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Cloning and characterization of a murine brain specific gene Bpx and its human homologue lying within the Xic candidate region

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The X inactivation centre (Xic) is a cis-acting locus thought to play a key role in the initiation of X-inactivation. We have cloned and characterized a new gene, Bpx, lying distal to the murine Xist. Bpx, which is specifically expressed in the brain, shows strong homology to genes encoding nucleosome assembly proteins and is normally X-inactivated in mice. Isolation and localization of BPX, its human homologue, has shown the gene to be located centromeric to XIST in man. The Xq13 region, whose orientation is apparently globally conserved between man and mouse, must therefore contain an inversion of at least 600 kb spanning the XIST sequence and including the CDX4 and BPX genes.

INTRODUCTION

X chromosome inactivation is the process which ensures the dosage compensation of X-linked gene products between male and female mammals. X-inactivation occurs early in development and is first seen in the mouse in extraembryonic tissues where the paternal X chromosome is preferentially inactivated. In embryonic tissues on the other hand, inactivation affects either of the X chromosomes in a random fashion. X-inactivation can be arbitrarily divided into three phases: initiation, spreading and maintenance. The presence of a cis-acting locus, the X inactivation centre (Xic), thought to act during the initiation step, is required for X-inactivation to occur (for recent review see ref. 1).

The existence of Xic and its localization have been deduced from studies on the inactivation status of the X chromosome in cells carrying structurally abnormal X chromosomes (2,3). In the mouse, Xic candidate region has been localized between the T16H translocation breakpoint (4) and a breakpoint characterized in the embryonic stem cell line HD3 (5). The existence of a larger number of X chromosome rearrangements in man than in mouse has allowed the present size of the candidate region for XIC in man to be restricted to between 700 and 1200 kb. The proximal boundary of XIC in man is defined by the breakpoint of an X;14 translocation (6) whilst the distal boundary is currently defined by the breakpoint of a late-replicating rearranged X chromosome [46,X r(14)] (7). Since the Xic/XIC candidate regions map to syntenic regions of the mouse and human X chromosome (8), it is generally thought that the more precisely defined limits of the XIC candidate region established in human will prove valid in the mouse.

The Xic/XIC candidate region is as yet poorly characterized for the presence of transcription units, although Lafrenière and colleagues have reported a novel gene on the human X, XPCT that lies 600 kb distal to XIST (9). Within the XIC/Xic candidate region itself, only two transcribed sequences have been characterized. One of these is the XIST/Xist gene (10–12). The XIST/Xist gene possesses several characteristics which make it a potential candidate for the X inactivation centre (13–15), including its unique property of being transcribed solely from the inactive X chromosome. A second gene lying within the XIC/Xic candidate region, CDX4/Cdx4, has been recently isolated (16).

As part of our ongoing studies of the murine Xic candidate region, we report the identification of a brain specific gene, termed Bpx, lying ~300 kb distal to Xist, which is normally X-inactivated in mice. Bpx, which shows striking homology to genes encoding nucleosome assembly proteins, is highly conserved between man and mouse. The human homologue has been mapped at least 600 kb proximally to XIST on the human X chromosome, indicating that the inversion of this region of the human and murine X chromosomes, previously found to involve both XIST/Xist (17) and CDX4/Cdx4 (16), includes Bpx and covers at least 300 kb in mouse and 600 kb in human.

RESULTS

Identification of a brain specific transcript lying 300 kb distal to Xist on the mouse X chromosome

