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ARTICLE

Physical map of a 1.5 Mb region on 12p11.2 harbouring a synpolydactyly associated chromosomal breakpoint

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Synpolydactyly (SPD) is a rare malformation of the distal limbs known to be caused by mutations in *HOXD13*. We have previously described a complex form of SPD associated with synostoses in three members of a Belgian family, which co-segregates with a t(12;22)(p11.2;q13.3) chromosomal translocation. The chromosome 12 breakpoint of this translocation maps to 12p11.2 between markers D12S1034 and D12S1596. Here we show that a mutation in the *HOXD13* gene is not responsible for the phenotype, and present a physical map of the region around the 12p11.2 breakpoint. Starting from D12S1034 and D12S1596, we have established a contig approximately 1.5 Mb in length, containing 13 YAC clones, 16 BAC clones, and 11 cosmid clones. FISH analysis shows that cosmid LL12NCO1–149H4 maps across the breakpoint, and Southern blot experiments using fragments of this cosmid as probes identify a rearranged *Bam*HI fragment in the patients carrying the translocation. A search for expressed sequences within the contig have so far revealed one CpG island, seven anonymous ESTs and three previously characterised genes, *DAD-R*, *KRAG* and *HT2I*, all of which were found not to be directly disrupted by the translocation. The gene represented by EST R72964 was found to be disrupted by the translocation. These findings lay the groundwork for further efforts to characterise a gene critical for normal distal limb development that is perturbed by this translocation. *European Journal of Human Genetics* (2000) **8**, 561–570.

Keywords: synpolydactyly; physical mapping; limb development; chromosome 12p11.2; *HOXD13*; *KRAG*; *HT2I*; *DAD-R*

Introduction

Synpolydactyly (SPD, OMIM No. 186000) is a rare dominantly inherited malformation of the distal limbs in which there is a combination of syndactyly (webbing of digits) and polydactyly (supernumerary digits). Typically, there is a syndactyly between the third and fourth fingers and between the fourth and fifth toes, with partial or complete digit duplication in the synpolydactyly web. In most cases this condition is caused by mutations in the *HOXD13* gene, which is located on chromosome 2q31. Classical SPD has been shown to be due to different sized expansions of an imperfect trinucleotide repeat sequence encoding a 15-residue N-terminal polyalanine tract in HOXD13.[1–3] An atypical form of SPD, associated with a novel foot phenotype, has been found in two families with two differing intragenic deletions in *HOXD13*.[4]

We recently described a Belgian family (Figure 1) in which three individuals have a complex type of SPD associated with metacarpal and metatarsal synostoses.[5,6] The phenotype in these patients is distinct from that in any previously reported case of SPD and appears to represent a novel dominantly inherited limb malformation. Interestingly, this malformation is not accompanied by an expansion of the HOXD13

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polyalanine tract, and seems to co-segregate in the family with an apparently balanced translocation t(12;22)(p11.2;q13.3).[7]

This co-segregation suggests that perturbation of a gene (or genes) located at 12p11.2 and/or 22q13.3 may underlie this unique form of SPD. Although no 'SPD gene' has previously been assigned to chromosome 12p11 or 22q13, a locus for dominantly inherited hypertension combined with the distal limb malformation brachydactyly has been shown to map to chromosome 12p11.[8] We have previously mapped the chromosome 12p11 breakpoint in our patients to the interval between markers D12S1596 and D12S1034, and the chromosome 22q13 breakpoint to the interval between markers D22S158 and N73F4.[7] Interestingly, the interval between D12S1596 and D12S1034, which is no larger than 1.7 Mb, maps to the region containing the hypertension/brachydactyly locus. This finding thus raised the intriguing possibility that different mutations affecting the same gene on 12p11 might give rise to two distinct distal limb malformations – brachydactyly and complex SPD.

In our current communication, we have formally excluded the possibility that a mutation elsewhere in *HOXD13* might nevertheless be responsible for the SPD-like phenotype by analysing the entire coding sequence in one affected family member. No mutations were found. We therefore directed our attention to the 12p11 breakpoint, using markers D12S1596 and D12S1034 as starting points for a chromosome walk. Here we describe a contig containing 12 YACs and 16 BACs spanning 1.5 Mb around the chromosome 12 translocation breakpoint region. Mapping of sequence tagged sites (STSs) and sequence tag connectors (STCs) together with restriction digests have allowed us to establish a long range physical map of the region. STCs derived from the ends of the appropriate BACs have been used to isolate cosmids for FISH studies, one of which (LL12NCO1–149H4) proved to span the chromosome 12p11 breakpoint. To date, three previously reported genes and seven anonymous ESTs corresponding to four cDNAs have been identified and positioned within the contig. The gene represented by one of the ESTs was subsequently found to be disrupted by the translocation.

