Isolation of BAC clones spanning the Xq22.3 translocation breakpoint in a lissencephaly patient with a de novo X;2 translocation

Naomichi Matsumoto, Daniela T Pilz, Judy A Fantes, Kirk Kittikamron, David H Ledbetter

Abstract
X linked lissencephaly and subcortical band heterotopia (XLIS/SBH) is a disorder of cortical development, which causes classical lissencephaly with severe mental retardation and epilepsy in hemizygous males and SBH associated with milder mental retardation and epilepsy in heterozygous females. Here we report the fine mapping of a breakpoint involved in a de novo X;autosomal balanced translocation (46,XX,t(X;2)(q22.3;p25.1)) previously described in a female with classical lissencephaly. We constructed a complete 490 kb BAC contig around the Xq22.3 breakpoint with 11 novel STSs and isolated three BAC clones spanning the breakpoint. This mapping information and BAC contig will be useful in the detailed characterisation of the XLIS gene and other contiguous genes which may also be involved in brain development or function.

Keywords: X linked lissencephaly; X;autosomal translocation; positional cloning

Malformations of neuronal migration such as lissencephaly are an important cause of mental retardation and epilepsy. Classical lissencephaly and cobblestone lissencephaly are two pathologically and radiologically well defined types of this disorder. Other types such as microlissencephaly (associated with severe microcephaly and cerebellar hypoplasia) are being increasingly recognised.1 Classical lissencephaly is characterised by gyral abnormalities ranging from agyria to pachygyria, a thick cerebro cortex, hypoplasia of the corpus callosum, and incomplete opercularisation. Its spectrum also includes subcortical band heterotopia. The cerebellum usually appears normal. It is associated with three major syndromes: Miller-Dieker syndrome (MDS), isolated lissencephaly sequence (ILS), and X linked lissencephaly (XLIS)/subcortical band heterotopia (SBH).1 Facial dysmorphism is recognised in MDS, but is mild or absent in ILS and XLIS. Additional malformations are also predominantly seen in MDS.1,3

Genetic causes in classical lissencephaly have been elucidated. MDS is associated with visible or submicroscopic rearrangements within chromosome band 17p13.3 in almost all patients. In ILS, submicroscopic deletions of chromosome 17p13.3 have been detected in approximately 40% of cases5 (Pilz et al, submitted). The L1SI gene has been mapped to the 17p13.3 deletion region8 and several mutations of the L1SI gene have been found in ILS patients without deletions.9,10 XLIS and SBH can be present with sporadic or familial mental retardation and epilepsy. The brain malformation varies from classical lissencephaly, which is observed in males, to SBH, which is observed primarily in females. The XLIS gene is located at Xq22.3 based on the observation of a female lissencephaly patient with a de novo X;autosomal translocation11 and two recent linkage studies on XLIS/SBH families.12,13 Physical mapping studies of the XLIS/SBH region using the t(X;2) patient showed that the Xq22 breakpoint lies between the anchor markers DXS1105 and DXS1072, which are located within 1 cM of each other on the Genethon map.13

Here we describe the isolation of BAC clones spanning the Xq22 breakpoint of t(X;2) in a lissencephaly patient and the construction of a 490 kb bacterial artificial chromosome (BAC) contig around the breakpoint.

Materials and methods

Cell lines
A lymphoblastoid cell line was previously made from a girl (XLI-01) with classical lissencephaly and the karyotype 46,X,t(X;2)
(q22.3;p25.1) de novo. A lymphoblastoid cell line with a normal karyotype was also used as a control. The patient's lymphoblastoid cell line and a somatic cell hybrid containing one of the breakpoints (the derivative chromosome 2, without the normal X or the derivative X chromosome present) were deposited in the NIGMS Human Genetic Mutant Cell Repository at the Coriell Institute (GM12514 and GM12515, respectively).

### BAC CLONE ISOLATION

All BAC clones were obtained by PCR screening of a BAC library (Genome Systems, St Louis, MO) according to the manufacturer's protocol. Region specific STSs between DXS287 and DXS1072 were obtained from an X chromosome map at 75 kb STS resolution. Primer sequences used here are shown in table 1. PCR was performed for 35 cycles at 94°C for 30 seconds, at 50-55°C for 30 seconds, and at 72°C for 30 seconds.

### FLUORESCENCE IN SITU HYBRIDISATION

Chromosome preparations were made from lymphoblastoid cell lines by conventional methods. Slides were incubated in 2 × SSC at 37°C for 30 minutes, serially dehydrated in 70%, 80%, and 95% ethanol at room temperature, denatured in 70% formamide/2 × SSC at 72°C for two minutes, then serially dehydrated in 70%, 80%, 90%, and 100% ethanol (at −20°C).