As part of a systematic search for transcribed sequences within the Xic candidate region in the mouse, we have isolated from the
Figure 1. Sequence analysis of Bpx. (A) Nucleotide and amino acid sequence of Bpx. The SspI site used to orientate the gene is underlined. The polymorphic nucleotide between T16H and Mai is indicated in bold. (B) Comparison of the Bpx and hNRP predicted protein sequences. The Bpx and hNRP amino acid sequences were aligned by computer analysis using the 'gap' program (GCG), with gaps (indicated by dashes) inserted to maximize identity. Identical residues are represented by vertical lines. These two proteins share 50% identity and 70% similarity. Boxes indicate stretches of residues conserved in yeast NAP-1 (KGGPDFWTL TV and DSFFNFSPH), a possible site for N-glycosylation (NVT) and a putative nuclear localization motif (IKKQRH). A bipartite nuclear address motif is underlined. Conserved serine and threonine residues at consensus sites for phosphorylation are indicated in bold. (C) Comparison of the nucleotide sequence of the Bpx and BPX genes. Alignment was performed as described before. The Bpx ORF begins at position 331 and extends through to nucleotide 1710. The identity is 80% over the whole sequence and 84% over the length of the ORF.
lambda clone IB8 lying ∼300 kb distal to Xist (17) a 4.5 kb XbaI genomic fragment which detects a 2.5 kb brain specific transcript on Northern blots (see below). A gridded brain cDNA library was screened with this genomic probe and a single positive cDNA clone isolated. A cDNA probe, P0324, derived from this clone, was used in turn to screen an adult brain cDNA library (Clontech), in order to isolate the full length cDNA. The 2.46 kb full length cDNA sequence is shown in Figure 1a. An open reading frame extends 1380 bp, starting at nucleotide 331 and running through to nucleotide 1710. The molecular mass of the corresponding 460 amino acid protein was calculated to be 52 109 kDa. The first in-frame ATG is flanked by a sequence which matches the consensus sequence for eukaryotic translation initiation sites (18) and probably represents the true initiation codon, since Kozak has observed that translation begins at the 5′ proximal AUG in 95% of the mRNAs studied. It should be noted that the 331 nt 5′ untranslated region is larger than that found in most eukaryotic mRNAs (18), suggesting a possible involvement in the regulation of expression. Two polyadenylation consensus motifs (AAUAAA) are present at positions −11 and −20 from the polyA tail. The gene has been named Bpx for brain protein, X-linked.

Cross-species conservation of Bpx was tested by hybridizing genomic DNA of several species including human, chinese hamster, rabbit, marmot, sheep, wallaby, trout, Xenopus, chicken, drosophila and yeast, with the cDNA probe P0324 at both high and low stringencies. At high stringency, a unique band can be detected in several mammalian species including hamster, rabbit, marmot and human. At lower stringency, signals are detected in species as distantly related as trout, drosophila and yeast (data not shown).

**Genomic organization of the Bpx gene**

In order to define the intron-exon structure of the gene, three primers pairs regularly dispersed along the cDNA sequence were used to amplify in parallel the cDNA and genomic DNA (see Material and Methods). No differences in size between cDNA and genomic DNA-PCR products were detected on 1.5% agarose gels (data not shown), indicating that no large introns are present in the gene. To discriminate between the complete absence of introns and the presence of very small introns, genomic DNA was amplified using primers which covered the whole cDNA and the amplification products sequenced. No differences were found between the genomic and the cDNA sequences (data not shown). These results indicate that Bpx is an intronless gene.

**Bpx expression profile**

When probed against a Northern blot containing multiple adult male tissues (Clontech), the P0324 cDNA probe detects a single 2.5 kb transcript in brain RNA (Fig. 3), as does the equivalent genomic probe IB8Xba (see above). To confirm the tissue-specificity of Bpx expression, brain, heart, spleen, liver, kidney and testis from adult male and female, 129/Sv mice were tested by RT–PCR using a set of primers (P0324S4 and P0324S5) located on either side of the stop codon. A PCR product of the expected size (147 nt) was only detected in both male and female brain samples. These results confirm that, in adult mice, Bpx is specifically transcribed from the brain of both males and females.

The developmental expression profile of Bpx was determined by hybridizing Northern blot of embryonic tissues (Clontech) with the P0324 probe. No signal was detected in RNAs of the 7 of embryonic life and is subsequently maintained in the adult.