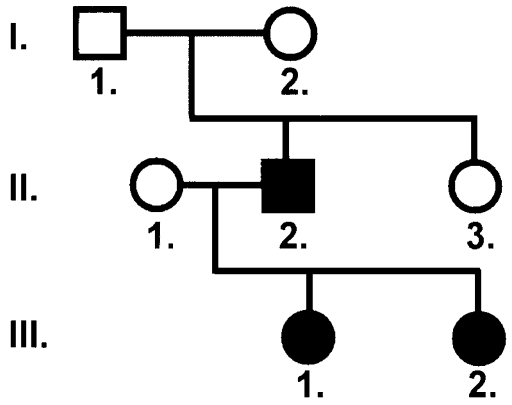

Figure 1 Pedigree of the family. Solid symbols represent the three affected individuals, who all carry an apparently balanced translocation t(12;22)(p11.2;q13.3).

Materials and methods

Subjects

A pedigree of the family is shown in Figure 1. The three affected family members, a father (II.2) and his two daughters (III.1 and III.2), all have a complex type of SPD associated with metacarpal and metatarsal synostoses, as reported previously.[5,6] These three individuals also share an abnormal karyotype t(12;22)(p11.2;q13.3) not present in the four unaffected family members (I.1, I.2, II.1 and II.3).

HOXD13 mutation analysis

For *HOXD13* mutation screening in individual II.2, the entire coding sequence of the gene was amplified from genomic DNA by PCR in three segments, using primers and reaction conditions described previously.[3] Products were subcloned into pCRScript (Stratagene, Amsterdam, The Netherlands) before being cycle sequenced (Applied Biosystems Prism Dye Terminator Kit, Cheshire, UK). For each product, a minimum of eight different subclones were sequenced.

Isolation of large genomic clones

YAC clones were isolated from the Centre d'Etude du Polymorphisme Humain (CEPH, Paris, France) Mark 1 YAC library[9] as previously described.[10] A genomic BAC library (Research Genetics, Huntsville, AL, USA) was also screened using probes D12S1034, D12S1596, PD56 and PD64. Isolated YAC and BAC clones were characterised by STS/STC–PCR, contour-clamped homogeneous electric field (CHEF) gel electrophoresis,[11] restriction mapping, hybridisation and FISH analysis. Using standard methods, cosmid clones were isolated from a chromosome 12-specific cosmid library constructed at the Lawrence Livermore National Laboratory (Livermore, CA, USA) (LL12NC01).[12]

Pulsed-field gel electrophoresis and Southern blot analysis

Pulsed-field gel electrophoresis and Southern blot analysis of YAC clones were performed as described previously.[11] For analysis of BAC clones, 0.5 μl of purified DNA was digested with rare-cutter restriction enzymes (*Bss*HII, *Not*I, *Sal*I, *Sfi*I, and a *Not*I–*Ksp*I double digest) in a total volume of 20 μl for 1 h using 10 units of each enzyme per reaction. Digestion products together with appropriate size markers (a γ DNA ladder obtained from Promega, Leiden, The Netherlands, and γ DNA digested with *Hind*III) were loaded on to a 1% agarose/0.25× TBE gel, and size-fractioned on a CHEF DRII or Mapper apparatus (BioRad, Eke, Belgium). Southern blotting and subsequent hybridisations were carried out as described previously.[10]

Generation of STCs from YAC insert ends, sequence analysis and primer design

STSs from YAC insert ends were obtained using vectorette-PCR combined with direct DNA sequencing analysis.[13] Throughout this paper, STSs/STCs are referred to by their abbreviated names. DNA sequencing was carried out using standard procedures. Sequence results were evaluated using the Lasergene software package (DNASTAR, London, UK) together with BLAST searches at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/blast.cgi).[14] After sequence analysis, primer pairs were designed using the computer program OLIGO.[15] Primers are listed in Table 1.

Results

HOXD13 mutation analysis

Using PCR amplification and direct sequencing, we have previously shown that the imperfect trinucleotide repeat in exon 1 of *HOXD13*, which encodes the 15-residue poly-alanine tract expanded in classical SPD, was normal in length in individual II.2.[7] In one of his alleles, however, alanine 12 of the tract was encoded by GCG rather than by GCA. This base change represents a single nucleotide polymorphism, resulting in a silent mutation, however, since the same finding has previously been reported in 5/20 normal and 8/20 expanded alleles,[3] and was also seen in the patient's unaffected mother (I.2). Here, we investigated whether some other mutation in *HOXD13* could be responsible for the novel complex type of SPD present in our patients. Using primer pairs designed to amplify the whole coding region of *HOXD13*, we identified no such mutations in individual II.2.

