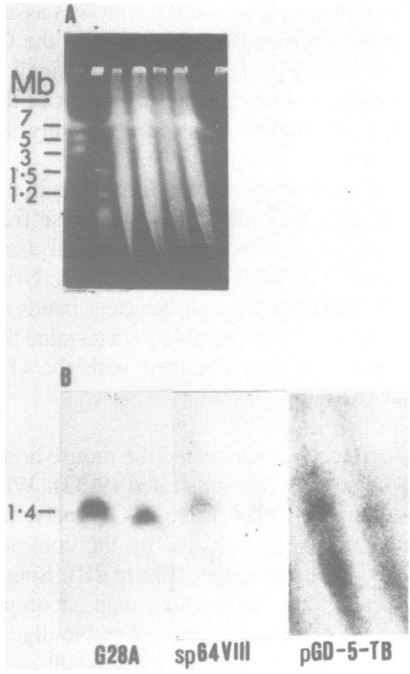


Figure 2. Resolution of very high molecular weight DNA
Ethidium bromide stained gel showing Mlu I digested DNA (10μg in lanes 1, 3 and 5, 5μg in lanes 2, 4 and 6) resolved in the size range 1–7 Mbp (A). Subsequent southern analysis (materials and methods) illustrates that the three probes G28A, sp64VIII and pGD-T-5B all detect a common 1.4 Mbp Mlu I fragment (B). PFGE was performed on the OFAGE apparatus. A ramped switching interval was used: 60 min (6 days), 30 min (5 days), 15 min (4 days). All three probes were hybridised separately to the same filter.

the 1.4 Mbp MluI fragment (figure 4A). The proposed gene order Cf-8-G6pd-P3 predicted a series of bands at 240, 320 and 370 Kbp. The experiment however clearly shows an additional band at 280 Kbp. Closer scrutiny of the 80 Kbp Bssh II band detected by G28A and pGD-T-5B revealed that this is in fact a doublet, a major band at 80 Kbp and a minor component at 40 Kbp. This is clearly demonstrated in figure 4B and 4C where the MluI/Bssh II partial digests were probed with G28A and pGD-T-5B. In both cases the doublet could be clearly seen in the first partial digest (lane 1), but became increasingly masked as the progress of the Bssh II digest increased. We conclude that there is a Bssh II site which lies in the centre of the 80 Kbp Bssh II fragment that is only partially digested in male DNA. It is not clear why this band was not apparent in figure 1D, although this may be due to the relatively weak signal obtained in that experiment. Figure's 4B and 4C also demonstrate further bands common to G28A and pGD-T-5B, confirming the proposed linkage.

The partial Bssh II digests shown in figure 4A also provided evidence of Bssh II sites at 420 Kbp and 570 Kbp from the Cf-8 gene (figure 5). It was not possible to position the Sac II sites produced by partial digestion (figure 3A) with certainty, so these sites have been excluded from the final map. A full summary of this data is shown in figure 5 and table 1.

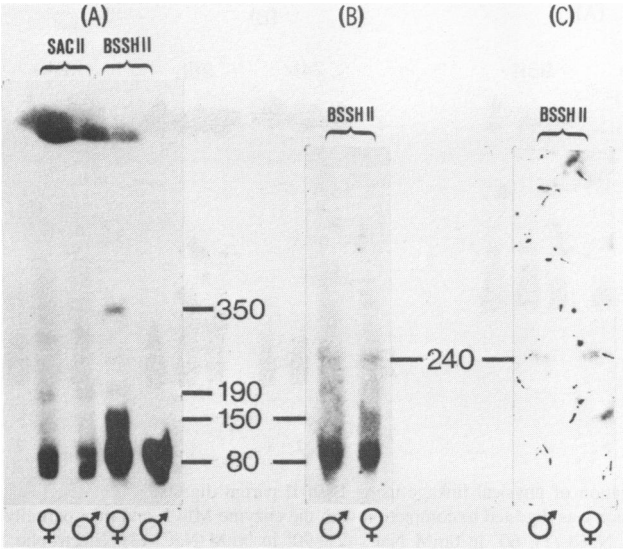


Figure 3. Physical linkage of the P3, G6pd and Cf-8 genes
DNA from male and female mice was digested with the enzymes Bssh II and Sac II. Electrophoresis was performed on the CFGE apparatus with a switch interval of 60 sec. Southern analysis was performed with the probes G28A (A), pGD-T-5B (B) and sp64 VIII (C). Fragment sizes are indicated in Kbp (referred to in the text). Common fragments are indicated by a double line. All three probes were hybridised separately to the same filter.

