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ABSTRACT

Autism is a neuropsychiatric disorder characterized by impairments in social interaction, restricted and stereotypic pattern of interest with onset by 3 years of age. The results of genetic linkage studied for autistic disorder (AD) have suggested a susceptibility locus for the disease on the long arm of chromosome 7. We report a girl with AD and a balanced reciprocal translocation t(5;7)(q14;q32). The mother carries the translocation but do not express the disease. Fluorescent in situ hybridization (FISH) analysis with chromosome 7-specific YAC clones showed that the breakpoint coincides with the candidate region for AD. We identified a PAC clone that spans the translocation breakpoint and the breakpoint was mapped to a 2 kb region. Mutation screening of the genes SSBP and T2R3 located just centromeric to the breakpoint was performed in a set of 29 unrelated autistic sibling pairs who shared at least one chromosome 7 haplotype. We found no sequence variations, which predict amino acid alterations. Two single nucleotide polymorphisms were identified in the T2R3 gene, and associations between allele variants and AD in our population were not found. The methylation pattern of different chromosome 7 regions in the patient's genomic DNA appears normal. Here we report the clinical presentation of the patient with AD and the characterization of the genomic organization across the breakpoint at 7q32. The precise localization of the breakpoint on 7q32 may be relevant for further linkage studies and molecular analysis of AD in this region.

KEY WORDS: autism; balanced reciprocal translocation; 7q31-q32; breakpoint mapping

INTRODUCTION

Autism is a prototypical pervasive development disorder (PDD) characterized by impairment in reciprocal social interaction and communication as well as restricted and stereotyped behaviors. Developmental abnormalities are apparent in the first 3 years of life with a prevalence of 5/10,000 [Lamb et al., 2000]. There is strong evidence for genetic components in the development of idiopathic autism. Epidemiological studies have demonstrated increased concordance rate in monozygotic twins versus dizygotic twins [Bailey et al., 1995] and a multilocus etiology has been suggested [Pickles et al., 1995; Risch et al., 1999]. Several independent genomic screen studies have revealed a susceptibility locus for autism on the long arm of chromosome 7 [International Molecular Genetic Study of Autism Consortium, 1998, 2001; Philippe et al., 1999; Risch et al., 1999]. Autistic patients with chromosomal rearrangements that involve the long arm of chromosome 7 have also been reported [Ashley-Koch et al., 1999; Warburton et al., 2000; Vincent et al., 2000; Yan et al., 2000]. Molecular studies of a gene interrupted by one of these rearrangements have been performed but mutation screening performed on sets of patients with autistic disorder (AD) did not reveal any alleles associated with the disease [Vincent et al., 2000]. However, the combined findings from linkage analysis and cytogenetic rearrangements suggest that the 7q31-q32 region contains a gene or regulatory sequences of importance for autism in a subset of patients. In this article, we present the clinical, cytogenetic, and molecular findings in a girl with AD and a balanced reciprocal translocation t(5;7)(q14;q32). Our findings support the hypothesis of an autism susceptibility locus on 7q and the precise mapping of the breakpoint provides additional information that may be useful for further studies of genetic mechanisms for AD.

MATERIALS AND METHODS

The patient is a 9-year-old girl and she was born after 40 gestational weeks in a normal delivery with a birth weight of 2,300 g. She has two younger twin sisters who are healthy at age 5 years of age. The father and the mother were 41 and 37 years old, respectively, at the time of delivery, and both parents are healthy and unrelated. The mother has six healthy siblings, of whom one has a child with an attention disorder. Two male first cousins of the mother have schizophrenia. The patient's psychological development during the first 2 years was normal, but before 3 years of age the girl presented with abnormal functioning in social interaction. Gross motor development was late and she walked without support at 16 months of age. She was retarded and at age 7 years she presented with a length of 112 cm (–2 SD) and a weight of 17,5 kg (–2 SD), whereas her head circumference was normal (51 cm; –0.5 SD). At the age of 7 years, she could not ride a bike, she took few initiatives, and she was shy. An Autism Diagnostic Observation Scales (ADOS) observation was performed at 7.5 years of age. Diagnosis of childhood autism was confirmed using the DSM-IV diagnostic criteria and diagnostic algorithm. The DSM-IV addresses three areas or domains of development: quantitative impairment in social interaction, qualitative impairment in interaction, and restricted repetitive and stereotyped patterns of behavior, interests, and activities.