**Bpx has homology with nucleosome assembly proteins**

Comparison of the deduced amino acid sequence of Bpx with those in GenBank revealed striking similarities with murine and human nucleosome assembly proteins (NAP-1) (20), with the mouse brain protein DN38 (21) and, to a lesser extent, with the yeast NAP-1 protein (22). The homologous residues are clustered in two domains (the first extending from residues 98 to 174 and the second from residues 230 to 418). Within these regions, the amino acid similarity between Bpx and mouse NAP-1 reaches 77% (Fig. 1b) and several distinct motifs found in hNRP (20), the human NAP-1 protein and NAP-1 from other species, are found to be shared by Bpx (Fig. 1b). Sequences DSFFNIFSSPH and KGPDPFWLT, which are highly conserved between yeast and mammalian NAP-1 proteins and probably represent functionally important domains, are for instance conserved in Bpx (238–246 and 368–377). Glycosylation may influence Bpx function since one putative site for N-glycosylation (at positions 339–341), present in hNRP, is found in Bpx. Other similarities include the presence of a threonine residue (position 162) and a serine residue (position 310) at consensus sites for phosphorylation by casein kinase II. This conservation is of interest as recent data suggest that the phosphorylation status of nucleoplasmin, an enzyme which also plays a role in nucleosome assembly, can affect its role in modulating chromatin structure (23). Such similarities are reinforced by the presence in the Bpx sequence of a IKKQRH motif (at position 346–352) which closely resembles the nuclear localization motif found not only in nucleosome assembly proteins such as nucleoplasmin and N1, but also in other nuclear proteins, including p53, c-abl and simian virus 40 T-antigen. A bipartite nuclear localization consensus motif (24) is found at position 138, which is however less conserved in hNRP.

Another similarity between the Bpx and NAP-1 proteins lies in their overall acidic nature, with 15% glutamic acid residues and 9.3% aspartic acid residues, some of which are clustered (see Fig.
1b, from residue 212 to 222). These polyacidic stretches would be expected to facilitate the binding of the molecule to histones or other basic proteins. However, although both NAP-1 and Bpx proteins are acidic overall, the acidic domains map in part outside the regions of homology of these proteins. Of the three acidic domains in the hNRP protein (between amino acids 13 and 26, 129 and 141 and 348 and 376), only the second is located within the first domain of homology with Bpx. Similarly, a particularly acidic domain is found in the middle of the Bpx sequence (4 Asp, 7 Glu), in a region which has no homology to either the nucleosome assembly proteins or to any other protein. The acidic nature of Bpx is reflected by its hydropathicity profile, indicating that Bpx encodes a highly hydrophilic protein.

A further similarity between Bpx and hNRP lies in their 3′ untranslated region. Both are AT-rich and large consisting of 309 nucleotides in hNRP and 755 in Bpx. These AT-rich 3′ untranslated regions are thought to influence transcript stability and half-life (25).

Outside of the two domains of Bpx which have homology to NAP-1 proteins (from residues 98 to 174 and 230 to 418), no significant homology with any known protein was found in the databases. The other, more specific regions may be implicated in the restricted expression profile of Bpx in the brain.

**Bpx is subject to X-inactivation**

Female mice carrying the T(X;16)16H translocation undergo non-random X-inactivation due to cell selection. In mice carrying the T16H translocation (derived from *Mus musculus domesticus*) and a normal X chromosome derived from the divergent mouse sub-species *Mus musculus musculus* (XMa), genes subjected to X-inactivation are expressed only from the domesticus X chromosome (26). Sequence analysis of the Bpx gene in the T16H and Mai strains permitted detection of an A to G transition in Mai mice, two nucleotides downstream of the stop codon. This single nucleotide polymorphism could be detected by single strand conformation polymorphism (SSCP) analysis, after PCR amplification using primers P0324S4 and P0324S5. To determine whether Bpx is normally inactivated in mice, brain RNA from a T16H male, a Mai female and a (T16H/Mai)F1 female were reverse transcribed and the corresponding cDNAs examined by
SSCP analysis (Fig. 4). As shown on the right part of Figure 4, only the T16H allele is amplified in the T16H/Mai heterozygous female. Control experiments using Xist primers have shown that, as expected, only the Mai allele of Xist is amplified in the T16H/Mai female (data not shown). Thus the Bpx gene appears to be, at least in the mouse, subject to X-inactivation and transcribed only from the active X chromosome.