Comparison of male and female DNA's

In the course of this study it became apparent that a number of sites for rare cutting restriction enzymes were partially blocked in female compared with male DNA. It is presumed that this occurs because of methylation of these sites on the inactive X chromosome. This effect was most noticeable with the enzyme Bssh II (figure 3A). Digests of male DNA probed with G28A produced an 80 Kbp and 40 Kbp doublet. These bands were also seen in female DNA, but in addition bands at 150, 190 and 350 Kbp were seen. In addition limiting mobility signal was detected only in female DNA. In other experiments some of this signal was resolved into a doublet of bands at 750 and 800 Kbp (data not shown). The 150 Kbp band was also detected by the pGD-T-5B probe, (figure 3B). No extra bands were seen with the sp64 VIII probe in female versus male DNA (figure 2C), thus confirming that the Bssh II digest of female DNA was complete. These observations were found to be reproducible using DNA from 3 male and 6 female mice, (data not shown). Taken together this data suggests that the Bssh II sites flanking the Cf-8 gene remain unmethylated on the inactive X chromosome. Partial methylation of the four Bssh II sites closest to the P3 gene could account for the appearance of bands at 150, 190 and 350 Kbp approximately. The larger bands detected (750 Kbp, 800 Kbp and limiting mobility) presumably arise through partial methylation of the Bssh II site 250 Kbp to the right of the P3 CpG rich island (figure 5). These putative sites have not been added to the map. A similar effect was also seen in digests of female versus male DNA with the enzymes SacII (figure 3A) and ClaI (data not shown). In both cases this was manifested as an increased signal in the limiting mobility region. These results are not unexpected, considering reports of increased methylation at certain sites on the inactive X chromosome (in particular

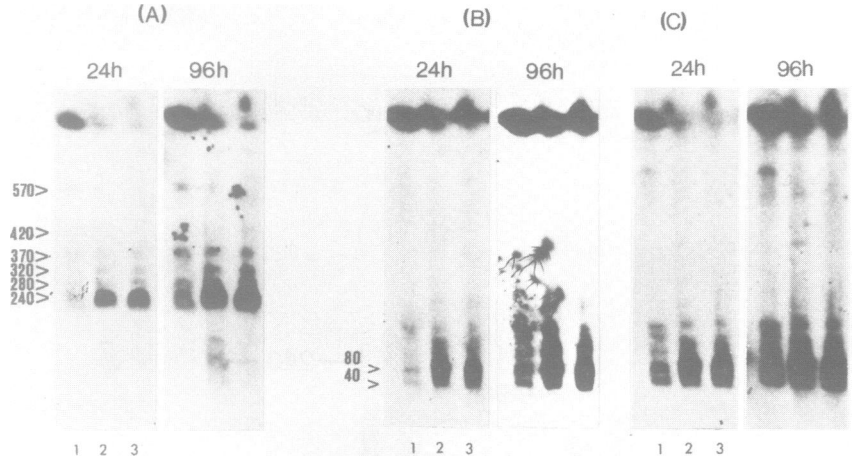


Figure 4. Confirmation of physical linkage using Bssh II partial digests.
DNA from male mice was digested to completion with the enzyme Mlu I, and then partially digested with Bssh II for 30′ in 0mM NaCl (1), 60′ in 0mM NaCl (2), 90′ in 0mM NaCl (3). Electrophoresis was performed on the CFGE apparatus with a switch interval of 90 secs (20 h) followed by 45 secs (20 h). Southern analysis was performed with the probes sp64 VIII (A), pGD-T-5B (B) and G28A (C). Fragment sizes are indicated in Kbp and are referred to in the text. All three probes were hybridised separately to the same filter. 24 h and 96 h exposures of the filters are illustrated.

methylation of CpG rich islands). The heterogeneity of bands seen with Bssh II was however unexpected. The most likely explanation is that within a given population of cells, there is a finite probability that a given CpG dinucleotide is methylated on the inactive X chromosome. We cannot rule out the possibility that partial digestion is a consequence of other changes in chromatin structure on the inactive X chromosome, or differences

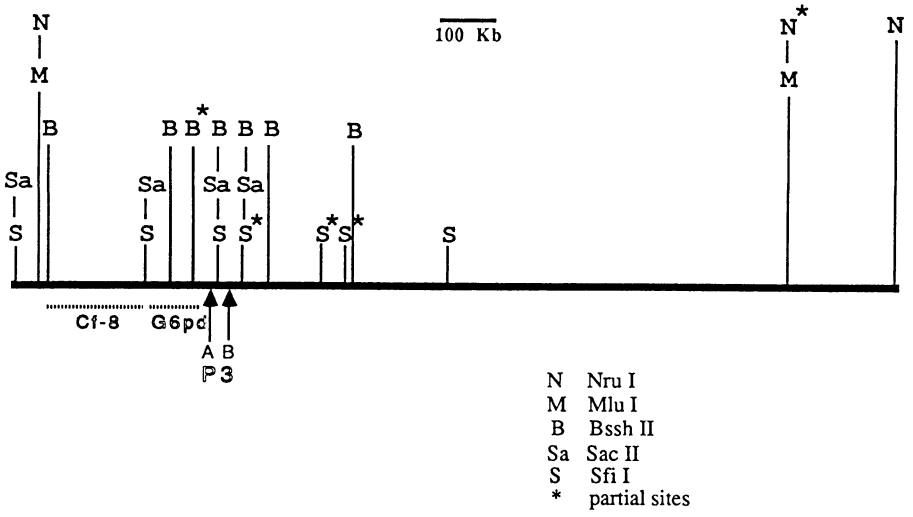


Figure 5. Physical map summarising PFGE data.