Psychological testing revealed a qualitative impairment in social interaction as manifested by marked impairment in the use of nonverbal behaviors, failure to develop peer relationships appropriate to developmental level, lack of spontaneous seeking to share enjoyment, interests, or achievement with other people, and lack of social or emotional reciprocity. The girl presented with qualitative impairment in communication, which was manifested as an impairment in the ability to initiate or sustain a conversation with others and the lack of varied or spontaneous social imitative play appropriate to the developmental level. The girl also presented with restrictive, repetitive, and stereotyped patterns of behaviors as well as a selective mutism. The characteristics did not fit with Rett syndrome or childhood disintegrative disorder.

Physical investigation was normal and no abnormal features were observed. Ophthalmological

examination and brain stem audiometry revealed normal vision and hearing. No abnormalities were observed with magnetic resonance tomography (MRI) of the brain. Fragile X syndrome was excluded by DNA analysis. Normal levels of amino acids and amino acid metabolites were found in urine, plasma, and cerebrospinal fluid.

Genomic Clones and PCR-Derived Probes

The chromosome 7-specific YAC clones were obtained from the National Human Genome Research Institute [Bouffard et al., 1997]. Clones were chosen from the YAC contig sWSS8 (http://genome.nhgri.nih.gov/chr7/YAC-STS/anchored.html). The relative order of the clones from centromere to the telomere and the corresponding Sequence Tagged Sites (STS) are shown in Figure 1. The PAC clones RP5-894A10 and RP-1154E9 were obtained from Resource Center/Primary Database (RZPD) of the German Human Genome Project (clone IDs RPCIP704A10894 and RPCIP704 E091154).

Genomic probes for Southern hybridization and templates for sequencing were obtained by PCR amplification under the following conditions: initial denaturation at 96°C for 4 min, followed by 35 cycles at 96°C for 40 sec, 58°C (PF1-PF7) or 61°C (MEST-5' and sequencing templates) for 30 sec, 72°C for 1 min, and extension at 72°C for 10 min after the last cycle. Probes PF1-PF7 were amplified from the PAC clone RP5-1154E9, whereas the probe MEST-5' and sequencing templates were amplified from genomic DNA. The list of primers used for the amplification of probes for Southern hybridization is presented in Table I and primers used for the amplification of sequencing templates is presented in Table II. The PCR fragments were separated in a 1% agarose gel and purified by Qiaquick gel extraction kit (Qiagen).

Conventional Cytogenetic and FISH Studies

Standard chromosome preparations were made from PHA-stimulated peripheral blood lymphocytes from the patient, both parents, and the twin sisters. Conventional cytogenetic analysis of G-banded chromosomes was carried out on 500 band metaphases. Fluorescent in situ hybridization was performed essentially as previously described [Pinkel et al., 1986; Lichter et al., 1988]. Purified PAC DNA was labeled with digoxigenin-16-dUTP (Boehringer Mannheim) by nicktranslation. Total yeast DNA containing a YAC was purified by the spheroplast method [Albertsen et al., 1998], digested by *Eco*RI, and labeled with digoxigenin by nick translation. The labeled yeast DNA (500 ng) was mixed with 5 µg sonicated salmon sperm DNA and 20 µg COT-1 DNA (Gibco BRL), ethanol-precipitated, air-dried, and dissolved in 18 µl hybridization buffer (50% formamide, 2 x SSC, 10% dextran sulfate, and 50 mM phosphate buffer, pH 7.0). Denauration of the probes was followed by preannealing for 2 hr at 37°C.

Metaphase chromosomes preparations were denaturated using 70% formamide/2 x SSC for 3 min at 70°C, followed by ice-cold ethanol series. After overnight hybridization, slides were washed three times in 50% formamide/1 x SSC at 45°C and twice in 0.5 x SSC at 60°C. The probes were detected by the application of a single layer of rhodamine-labeled antidigoxigenin (Boehringer Mannheim). Chromosomes were counter-stained using DAPI (Serva) and mounted in Vecta-shield antifading solution (Vector Labs). The slides were analyzed with a Zeiss Axioscope microscope. Images were merged using a cooled CCD camera (Photometrics) and the IPlab software (Vysis).

