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

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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.
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Keywords: X linked lissencephaly; X;autosome 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 cerebral 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).² Facial dysmorphism is recognised in MDS, but is mild or absent in ILS and XLIS. Additional malformations are also predominantly seen in MDS.³⁻⁵

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 cases⁶ (Pilz *et al*, submitted). The *LIS1* gene has been mapped to the 17p13.3 deletion region^{7,8} and several mutations of the *LIS1* 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;autosome translocation^{4,11} 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)

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Table 1 Published STSs

Primer name	Sequences (5'→3')	Product size (bp)	Ta (°C)
sWXD500a	GGC CGT CAT TAG TGC TGG	440	55
sWXD500b	GCA AGA TAT GAA TCC TGG AG		
sWXD1665a	GGG GAA GGA AGG GAA AAA	97	55
sWXD1665b	GTA TGA TAA GCA TAA TAC AAA TGA CC		
sWXD1198a	CCT AAT AAA CAC CAG GCA C	71	50
sWXD1198b	GTA TGA TAA GCA TAA TAC AAA TGA CC		
sWXD2961a	CAT CTG GCA ACT TAA AGT GG	141	55
sWXD2961b	CTG GAC TAG GAT TTG ATT CTT GG		
sWXD1689a	GGA GAA ATG GGG AGT AAC TG	276	55
sWXD1689b	ACA CAT GGC TGG CTA GCT T		
sWXD1607a	TAT TTA CAG CAT GGT TTA CTT GAA	123	55
sWXD1607b	ATA CAT CTA TCC CTA CAC AGT TAC G		
sWXD1880a	CTT GGG GAG CCT GCT AA	184	55
sWXD1880b	TGC TAT CAA CCA GTT CAG CC		
sWXD2045a	GGG GGG TTA ATG AAT AAA TGG	171	55
sWXD2045b	TAG AAG GGG ATG CTG AGT G		
sWXD2044a	GGT AGG ATG AGG AAA GTA ATG	120	55
sWXD2044b	CCT CTG TAG GCT TGC TTA ATG		
sWXD1961a	CTA TTC TAG GTC TTC TGC ATT TC	60	55
sWXD1961b	TCT TAG GAA TTG ATG AGC AGG		
sWXD497a	GTA TGC AAC ACC ACC ATC C	66	55
sWXD497b	GTA TTT CCA CAG CCT TCT TGA G		
sWXD2043a	CCC AAA TAT CCT GGA AAA CC	79	55
sWXD2043b	CCG TGT AGT TAA AAT TAT GTT GTT C		
sWXD2425a	GGT TTT CCT TTG GCG TAT GAC	101	55
sWXD2425b	CTA CTA TCA ATC CTA CCC AGA G		

Ta: annealing temperature.

Table 2 Isolated BAC clones with region specific STSs and FISH localisation

BAC clone	STSs	FISH analysis
47O7	sWXD500	P
	sWXD1665/1198	
	sWXD2961	
76P19	sWXD500	P
	sWXD1665/1198	
	sWXD2961	
90P19	sWXD2961	P
97N17	sWXD1689	P
175L4	sWXD1689	P
	sWXD1607	
268L20	sWXD1880	P
37G4	sWXD2045	P
278H4	sWXD2045	P
35L6	sWXD2044	B
163I7	sWXD2044	B
183N12	sWXD2044	B
127H8	sWXD2044	D
162M18	sWXD1961	D
	sWXD497	
115I2	sWXD497	D
	sWXD2043	
	sWXD2425	
75C8	sWXD2425	D
262J17	sWXD2425	D

P: clone mapped proximal to the breakpoint. B: clone spanning the breakpoint. D: clone mapped distal to the breakpoint.

(q22.3;p25.1) de novo.^{4 13} 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 Insititute (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 DXS1072¹³ were obtained from an X chromosome map at 75 kb STS resolution.¹⁴ Primer sequences used here are

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.

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 10 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.⁷ 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 (KA-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 16 ml of P1, 10 ml of P2, and 10 ml of P3 solutions as described elsewhere.¹⁵ 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.¹⁶ 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.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

Table 3 New STSs

Primer name	Sequences (5'→3')	Product size (bp)	Ta (°C)
136I14-T7a	CAT GTA TGA CAA ACC CAC AGC	101	55
136I14-T7b	GTG GCA AGA GTG GAC ATC CT		
253G2-T7a	AGC AAA CAG AAA AGC CCA GA	161	55
253G2-T7b	TGG CAA ATG TAC CTG AAG CA		
253G2-SP6a	ATT GTC TCA CAG CTC CCT GG	155	55
253G2-SP6b	CAC TCA CTG AAT GGG CTC AA		
278H4-T7a	TTG AAC TTC AGC CGT CCT TT	284	55
278H4-T7b	TTG GCT GAA CAT TGA CTT GC		
278H4-SP6a	CCA GCA ATT TGG TAA ACT GGA	125	55
278H4-SP6b	TGC ACT TAC ACA AAG GTA CAA AA		
35L6-T7a	TGG GGC TTA TAT CCC CTT TC	199	55
35L6-T7b	ATA CCC TGC CAA TAG TCC CC		
35L6-SP6a	GGG ATA TCG CTG GCA ATT TA	244	55
35L6-SP6b	GCC ACA AGT CCA GTA GCT GA		
127H8-T7a	CCA ATC ACT TTT TAT TGT ACC A	196	55
127H8-T7b	TGA ATG CAA TGT TTT ATT GAA CC		
127H8-SP6a	ATT TGA ACA AGA TGG CAG GC	60	55
127H8-SP6b	GCC TCC CAA AAG TGC TGA G		
162M18-T7a	TAA CTT GAG CAA TTC CCG GT	124	55
162M18-T7b	AGA TTC CTG CAG AGC CTA GC		
162M18-SP6a	TTC CCA ATG TTT TCT GTG TCC	158	55
162M18-SP6b	TGC TTT TTG GGA GGT TAA GAG		

Ta: annealing temperature.

used for PFGE. BAC DNA was extracted by an automatic nucleic acid isolation system (AutoGen 740, Integrated Separation 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 X;2 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 X;2 breakpoint showed that the breakpoint was located between markers DXS1105 and DXS1072, which are within 1 cM of each other.¹³ 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 sWSS2044, spanning the breakpoint. The availability of high resolution physical maps, such as the 75 kb STS resolution maps now available for chromosome 7¹⁷ and the X chromosome,^{14,18} 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 novel 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.*¹³ 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 mosaics. 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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