Construction of a 1.5-Mb YAC contig and physical map

In a previous study,[7] we established that the 12p11.2 breakpoint in our patients was located between markers D12S1034 and D12S1596. These markers both reside within YAC 774E2, which is 1.7 Mb in length. By FISH analysis, which was performed essentially as described previously,[16,17] this YAC was shown to map across the breakpoint.[7]

Table 1 STS primer sequences, annealing temperatures and expected PCR product sizes

STS name	*Nucleotide sequence 5'–3'*	*Product size (bp)*	T_{ann}	*STS name*	*Nucleotide sequence 5'–3'*	*Product size (bp)*	T_{ann}
D12S1596	TCATGTGGCTGGTAGAGAAG TTTGGTAGTGCTGTTTTCCA	123	56	PD73	AAAGCATGGTGACTAAGGGCACAG CCTGGGGATCTTTATCCCCAAATA	111	56
D12S1411	ATTATCTGCCCTTGTCATATATTGG ACGCACTATCAAAACCAGATAGC	227	56	PD81	CAGTCTCGCCTTTCCTTG CACCAGGGTCAATGGACT	69	58
D12S1034	GTGTGACTGTGCTTGCTAGG CTGTCCTGATCTTCCAAACC	195	56	PD92	CGTGGAACAGGTTGAAATAG CCATGACACAGTCTGGAAAA	240	58
z38802	GGTATTTTACAGGTCCTCATCA GATAAAACACCCGATTAACAGA	196	56	PD97	GAGCGTCACATGATAACACG ACAGCACTGAGCATCCTTAGA	124	58
5'KRAG bis	GTTCCCGCTGCTGCTCG TGATCCCAGCCCAAAATG	125	60[a]	PD100	CCCAAATTTTCTCATTCTC AATCCCAGAAAAATTGAAC	107	58
P24/25[b]	GAGAAGGTATCCATGGGGAAGG CCAACAATAAAGGCTCTTTCAGAT	(~1.2kb)	58	PD108	CGTTGCAATTTTTCTGCTTC TCCATTGAGAAACAGCTGGA	302	58
DAD-R	CTAGCACCATCTTGTACCTTATC ACTTAATTGAAGGCAGAAGTCA	277	52	PD109	TCTCCATTACCAGCATGTCT CTTATGGTTTTAGCCACCAG	208	58
PD30	CATGTTGAAAAGGACAAAACA GTAACATTGGAATGGGATTGT	174	58	PD118	GTGGTCATTTACTGGCAGAC TTACCCACTGGTTAAAGGAA	166	56
PD52	CCAATCAAAATTACCACTA GGAATCATTTCATCACTCT	173	56	PD119	CAGATTTTGAGACAGGTTTGG CATTCTCGAATCAGAGCTTCA	160	56
PD53	GCTTGCCACAGTATATGACA CATTTGGGGTGACTTTAATA	152	56	PD120[c]	CTAGGAGCTGTAACCCTGAT AGTCTGAACGATGACCAATC	129	58
PD55	GCCAGTTATCTATGAGAAGT TGGAGTCTTAGATCATTCTC	174	56	PD122	TCGACCAGACCCCAGCAAGC CCACCTCACTCCAGCCCGTTC	150	58
PD56	TGCCAATGCCTATTTTTATT GATACATGCTCATGCCAGTT	155	56	PD168	AATCACATCCCTGTTAAGCCTTC TAGTTAGGCTCCTTTCCTGGAATA	111	56
PD57	CAGTCCCGGAGAGCTGCTAACA CTTCCTGGGCTTGGGACGTTAT	148	56	PD169	ACCAACTTCGCCGTTTTGATAAG TCACACCCACAGCCATACTGTTC	260	56
PD64	TCAATTCTGGGACTGGGCTGAG CACATCACGTGCAACCTGTGTG	160	56	149H4-B5-SB	TAATACCCTATACCACAT ACATTTTTATAAGTGTCC	192	52
PD70	GAACCCTTCCCCTAGGCATG TCCTGCCTTGGGATGTTCAG	290	56	149H4-B7-SB	TTCTTCAGTAAAGCCTCATCTCATC AGAGACATCCTGAGCACATTACAAT	355	56
PD71	TATGTGGCTGGTGGGCCTTCAT CAGGCTAGCTGGCTAGGGCACA	195	58	HT2I-int	TCTGTGTGCAGAATAAAAAGTG TCATAGGGTATCTGCAGAAGAT	100	58
PD72	TGAATCACATTGGCCTTAATCA CGGCCTTAATAAGCATTTTTTG	136	58				

[a]add 10% DMSO; primer used to amplify exon 1 from *KRAG*; [b]primers used to amplify exon 5 from *KRAG* (Heighway *et al*., 1996)[22]; [c]STS PD120 is made on single copy sequences from EST AA190175 which was isolated with end-sequences of LL12NCO1–93A1.