Southern Blot and Methylation Analysis

Genomic DNAs were isolated from peripheral blood from AD patients and healthy individuals using standard procedures. Southern blot and hybridization were carried out essentially as described [Sambrook et al., 1989]. Ten µg of DNA was digested with an appropriate restriction enzyme, fragments were separated on 0.8% agarose gel, transferred to Hybond-N^+ membrane (Amersham), and hybridized with probes labeled with ^{32}P-dCTP by random priming.

Methylation analysis of genomic DNA obtained from blood of vt(5;7) patient and normal control individuals was performed as described previously [Riesewijk et al., 1997]. Southern blots with genomic DNAs (10 µg of each) digested by methylation-sensitive restriction enzyme *Hpa*II or its methylation-resistant isoschizomer *Msp*I were hybridized with the chromosome 7 probes MEST-5' and PF4 in order to determine the methylation patterns.

Sequencing of DNA

All sequencing reactions were performed using the Big Dye terminator cycle sequencing kit (PE Applied Biosystems) according to the manufacturer's instructions. Reactions were analyzed on an ABI 377 automated sequencer. Primers used for sequencing reactions are presented in Table II.

RESULTS

Mapping Chromosome 7 Translocation Breakpoint

The cytogenetic investigation of the patient showed an apparently balanced translocation between chromosome 5 and chromosome 7. The resulting karyotype was 46,XX,t(5;7)(q14;q32-q35). The sisters and the mother were found to carry the same translocation, whereas the paternal karyotype was normal. The breakpoint region on chromosome 7 was further mapped by FISH analysis. Chromosome 7-specific YAC clones were used as probes, of which the 800 kb YAC yWSS1954 is positive for the marker D7S2513 (Fig. 1A). The clone yWSS1954 hybridized to both der(5) and der(7), which indicates that the clone spans the chromosome 7 breakpoint (Fig. 2). A BLAST search using the sequence from D7S2513 and other STSs (http://genome.nhgri.nih.gov/chr7/YAC-STS/CONTIGR/ index.html) mapped within the YAC yWSS1954 was carried out, and several PAC clones were identified. Further FISH analysis with these PAC clones revealed that the PAC clone RP5-1154E9 spans the translocation breakpoint. A sequence of 82 kb (GenBank AC004979) corresponding to this PAC clone contains a cluster of candidate taste receptor genes (T2R3, T2R4, T2R5) as well as the 3' exon of the gene encoding mitochondrial single stranded DNA binding protein (SSBP; Fig. 1B).

In order to map the breakpoint in detail, we performed Southern blot analysis with genomic probes derived from the sequences in GenBank (Fig. 1C). The probes were hybridized to *Bam*HI-or *Eco*RI-digested DNAs from the patient and normal controls. Rearranged bands were detected in the patient DNA digested with both enzymes when hybridized with the probe PF6. This indicates that the breakpoint is located within a 2.3 kb *Eco*RI fragment between the T2R3 gene and the adjacent L1 sequence (Fig. 1B and 1C).

Mutation Screening

No gene was shown to be disrupted by the chromosome 7 breakpoint and a positional effect of the translocation on a flanking gene remained possible. The previously described 7q rearrangements associated with autism are located centromeric to our breakpoint, and we therefore chose to perform a mutation screening of coding regions of the genes SSBP and T2R3 located immediately centromeric to our breakpoint. Mutation screening was performed on genomic DNA from 29 independent autistic sibpairs [Philippe et al., 1999], which shared one or two parental chromosome 7 regions corresponding to the translocation breakpoint. Two nucleotide polymorphisms (–44 nt and 807 nt from ATG codon) were found within the T2R3. Three genotypes (–44T/T, 807C/C; –44T/C; 807C/T; –44C/C, 807T/T) were observed in autistic patients. In order to check possible association of the polymorphism with autistic phenotype, we screened 29 healthy control individuals. No association was observed between any polymorphic variant and autism (Table III). No polymorphisms were identified in the SSBP gene and no nucleotide change

predicting an amino acid substitution was identified in either SSBP or T2R3 genes.