BAC DNAs were labelled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation, precipitated in ethanol with a 50 × excess of human Cot-1 and herring testis DNA (Gibco-BRL, Gaithersburg, MD), and resuspended to a final concentration of 20 ng/μl in hybridisation solution (50% formamide, 2 × SSC, 10% dextran sulphate). Probes were denatured at 76°C for 10 minutes and preassociated at 37°C for 15 minutes before hybridisation.

The probe-hybridisation mix (10 μl) was applied under a 22 × 22 mm coverslip and slides were incubated in a moist chamber for 16 hours at 37°C, then washed as previously described. Probes were detected with 50 μl of rhodamine anti-digoxigenin (Boehringer-Mannheim) at 1 μg/μl. Slides were washed three times in 4 × SSC, 0.1% Tween-20 at 45°C and mounted in antifade solution (Vector) containing DAPI. Analysis was performed using a Zeiss Axiophot microscope equipped with filters to detect DAPI and rhodamine separately, as well as a triple band pass filter (Chroma Technology Corporation) to detect signals simultaneously. Images were collected and merged using a cooled CCD camera (KAF 1400, Photometrics) and IP Lab Spectrum software (Signal Analytics Corporation).

### BAC END SEQUENCING AND STS DEVELOPMENT

Purified BAC DNA was isolated using Qiagen Midi-Prep columns (Chatsworth, CA) according to the manufacturer's protocol, with the following modifications: Qiagen-Tip 100 columns were used for a 250 ml overnight culture with 10 ml of P1, 10 ml of P2, and 10 ml of P3 solutions as described elsewhere. One μg of BAC DNA as a template and 40 pmol of T7 and SP6 primers were used for sequencing reactions with ABI Prism™ Dye or dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA). T7 and SP6 primer sequences have been described previously. The sequencing reaction was cycled 50 times at 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 60 seconds. The Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) was used for generating new STSs from the sequences obtained. All clones used here were confirmed to contain the new STSs by PCR using 0.1 μg of BAC DNA as a template.

### PULSED FIELD GEL ELECTROPHORESIS (PFGE)

The Chef Mapper™ pulsed field electrophoresis system (BIO RAD, Richmond, CA) was

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**Figure 1** FISH analysis of the translocation patient's chromosomes using 183N12 as a probe. Arrow indicates a signal on a normal chromosome X, and arrowheads indicate signals on both the der(X) and the der(2) chromosomes.
Table 3 New STSs

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5'-3')</th>
<th>Product size (bp)</th>
<th>Ta (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>136I14-T7a</td>
<td>CAT GTA TGA CAA ACC ACC AGC</td>
<td>101</td>
<td>55</td>
</tr>
<tr>
<td>136I14-T7b</td>
<td>GTG GCA AGA GTG GAC ATC CT</td>
<td>161</td>
<td>55</td>
</tr>
<tr>
<td>253G2-T7a</td>
<td>AGC AAA CAG AAA AGC CCA GA</td>
<td>155</td>
<td>55</td>
</tr>
<tr>
<td>253G2-T7b</td>
<td>TGG CAA ATG TCC CCA AAG CA</td>
<td>284</td>
<td>55</td>
</tr>
<tr>
<td>253G2-SP6a</td>
<td>ATT GTC TCA CAG CTC CCT GG</td>
<td>125</td>
<td>55</td>
</tr>
<tr>
<td>253G2-SP6b</td>
<td>CAC TCA CTG AAT GGG CTC AA</td>
<td>199</td>
<td>55</td>
</tr>
<tr>
<td>278H4-T7a</td>
<td>TTG AAC TGC ACC GCT CCT TT</td>
<td>124</td>
<td>55</td>
</tr>
<tr>
<td>278H4-T7b</td>
<td>TTC GCT GTA CAA TAG TTA GC</td>
<td>136</td>
<td>55</td>
</tr>
<tr>
<td>278H4-SP6a</td>
<td>GAG ATA CTG CTC CCA ATT TA</td>
<td>60</td>
<td>55</td>
</tr>
<tr>
<td>278H4-SP6b</td>
<td>GCC ACA ATG CCA GTA GCT GA</td>
<td>196</td>
<td>55</td>
</tr>
<tr>
<td>127H8-T7a</td>
<td>TGA ATG CAA TGT TTT ATT GAA CC</td>
<td>60</td>
<td>55</td>
</tr>
<tr>
<td>127H8-T7b</td>
<td>TGG GCC TTA TAT CCC TCT TC</td>
<td>124</td>
<td>55</td>
</tr>
<tr>
<td>127H8-SP6a</td>
<td>ATA CCC TGC GAA TAG TTA GC</td>
<td>158</td>
<td>55</td>
</tr>
<tr>
<td>127H8-SP6b</td>
<td>GGC ACA ATG CCA GTA GCT GA</td>
<td>196</td>
<td>55</td>
</tr>
<tr>
<td>162M18-T7a</td>
<td>TAA CTT GAG CAA TTC CCG GT</td>
<td>125</td>
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<tr>
<td>162M18-SP6b</td>
<td>TGC TTT TGT GGA GGT TAA GAG</td>
<td>124</td>
<td>55</td>
</tr>
</tbody>
</table>

Tk: annealing temperature.

used for PFGE. BAC DNA was extracted by an automatic nucleic acid isolation system (AutoGen 740, Integrated Separations Systems, Natick, MA). One μg of DNA was digested with NotI for three hours and electrophoresed in a 1.0% SeaKem GTG agarose gel (FMC Bioproducts, Rockland, ME) with run conditions set by the auto algorithm to separate the size range from 5 kb to 300 kb. The gel was stained with ethidium bromide.