**Cloning and localization of Bpx, the human homologue of Bpx**

When a human Northern blot was screened with the mouse P0324 probe, a single 2.5 kb band was detected specifically in the brain (data not shown). A human brain cDNA library was screened with probes corresponding to different murine cDNA clones spanning the entire cDNA sequence, allowing the sequence of Bpx to be established (Fig. 1c). The Bpx and BXP sequences are highly conserved, with 79.8% identity over the whole cDNA sequence. Within the ORF, the homology is 84.6% at the nucleotide level. At the amino acid level, the deduced proteins are 82.5% identical and 91.5% homologous. A stretch of nine GAA/GAG triplets coding for glutamic acid is found in the middle of the ORF. This region is not perfectly conserved in the murine cDNA which has only seven GAA/GAG. As trinucleotide repeats have been shown to be unstable in the human genome and are associated with several diseases, we analyzed this region for potential polymorphisms using DNA from 12 unrelated individuals. This analysis did not show any length variation in the repeated region (data not shown).

The Xist transcription unit was shown sometime ago to be oppositely orientated in man and mouse (17). More recently, Horn and Ashworth have reported that the Cdx4 gene, which lies 100 kb distal to Xist in mouse, is located 400 kb proximal to XIST on the human X chromosome (16). We were therefore interested in the localization of the human homologue, Bpx. Bpx was initially shown to map on the human X chromosome by the use of a hybrid containing the human X chromosome on a mouse background (data not shown). To refine the localization of Bpx, a human YAC contig spanning 1.8 Mb around XIST was then screened with the Bpx probe (27). The results are shown in Figure 5. YACs 4566, 8028, 8038, 4565 and 8039 were shown to hybridize with the Bpx probe, whereas YACs 8027, A39G7, B245H8 and 8012 remained negative. These results indicate that Bpx is located 600–800 kb proximal to XIST on the human X chromosome. Thus, the size of the inversion between man and mouse is at least 300 kb in mouse and 600 kb in human.

**DISCUSSION**

The Xic candidate region remains poorly characterized for the presence of transcription units. Apart from Xist, only one other gene, Cdx4, had been found to map within the Xic region (16) prior to this report on the identification and characterization of a new brain specific gene, Bpx, lying 300 kb distal to Xist in the mouse.

The Bpx cDNA encodes a predicted protein which shows homology to nucleosome assembly proteins (NAP-1). Like the NAP-1 proteins, Bpx is highly acidic, containing 24.3% of negatively charged amino acids (aspartic and glutamic acids). The overall acidic nature of Bpx, as well as the presence of two nuclear address motifs provide arguments supporting the hypothesis that Bpx is a nuclear protein. The homology with the NAP-1 protein indeed suggests that Bpx may interact with core histones in the nucleosome.

Whilst the presence of a gene coding for an eventual nucleosomal protein lying within the Xic candidate region is highly provocative, Bpx appears to be restricted in its distribution in the adult and in the embryo to postmitotic neurons (Rougeulle et al., in preparation). The distribution of Bpx is in contrast to that of hNRP, which is ubiquitously expressed and is thought to play a role in cell proliferation. Rather than having a ubiquitous role in nucleosome assembly during DNA replication, the histone interaction of Bpx could therefore be involved more in local chromatin modifications that might be implicated in the regulation of gene expression specific to neural function. It is of interest however to note that the Drosophila scute gene, which plays an important role in the development of both the embryonic central and peripheral nervous system of larvae and adult flies, is also involved in sex determination and dosage compensation (28).

Indeed, most of the genes involved in chromosome counting in Drosophila have been shown to play an important function in neurogenesis (29). Outside of those domains showing homology to NAP-1, the unique regions of the Bpx protein may be involved in its functional specificity via interactions with other proteins or DNA which permit the specific regulation of the expression of genes or domains by modifying chromatin structure.