We therefore screened the CEPH human Mark 1 YAC library with D12S1034 and D12S1596, isolating 8 YACs (Table 2). Restriction maps of each of these YACs were aligned and a consensus restriction map of the region established, as illustrated in Figure 2. Interestingly, YAC 368D4 included both D12S1034 and D12S1596, thereby narrowing the region containing the breakpoint to the size of this YAC (350 kb). Several YACs isolated with markers D12S1411 and EMBL z38802 (which are both known to lie centromeric to the breakpoint on chromosome 12p) were also found to overlap with YACs containing D12S1034. An additional 5 YACs (Table 2a) could thus be integrated into our contig so that it eventually comprised 13 YACs spanning approximately 1.5 Mb of DNA.

Construction of a BAC and cosmid contig

To pinpoint the breakpoint region further, we constructed a BAC contig anchored on D12S1034 and D12S1596. By screening the BAC library with these two markers, we obtained seven D12S1596-anchored BACs and three D12S1034-anchored BACs (Table 2b). Restriction maps of each of these BACs were constructed and aligned into two separate contigs, centered on D12S1034 and D12S1596, respectively. The chromosomal orientation of these contigs was determined by FISH analysis using cosmids mapping to isolated BAC ends. Thus, LL12NCO1–247C2, containing PD56 (derived from BAC 549C24) mapped telomeric to the breakpoint (positive signal on the normal chromosome 12 and the der(22)), whereas LL12NCO1–71E7, containing PD64 (derived from BAC 570H2) mapped centromeric to the breakpoint (positive signal on the normal chromosome 12 and the der(12); results not shown).

Since the two BAC contigs anchoring at D12S1034 and D12S1596 did not overlap, the BAC library screen was repeated, using probes PD56 and PD64. Six additional BACs (Table 2b) were isolated, allowing the two contigs to be merged (Figure 3). In addition, BAC 264F23 was found to span the breakpoint on FISH analysis (positive signal on the normal chromosome 12, the der(12) and the der(22); results not shown).

Based on STS/STC-content mapping, 11 cosmids (Table 2c), isolated to position the various BAC STCs, could also be grouped into a single contig. The positions of these cosmids relative to the t(12;22) breakpoint were independently confirmed by STC-content mapping of the newly generated STCs as well as by FISH.

Physical mapping of STSs, STCs, ESTs and known genes

By sequencing the ends of the isolated BACs, we generated multiple novel STCs which were subsequently integrated into the contig described above. STCs were also generated from the ends of cosmids which had been isolated to position the BAC ends more precisely relative to the translocation breakpoints, and in a few cases from the ends of YACs. A total of 23 STCs were generated in this way. An additional STC (PD81) was derived from the left end of YAC 170A5. This STC was isolated as a suitable starting point for a sequence-walking approach aimed at isolating CpG island sequences, as it maps close to a cluster of sites recognised by rare-cutter restriction enzymes, which is generally indicative of a CpG island.[18] All sequence data obtained were analysed for the presence of repeats and transcribed sequences. This approach revealed that the contig contained exons 1 and 5 of *KRAG* (Kirsten-ras associated gene – GenBank accession no. X89105); and STS marker D12S1411, corresponding to the 3'UTR of *HT2I* (encoding the type 2 inositol, 1,4,5-triphosphate receptor (IP_3R) – GenBank accession no. D26350). Our previous FISH studies had shown that D12S1411 maps centromeric to the breakpoint. To determine the orientation of the *HT2I* gene relative to the breakpoint, an EST containing *HT2I* sequences upstream of the 3'UTR (*HT2I*-int, position 3993–4092, Figure 2) was mapped within the contig. Cosmids isolated using probes derived from the 3' and 5' ends of the gene all mapped centromeric to the breakpoint on FISH analysis, indicating that the entire *HT2I* gene lies centromeric to the breakpoint (data not shown). Finally, the *DAD1*-homolog *DAD-R* was recently isolated and mapped to 12p12.1–11.2 by Kuittinen *et al*, following a positional cloning effort focusing on recurrent chromosome 12 aberrations (manuscript in preparation). The use of *DAD-R* specific primers initially allowed us to position this gene within YAC 292C10 (by STS-content mapping approach). Subsequent Southern blotting experiments showed that the gene was situated within a *Sal*I-*Bss*HII restriction fragment of approximately 10 kb, located at the telomeric part of our contig (Figure 2).