Results

BAC clone isolation and FISH analysis

One to three BAC clones were isolated from a Genome Systems BAC library with region specific STS primers on the X chromosome as shown in table 2. Each clone contained one to three STSs. FISH using these clones on normal metaphases showed that they mapped uniquely to Xq22.3 with no evidence of chimerism (data not shown). FISH analysis of the same clones on metaphase preparations from the translocation patient showed that eight clones mapped proximal to the Xq22.3 breakpoint, five clones mapped distal to the breakpoint, and three clones mapped both proximal and distal to the breakpoint. The latter three clones therefore span the translocation breakpoint. An example of the FISH results with a breakpoint clone is shown in fig 1.

Contig assembly around the breakpoint

Chromosome walking was performed by generating new STSs from BAC clones 278H4, 35L6, 127H8, and 162M18. These STSs were used to isolate the additional BAC clones 136I14, 253G2, and 260M7. Of these, 136I14 and 253G2 mapped proximal to the breakpoint and 260M7 mapped distal to the breakpoint on Xq22.2 by FISH, with no evidence of chimerism. 136I14, 253G2, 278H4, 35L6, 127H8, and 162M18 were sequenced from both ends of the insert DNA from the T7 and SP6 vector sequences. All ends produced 300–400 bp of sequence, which was used to design specific primers (table 3), with the exception of the 136I14-SP6 end which contained L1 sequence. A complete contig around the breakpoint was constructed with 13 BAC clones (fig 2). PFGE analysis showed the clone sizes ranged from 60 kb to 180 kb. The contig spans at least 490 kb as estimated from the sizes of non-overlapping clones within the contig. The breakpoint is located between STSs 278H4T7 and sWXD2044.

Discussion

The first linkage study of XLIS/SBH showed a putative XLIS/SBH locus either at Xq22.3-q23, between DXD8020 and DXS1072, or at Xq27. 12 Though the latter region was not excluded, the mapping of the breakpoint involved in the X2 balanced translocation to Xq22.3 strongly suggested that Xq22.3-q23 was the probable locus for XLIS/SBH. DXS8020 in Xq22.2 and DXS1072 in Xq23 defined a 9.2 cM candidate region. A second linkage study indicated that the critical region was between Xq21.3 and Xq24, flanked by markers DXS990 and DXS1001 with a genetic distance of 22 cM. 13 Furthermore, physical mapping of the X2 breakpoint showed that the breakpoint was located between markers DXS1105 and DXS1072, which are within 1 cM of each other. 11 For isolation of clones spanning the breakpoint, we chose 14 STSs between DXS1105 and DXS1072 using the 75 kb resolution map of Nagaraja et al. 14 We identified one to three BAC clones corresponding to each STS. FISH analysis of these clones showed three clones, corresponding to sWXS2044, spanning the breakpoint. The availability of high resolution physical maps, such as the 75 kb STS resolution maps now available for chromosome 7 15 and the X chromosome, 16 greatly facilitates the construction of clone contigs using BAC libraries with an average insert size of 120 kb. These resources make it possible to construct large contigs, such as the 490 kb contig described here, with relatively few chromosome walking steps. The resulting physical map and clone

Figure 2. Physical map of a 490 kb BAC contig of the region around the Xq22.3 breakpoint of t(X;2) in a lissencephaly patient. Anchor markers and 11 noted STS (short vertical line) are indicated at the top of the map. The breakpoint is estimated to be located in a region between STSs 278H4T7 and sWXD2044.
contig, including three clones spanning the translocation breakpoint, will be useful for the isolation and complete genomic characterisation of the XLIS gene as well as other contiguous genes which may play a role in brain development or function.

As previously hypothesised by Ross et al., it is likely that the phenotypic differences between males and females with mutations in the XLIS gene are the result of Lyonisation in females. Hemizygous males have lissencephaly while heterozygous females are mosaic. Neurons with the normal allele active migrate normally, while cells with the mutant allele active fail to migrate and form the subcortical band. The thickness of the band, and consequently the clinical severity, would be dependent on the degree of skewing of the X inactivation pattern.

Isolation of the XLIS/SBH gene and determination of its function and relationship to LIS1 will provide valuable insights into normal cortical development as well as the pathophysiology of human neuronal migration disorders.

Note added in proof
While this manuscript was under review, the XLIS gene was successfully isolated by two independent groups. 19 20

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