Table 1. Summary of PFGE Fragment Sizes

Probe	Enzyme SfiI	SacII	BsshII	NruI	MluI	SfiI + SacII	SfiI + BsshII	SfiI + NruI	SfiI + MluI	SacII + BsshII	SacII + NruI	SacII + MluI	BsshII + NruI	BsshII + MluI	NruI + MluI	BsshII Female
G28B	50* 190* 240* 430	50	50	1400* 1600	1400	50	50	50* 190* 240* 430	50* 190* 240* 430	50	50	50	50	50	1400	50* 150* 190* 350* >700
G28A	50* 190* 240* 430	50	40* 80	1400* 1600	1400	50	80	50* 190* 240* 430	50* 190* 240* 430	-	50	50	80	80	1400	80 150 190 350 750 800 >1000
Sp64VIII	240	240	240	nt	1400	240	180	200	200	180	240	240	240	240	nt	240
pGD-T-5B	nt	nt	40* 80	nt	1400	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	40* 80* 150

Fragment sizes are indicated in Kbp. The degree of error relative to markers of known size is possibly as much as 20%. Comparative data using different probes is however accurate as filters were re-used in successive experiments. Fragments produced by partial digestion are indicated (*). When signal was seen in the limiting mobility region and not subsequently sized this is indicated by e.g. >1000.

in the sensitivity of Bssh II to one or both CpG's in its recognition sequence being methylated.

DISCUSSION

There remain certain difficulties in establishing accurate and representative physical maps by PFGE over long regions of mammalian chromosomes; methylation differences between tissues, between cell lines, and in the case of the X chromosome, between chromosomes must be taken into account. In this study we have utilized DNA from male mice to establish a physical map and have used a homogeneous cell population to prepare DNA. With certain restriction enzymes (e.g. Sac II) the sequence environment surrounding a given recognition site appears to influence the relative rate of cleavage at that site (31). Certain regions of the mammalian genome appear to contain a high density of CpG rich islands (16). The frequent occurrence of rare cutter sites can restrict attempts to obtain long range mapping information with relatively few probes. The region we have mapped appears to fit into this category and we have had to use partial digestions to overcome this problem.

The demonstration of physical linkage of P3, G6pd and Cf-8 confirms evidence from a number of sources suggesting genetic linkage of these loci (7,9,28,33). It was recently demonstrated that these three genes are physically linked within 600 Kbp on the human X chromosome (22). The human physical map is not sufficiently detailed to allow accurate comparisons with our map. However, it is apparent that the mouse Cf-8 and G6pd loci are approximately 200 Kbp closer together than the human equivalents. The ability to examine the syntenic relationship between X chromosomes from man and mouse at the physical level should provide valuable information concerning the mechanisms involved in chromosome evolution.

In common with a number of other PFGE maps, clusters of rare cutter sites have been identified, indicating the presence of CpG rich islands (figure 5). It has recently been suggested that regions of the genome rich in CpG islands correspond to Giemsa-light bands, and that regions with few CpG islands (e.g. the Duchenne Muscular Dystrophy locus) correspond to Giemsa-dark bands (20). Furthermore, *in situ* hybridisation studies were used to demonstrate that the relatively GC rich Alu repeat is found in G-light staining regions and that the relatively AT rich L1 repeat is found in G-dark staining regions of the human karyotype (36). *In situ* hybridisation localizes the mouse P3 gene to the X_B (G-light)$-X_c$ (G-dark) interface (Mattei, M.G. and Avner, P., manuscript in preparation), while the DMD gene in mouse which lies distal to P3 has been localised to the X_c (G-dark) band (E.P. Evans, manuscript in preparation). PFGE studies using a mouse X chromosome specific anonymous clone, DXSmh 141, localised to the A3 dark band, indicate that it lies within a region spanning at least 1 Mbp that is devoid of CpG rich islands (S.D.M. Brown, manuscript in preparation). Extension of the current PFGE maps will help to confirm or deny this possible relationship between chromosome structure and underlying sequence organisation.

Our findings concerning methylation on the inactive X chromosome support accumulating evidence that methylation occurs in the CpG rich islands associated with X-linked genes (12,13). X-linked genes in the human X-Y pairing region escape inactivation and it has recently been shown that CpG rich islands associated with these genes remain hypomethylated on the inactive X chromosome (14,15,16). The apparent heterogeneity of methylation at Bssh II sites within our map is probably due to variation in the methylation pattern produced when X-inactivation occurs during early embryogenesis. There is strong

evidence that methylation patterns are stably inherited within a given cell lineage (32). PFGE therefore provides a powerful tool for the study of X-inactivation over large regions of the X chromosome.

In conclusion, we have constructed a detailed physical map encompassing approximately 1% of the mouse X chromosome. We are currently attempting to extend this map using other genetically localised probes (1), and this work should allow us to orientate the map with respect to the centromere and to correlate the PFGE map with the standard Giemsa stained karyotype.

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