Analysis of 7q Methylation Pattern

The 7q methylation pattern was analyzed on genomic DNAs from the t(5;7) patient and normal controls by Southern blot analysis. The DNA was digested by either *Hpa*II or *Msp*I. The restriction enzymes have recognition sites, which flank a 4.95 kb fragment, detected by the probe PF4 (Fig. 1C). No difference in size or intensity of bands were observed between DNA from the t(5;7) patient and normal controls (data not shown). We also analyzed the methylation pattern of the PEG1/MEST gene in a more centromeric region of chromosome 7 to clarify a possible long-distance effect of the breakpoint. The PEG1/MEST gene is known to be imprinted in human and mouse [Riesewijk et al., 1997] and an altered PEG1/MEST gene activity is associated with behavioral disturbances in mice. A 1.27 kb PCR-derived probe located within the first intron of PEG1/MEST (Fig. 3) was hybridized to genomic DNA digested by *Hpa*II or *Msp*I. No difference of the hybridization pattern was detected between DNA from the t(5;7) patient and from normal control individuals (Fig. 3). Our results were consistent with the normal methylation pattern of PEG1/MEST [Riesewijk et al., 1997].

DISCUSSION

An intensive search for autism-responsible genes has been currently performed by different groups. Four full genomic screens were recently published and the combined results indicated several susceptibility loci for AD [International Molecular Genetic Study of Autism Consortium, 1998; Collaborative Linkage Study of Autism, 1999; Philippe et al., 1999; Risch et al., 1999]. However, the regions defined by the linkage studies are large, and positional cloning of candidate genes based on linkage data only may be a very difficult task. A refined mapping and cloning of candidate genes may require complementary approaches. The identification and molecular exploration of chromosomal rearrangements associated with AD may provide key information. In this article, we present clinical, cytogenetic, and molecular findings in a girl with AD associated with a balanced reciprocal 5;7 translocation. The chromosome 7 breakpoint was mapped to a region close to the marker locus D7S2513. This translocation may have occurred by chance. However, the results from previous genomic screens were consistent with linkage to a region on chromosome 7 between marker D7S486 and D7S661 [Philippe et al., 1999; Risch et al., 1999], which coincides with the 7q breakpoint in our patient. No evidence of an autism susceptibility locus on proximal 5q has been found in genomic screenings, which makes the 5q breakpoint less likely as a candidate region.

Recently, a familial paracentric inversion in 7q and other 7q rearrangements associated with autism were reported [Ashley-Koch et al., 1999; Vincent et al., 2000; Warburton et al., 2000; Yan et al., 2000]. Linkage for this region to AD was demonstrated in the first study, and linkage disequilibrium analysis indicated an association with paternal inheritance. Such a parental effect suggests the involvement of an imprinting mechanism. The paracentric 7q inversion was inherited by affected siblings from the healthy carrier mother. Similarly, our patient inherited the translocation from the carrier mother who did not express AD. Thus, the linkage disequilibrium and the cytogenetic abnormalities may suggest a parental specific effect with different inheritance, and the molecular mechanism behind this contradiction remains to be explained. Interestingly, similar results were obtained from the analysis of a family with a 15q duplication associated with AD [Cook et al., 1997]. The effected female inherited the duplication from the healthy mother who had received it from her father. The proximal 15 q and 7q21-q36 regions are known to be subject to imprinting [Hannula et al., 2001], and similarities in parental effects may exist for autistic individuals with 15q11-q13 and 7q rearrangements. The cytogenetic abnormalities may change a normal imprinting pattern of a single gene or a number of genes, which, in turn, causes the disease. However, the

fact that two healthy twin sisters carry the translocation does not support an imprinting effect as major etiological mechanism for AD in our patient. We did not observe any abnormalities in the methylation pattern of the PEG1/MEST gene and in the 7q breakpoint region in our patient, but this does not exclude an effect of the translocation on other regions of 7q. A more thorough molecular analysis of the chromosome 7 breakpoints regions in patients with 7q rearrangements is required in order to prove such a mechanism. Important information may also be obtained from a detailed investigation of the expression pattern of genes located in the 7q autism susceptibility region. A comparison of the gene expression pattern in autistic patients and normal individuals may reveal genes, which contribute to the autistic phenotype. Taken together, our study in combination with previous studies supports the existence of a susceptibility region for AD on 7q31-q32. A detailed study of the expression of genes located in 7q31-32 may reveal candidate genes or regulatory sequences involved in the disease.