Sequencing of the Bpx cDNA and the corresponding genomic DNA has revealed that Bpx is an intronless gene. Several characteristics indicate however that Bpx is a functional gene rather than a pseudogene. First, at high stringency, a single band is detected by Southern blot analysis on mouse genomic DNA. Secondly, Bpx is highly conserved in man, with the human homologue mapping to the syntenic region of the human X chromosome. Thirdly, when Bpx expression was assessed by SSCP in T16H/Mai heterozygote females, the T16H derived allele and only the T16H allele was detected, indicating that the gene located on the X chromosome is indeed expressed. Taken
Figure 5. Localization of the human \textit{BPX} gene. Schematic diagram of the region surrounding \textit{XIST} on the human X chromosome and the position of various YACs used in this study (modified from ref. 26). X chromosome derived YAC DNA is shown as open boxes. Lines indicate chimaeric sequences. The previously noted inverted repeat structures present in the region are indicated by arrows. Open boxes indicate the location of genes in the area. The shaded area on some of the YACs indicates the presence of \textit{Bpx} hybridizing sequences. The minimal region to which \textit{XIC} has been localized is indicated at the top of the figure. Since \textit{BPX} has not yet been mapped by pulse field analysis, its position on the X chromosome is approximate.

Together, these data strongly suggest that the \textit{Bpx} gene is a functional gene rather than a pseudogene. \textit{Bpx} is not the only intronless gene known to lie on the X chromosome. Other X-linked intronless genes such as the brain specific POU-box gene \textit{Brn4} (30), the glutamate dehydrogenase 2 gene GLUD2 (31), a member of the glycerol kinase gene family (32) and the MYCL2 gene (33) have been previously reported in man and mouse. The genomic organization of the NAP-1 related genes has not been extensively studied and the presence of introns in these genes has yet to be determined. It is however of interest to note that the gene family coding for histone proteins is also intronless. Whilst the origin of the absence of introns in \textit{Bpx} remains to be determined, assuming that primordial genes evolved with introns, the lack of introns in genes such as \textit{Bpx} in higher eukaryotes must be due either to intron loss or to reinsertion into the genome of genetic material copied from mRNA by reverse transcription.

We have shown by SSCP analysis using the T16H translocation system, that \textit{Bpx} is subject to X-inactivation in mice. It is the second gene with \textit{Cdx4}, located within the present \textit{Xic} candidate region, that has been shown to be normally X-inactivated (16). This result supports the hypothesis that the \textit{Xist} expression profile, that is exclusive expression from the inactive X, is either specific to \textit{Xist}, or to a highly restricted chromosomal domain including \textit{Xist}. It will be of great interest to determine whether genes located proximally and close to \textit{Xist} show a similar profile.

Sequence comparison of \textit{Bpx} and its human homologue \textit{BPX} has revealed striking homology, with 79.8% identity at the nucleotide level for the entire cDNA, which contains >1 kb of untranslated sequences. Since the homology within the ORF is 84.6%, this suggests that the 5' and 3' untranslated regions are themselves highly conserved. These regions may well be involved in the regulation of expression and/or stability of the mRNA. PCR analysis of both \textit{BPX} genomic DNA and cDNA using primers which cover the whole cDNA sequence also suggest that \textit{BPX}, like its murine homologue, is an intronless gene. Of note is the presence of a stretch of nine GAA/GAG triplets coding for glutamic acid found in the middle of the ORF. Such amino-acids homopolymers (poly-glu in \textit{Bpx}/\textit{BPX} case) are frequently found in DNA binding proteins which act as transcription factors. Such repeats have been found in the NH2 terminus of the Xnp protein which is similarly encoded by a gene lying slightly distal to this region of the X chromosome (34).