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TABLE I. Primers Used for Amplification of PCR-Derived Probes Applied for Mapping of the 7q Translocation Breakpoint and Methylation Studies by Southern Blot Hybridization

Probe name	Fragment size (kb)	Forward primer (5'-3')	Reverse primer (5'-3')
PF1	0.46	GTTTATTCTTCTAAGGGTGG	GCTACTAATGAAACTGTC
PF2	0.41	ACCACCCTCTGTCAGTGC	AACTAAGCTACAGTTAGC
PF3	0.9	CTTGCTCAGGAACAGTAG	ACGCATAATCACCCTCTC
PF4	0.86	TCATAGCTCACTGCAGCC	TGTGCACCCTGTCACATC
PF5	1.03	CACATGGGAAGTGCTTAG	TCCACAGCAGAGATCCGT
PF6	0.88	AAGTCTTGCAATGGCTCTCC	CTGAGCTCTTTCTACTACAC
PF7	0.99	CCCTTGATCATGAATGGC	GAGAACAGAATGTCCTGG
MEST-5'	1.27	ACTCTACCGACAGGCGGCAC	GAATCTCTGCAGTGACTCAGC

TABLE II. Primers Used for Amplification and Sequencing of Candidate Genes

Gene	Amplified sequence	Fragment size (kb)	Forward primer (5'-3')	Reverse primer (5'-3')	Sequencing primer forward (5'-3')	Sequencing primer reverse (5'-3')
SSBP	Exons 1 and 2	0.92	TTTCCTCTGCGAGCTTTGCG	TCTGATCACTGTGGCTGATAG	TTTCCTCTGCGAGCTTTGCG	TCTGATCACTGTGGCTGATAG
	Exon 3	0.22	TGAACGTGAGTAATTCTTCTG	GCAGCATACCTCTTCTACAAC	TGAACGTGAGTAATTCTTCTG	GCAGCATACCTCTTCTACAAC
	Exons 4 and 5	0.61	CAGTCTATAGCAGGTTGTCTC	GACTACACACTCCTTGCCATG	GGTTGTCTCATTTGGCTCTG	ATGAGAACACTTCTTATCGG
	Exon 6	0.25	TATACTCAGTACCACCCTGAC	TTAGTTACAGTGTAGCCAAGC	TATACTCAGTACCACCCTGAC	TTAGTTACAGTGTAGCCAAGC
	Exon 7	0.26	AGAGGAGAATTATTGAATGAG	AAGGAGAGCGAGAACGACAG	AGAGGAGAATTATTGAATGAG	AAGGAGAGCGAGAACGACAG
T2R3	Coding region	1.41	GAGTCCAAGTCCTCCATCATC	CAGTCAGTGTTTCTCTCCTAC	GACCTAGATCTCTTCCTTCTC	GCTGCTACCATTGACCAACTC
					GTGTTCCTGATTCTGTCTGGC	CTGAAGTGTTCAGTCACATTCC
					GGAATGTGACTGAACACTTCAG	AGAGTCCTGTAGTCTTGAGCC

TABLE III. Number of Autistic and Control Individuals Who Share Different Genotypes of T2R3 Polymorphisms Analysis of 7q Methylation Pattern

	–44T/T: 807C/C	–44T/C: 807C/T	–44C/C: 807T/T
Autistic patients	8	11	10
Normal controls	12	13	4