Using a YAC contig spanning 1 Mb on the human X chromosome (27), \textit{BPX} has been localized 600–800 kb proximal to \textit{XIST} (Fig. 5), suggesting that the inversion between man and mouse in this part of the X chromosome covers at least some 300 kb in mouse and 600 kb in man. It is likely therefore that \textit{BPX} maps to within the same region as the proximal limit of the \textit{XIC} candidate region (6), although testing against the X;14 translocation breakpoint carried by the hybrid W4-1A will be necessary to confirm this interpretation. If we assume, since the X-inactivation processes are similar in human and in mouse, that the limits currently defined for the human \textit{XIC} are valid in mouse, then the distal limit of the actual murine \textit{Xic} candidate region would be located some 300 kb distal to \textit{Xist}, very close to the \textit{Bpx} gene itself. A striking feature is the difference in the physical distance between \textit{Bpx} and \textit{Xist} in the mouse and \textit{BPX} and \textit{XIST} in human, of 300 and 600 kb respectively. Such a difference has already been reported for \textit{Cdx4}, which is located 100 kb distal...
to Xist in mouse and ~400 kb proximal to XIST in human (16). Thus, some 300 kb of the human X chromosome, lying between XIST and CDX4, seem to be absent from the murine X, suggesting that these 300 kb are not necessary in the X-inactivation process, or at least for Xic function itself. Whilst the identification of further conserved transcription units lying close to Xist will be necessary to allow the complexity of this chromosomal rearrangement to be verified, the possibility exists that the inversion is associated with the generation of the large (125 kb) inverted duplication previously identified by Lafreniere et al. (27) as lying between PHKA1 and DXS2227. In this case, the inverted duplication may well represent the proximal boundary of the inversion.

Several human inherited diseases have been localized to the Xq13 region of the human X chromosome. Amongst these are genes involved in mental retardation. Schwartz has noted for example that 35% of mapped mental retardation genes on the X chromosome were linked to markers in the region Xq12-q21 (35).

Case reports have shown that absence of BPX is involved in human diseases which have been mapped to the Xq13 region. The localization of BPX in Xq13, as well as its restricted expression to neurons makes it a good candidate for involvement in brain disorders such as mental retardation.

MATERIALS AND METHODS

Isolation of Bpx and BPX cDNAs

Genomic probe IB8Xba was used to screen 20 000 clones from a gridded adult mouse brain cDNA library kindly provided by Hans Lehrach. The P0324 cDNA probe derived from the clone isolated, which contains 1.3 kb of the 3′ cDNA sequence, was used to screen an adult mouse brain 5′-stretch plus cDNA library in the λgt11 vector (Clontech) and a human brain cDNA library in the λZapII vector (Stratagene). 5 × 105 recombinant clones from each library were plated onto 210 mm plates at a density of 105 phages per plate and colony lifts were taken with Hybond N + membranes.

Probe labelling and hybridizations

Double stranded DNA probes were labeled by random priming (36).

High stringency hybridizations were carried out with 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA and 7% SDS at 65°C, modified from Church and Gilbert (37). Filters were washed in 40 mM sodium phosphate, pH 7.2, 1% SDS at 65°C. Low stringency hybridization were carried out with 25% deionized formamide, 5X Denhart, 5% dextran sulfate, 1% SDS, 50 mM sodium phosphate, pH 7.2, 5 mM EDTA, 1 M NaCl and 100 µg/ml sonicated salmon sperm, at 42°C for 15 h. Filters were washed in 25% deionized formamide, 1% SDS, 50 mM sodium phosphate, pH 7.2, 5 mM EDTA, 1 M NaCl, at 42°C.

Northern blot hybridizations were carried out using the Express-Hyb solution provided from Clontech, as recommended by the manufacturer.

Sequencing and sequence analysis

Double strand plasmid clones were sequenced using the Sequenase version 2.0 sequencing kit. Search for homologous sequences was carried out using the BLAST server (non-redundant DNA and protein sequence databases) at the National Center for Biotechnology Information (NCBI).