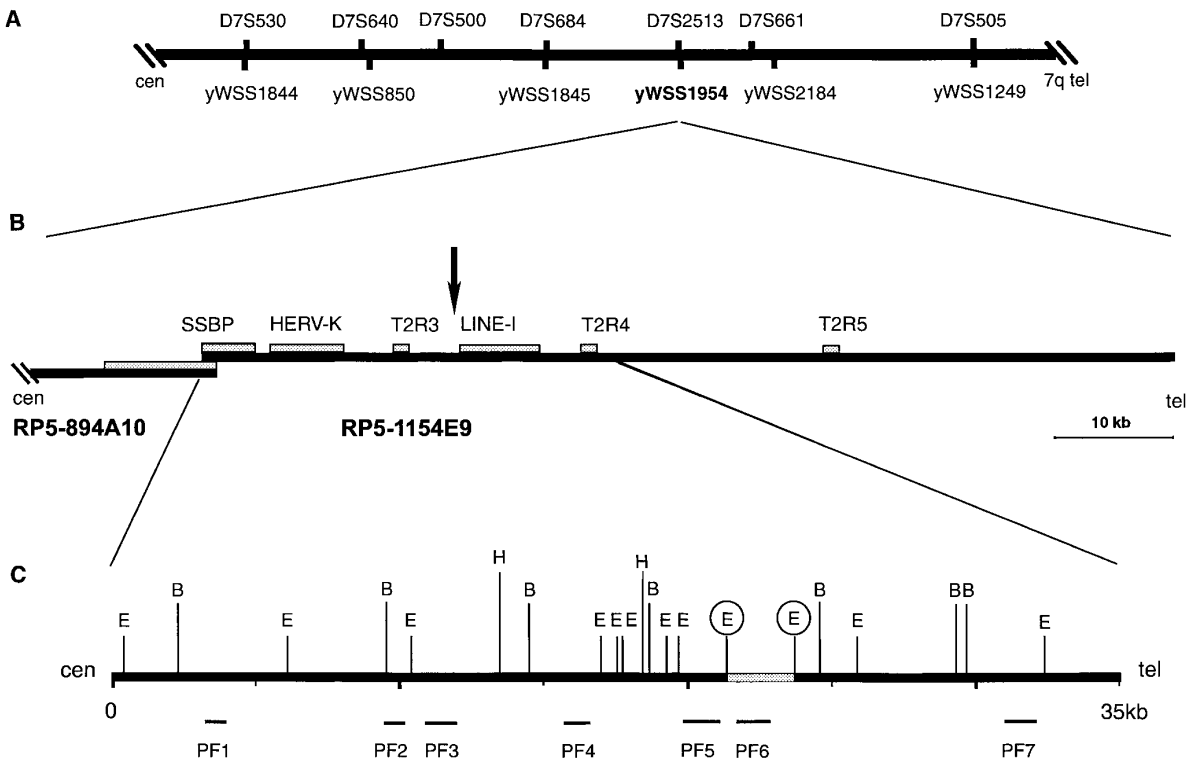

Fig. 1. Map of the 7q32'q35 region spanning the breakpoint. A: The relative position of DNA markers are indicated (top) as well as the position of YAC clones used for FISH mapping (below). The YAC yWSS1954 (bold) spans the breakpoint. B: Two PAC clones in the 7q breakpoint region, of which RP5-1154E9 spans the translocation breakpoint. The breakpoint position is indicated by an arrow. Gray boxes denote genes as well as the long repeat sequences HERV-K and LINE-1. C: Restriction enzyme map from part of the RP5-1154E9 derived sequence (GenBank AC004979) around the 7q translocation breakpoint (B, *Bam*HI; E, *Eco*RI). The PCR-derived probes used for the breakpoint mapping and determination of the methylation pattern by Southern analysis are indicated below. The gray bar indicates the *Eco*RI fragment spanning the breakpoint. The two sites *Hpa*II/*Msp*I (H) utilized in the methylation analysis are shown.