RNA isolation and RT-PCR

RNA from brain, heart, liver, spleen, kidney and testis were prepared according to the method described by Chomczynski and Sacchi (38). First strand cDNA was synthesized from 10 µg of total RNA using the AMV reverse transcriptase (Promega). One-thirtieth of the cDNA was used for PCR reactions using primers P0324S4 (ATTATTATGATGATTGAGTC) and P0324S5 (TGGTTATGCTCTCAGTAACTGC). PCR reactions were performed in 50 µl containing 0.5 µM of each primer, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 1.25 mM each dNTP and 2.5 U of Thermus Aquaticus Polymerase (Amersham). Fragments were amplified under 50 µl of mineral oil in an automated thermal cycler using a denaturation step of 5 min at 94°C followed by 30 cycles consisting of a 94°C denaturation (30 s), a 52°C annealing (30 s) and a 72°C extension (1 min) step completed by a final 5 min extension step at 72°C. The integrity of all RT-products was controlled by performing PCR using primers for the hoeskupping gene Hprt HPRTNAF/HPRTNAR (15).

Analysis of exon-intron structure of Bpx/BPX

PCR reactions were carried out as described above (except for annealing temperatures) on 300 ng of genomic DNA and cDNA, using the following primers.

**Bpx analysis.** P0324S1 (ACACAGGACAATTATTTGCAAC)/P0324S2 (CACTGGCTGCGACATAGA): 50°C.

MB1b2KS0 (GAAGAATCTCCAGTTGAAAGTT)/P0324S3 (ACACTCTATAATATCCAGGCC): 52°C.

MB3bT7-1 (CCCCACTGTTTGCTGCC)/MB1b2inv (AATTTCACCTGGGAGACTT): 55°C.

**BPX analysis.** HB1aT3–1 (TGATTAAGAATATATGTAGG)/HB1eT3–1 (ACTAACACTTCCACCCAACA): 50°C.

HBR1 (GCTGCTTGCGGAGGAGG)/HB4bT3-2 (CTTTCACTAGTGGACTTTAACA): 57°C.

HB5r (GGACATTCGGTATGTATTTTAGA)/HB1aj (CATTAGCCAAAATCAGGA): 52°C.

**Sequencing of Bpx genomic region**

YAC PA-2 DNA was PCR amplified using a denaturation step of 5 min at 94°C followed by 30 cycles consisting of a 94°C denaturation (30 s), a 55°C annealing (30 s) and a 72°C extension (2 min) step completed by a final 10 min extension step at 72°C, using the following primers.

MBr1 (CACCAGTTCCTCACTCC)/MB1b2inv

MB3bT7-1/P0324S5 (GTTATTGTCCTGTAATCAGT)

P0324S4/MBr2 (CGTGAATGCAAGAATATAGAAA)

PCR products were cloned into the pGEM vector (Promega) according to the instructions of the manufacturer. Clones were double strand sequenced as described above.

SSCP analysis

First strand cDNA was synthesized from 5 µg of total brain RNA from the T16H, Mai and (T16H/Mai)F1 strains as described above. One twenty-fifth of the cDNA was used for PCR amplifications using primers P0324S4 (ATTATTATGATGATTGAGTC) and P0324S5 (TGGTTATGCTCTCAGTAACTGC). Labelled PCR was performed in a 10 µl reaction containing 1 µl of 10 × PCR buffer (Amersham), 100 ng genomic DNA or 2 µl cDNA, 0.25 mM primers, 200 mM dNTPs, 0.1 µl PCR.
30 cycles at 94, 52 and 72 °C for 1, 1 and 0.5 min respectively. Nine microlitres of the reaction was mixed with 9 μl of 95% formamide, 20 mM EDTA 0.05% bromophenol blue and 0.05% xylene cyanol, denatured at 95 °C for 5 min and cooled on ice. Two microlitres were loaded on 5% acrylamide gels containing 5% glycerol at 4°C in 0.5x TBE for 4 h at 30 W. Gels were dried and exposed for 1–5 days at –80 °C.

The Xist primers cX8 (CAAAACCTCCTGACCTG AAA) and cXL8 (TGAGGGTTAGGATCTTGTTT) were used as control using the same conditions as those for Bpx primers.

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