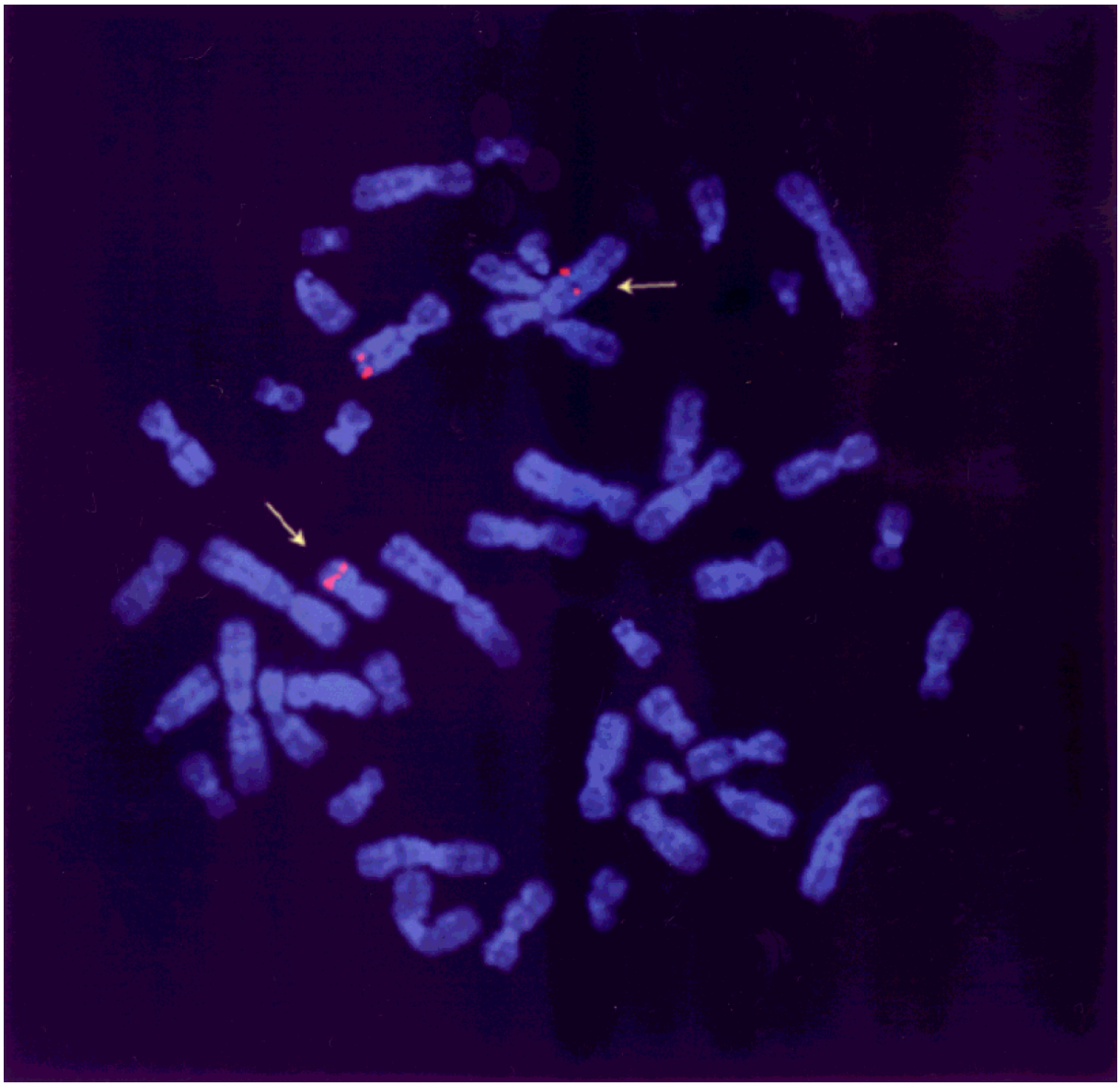

Fig. 2. Fluorescent in situ hybridization of the YAC yWSS1954 to metaphase chromosomes from the patient with the 5;7 translocation. The YAC yWSS1954 (red signals) is detected on both chromosome derivatives (arrows) and on the normal chromosome 7.

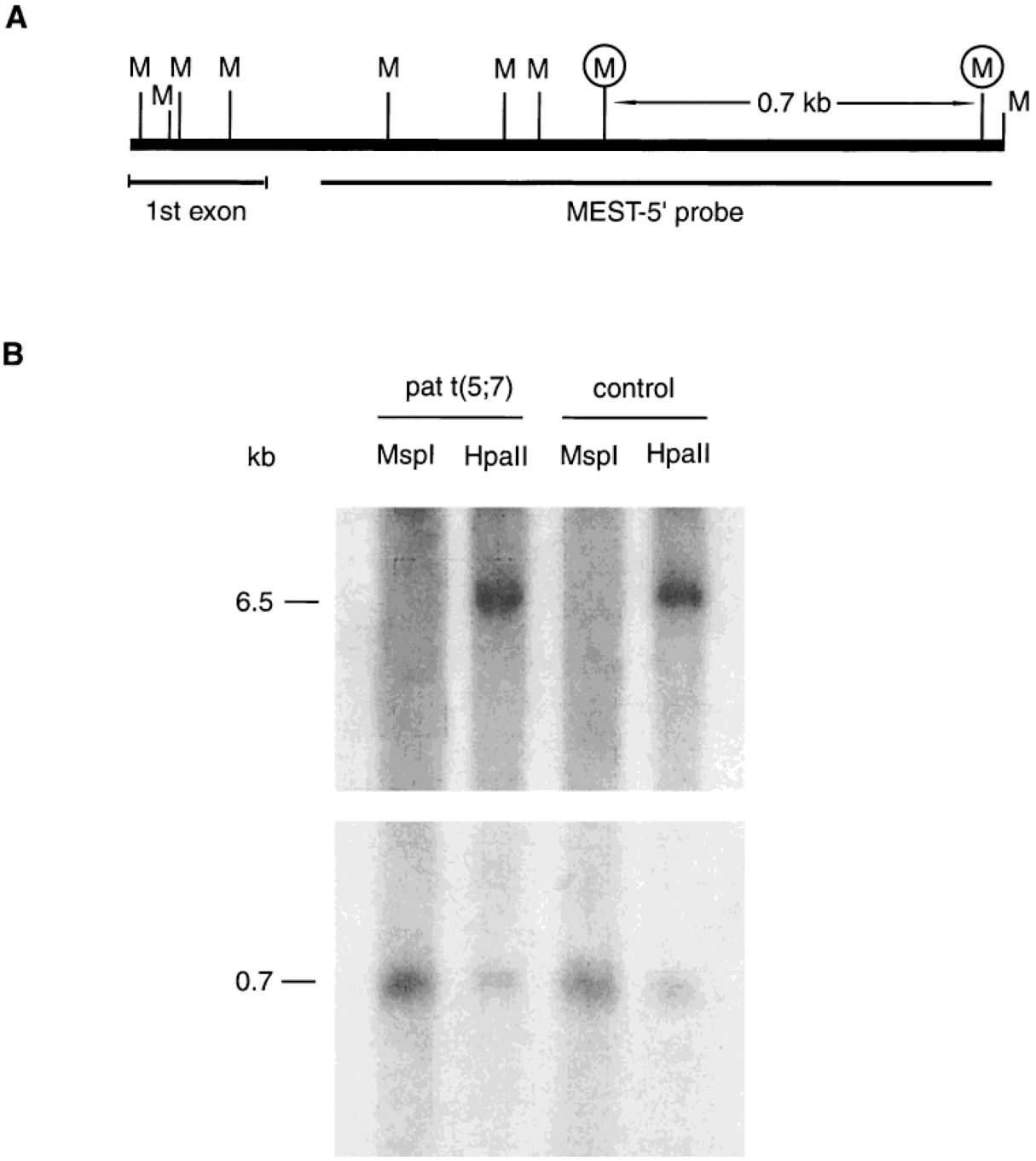

Fig. 3. Southern blot analysis of the PEG1/MEST gene digested with either *Msp*I or the methylation-sensitive isoschizomer *Hpa*II. A: Map of part of the PEG1/MEST gene including exon 1 and a part of the intron 1 (GenBank AC007938). The *Hpa*II/*Msp*I (H) sites, which have been analyzed for their methylation status, are circled. The probe used for Southern hybridization is shown below. B: Autoradiogram from Southern blot analysis of DNA from the patient with the translocation and a healthy control. The hybridization patterns after *Msp*I and *Hpa*II digestion are identical, which indicate a similar methylation pattern detected by the MEST-5' probe in the two individuals. The lower bands correspond to a 710 bp fragment restricted by the *Hpa*II/*Msp*I sites investigated (A). The upper bands correspond to the DNA fragment from the allele with the methylated 5' region of the PEG1/MEST gene.