The contig also contains several non-overlapping ESTs which do not correspond to any known genes (AA190175, isolated using STC PD120; T48235, isolated using STC PD122; and R72964, isolated using STC PD108), as well as the overlapping ESTs AA701549, AA380212, AA782961, and AA532445 (isolated using STC PD168). Based on northern blot analysis of multiple adult human tissues, AA190175 appears to be exclusively expressed in testis as a 2.4 kb transcript (Figure 3B), whereas T48235 is widely expressed as a 2.2 kb transcript, with particularly high expression levels in prostate and placenta (Figure 3C). R72964 is homologous to a putative regulator of cell growth and differentiation (Swiss-Prot accession no. B44478). It is widely expressed as a transcript of 6.2 kb, with particularly high expression levels in skeletal muscle, although it is apparently not expressed at all in peripheral blood leukocytes (Figure 3A). A 2.2 kb transcript (possibly resulting from alternative splicing) is also expressed exclusively in testis. The remaining four overlapping ESTs are exclusively expressed in testis as a 1.8 kb transcript (Figure 3D).

Isolation of a cosmid spanning the breakpoint

During our chromosome walk, cosmid clone LL12NCO1–149H4 was shown by FISH to map across the chromosome 12

Table 2 Analysed genomic clones

a Analysed YAC clones

CEPH code	*Size (kb)*	*Probe*	*Landmark left*	*Genbank acc. no*	*Landmark right*
292C10	510	D12S1596	ND[b]		ND
368D4	350	D12S1596 D12S1034	ND		ND
68F12	260	D12S1034	ND		ND
374D10	310	D12S1034 z38802	ND		ND
169E1	310	D12S1034	ND		ND
170A5	230	D12S1034	PD81	AJ012002	ND
320H7	290	D12S1034 z38802	ND		ND
351H6	220	D12S1034	ND		ND
15F3	330	D12S1411	ND		ND
15B11	330	D12S1411	PD30	AJ011993	ND
336H10	340	D12S1411	ND		ND
433B12	480	D12S1411 z38802	ND		ND
513B6	230	D12S1411	ND		ND

b Analysed BAC clones

BAC code	*Size (kb)*	*Probe*	*Landmark left*	*Genbank acc. no*	*Landmark right*	*Genbank acc. no*
456N13	110	D12S1034	ND		ND	
5H3	180	D12S1034 PD64	PD71	AJ012001	PD70	AJ012002
570H2	210	D12S1034 PD64	PD64	AJ011998	ND	
526G26	125	D12S1596	PD52	AJ011994	PD53	AJ011995
156L15	80	D12S1596	ND		ND	
542J7	160	D12S1596 PD56	PD55	AJ011996	ND	
549C24	120	D12S1596 PD56	PD57	AJ012450	PD56	AJ011997
312G2	180	D12S1596	ND		ND	
567H24	150	D12S1596	ND		ND	
438C7	110	D12S1596	PD72	AJ012451	PD73	AJ012453
479I21	125	PD56	PD92	AJ012003	ND	
177H19	50	PD56	ND		ND	
554E4	130	PD56	ND		ND	
520I17	180	PD56	ND		ND	
241P15	115	PD64	PD97	AJ012004	ND	
264F23	115	PD64	ND		ND	

c Analysed cosmid clones

Code[a]	*FISH*	*Probe*	*Landmark left*	*Genbank acc. no*	*Landmark right*	*Genbank acc. no*
c113B3	above	D12S1596	ND		ND	
c135F7	below	D12S1034	ND		ND	
c247C2	above	PD56	ND		ND	
c71E7	below	PD64	PD109	AJ012259	PD108	AJ012258
c139H2	ND	PD64	PD122	AJ012262	ND	
c48H9	above	PD81	PD100	AJ012257	ND	
c93A1	ND	PD108	PD120		ND	
c202B11	below	PD108	ND		ND	
c219E2	below	PD109	PD119	AJ012261	PD118	AJ012260
c149H4	across	PD119	ND		ND	
c50G3	ND	PD100	PD168	requested	PD169	AJ012263

[a]abbreviated names; [b]not determined.

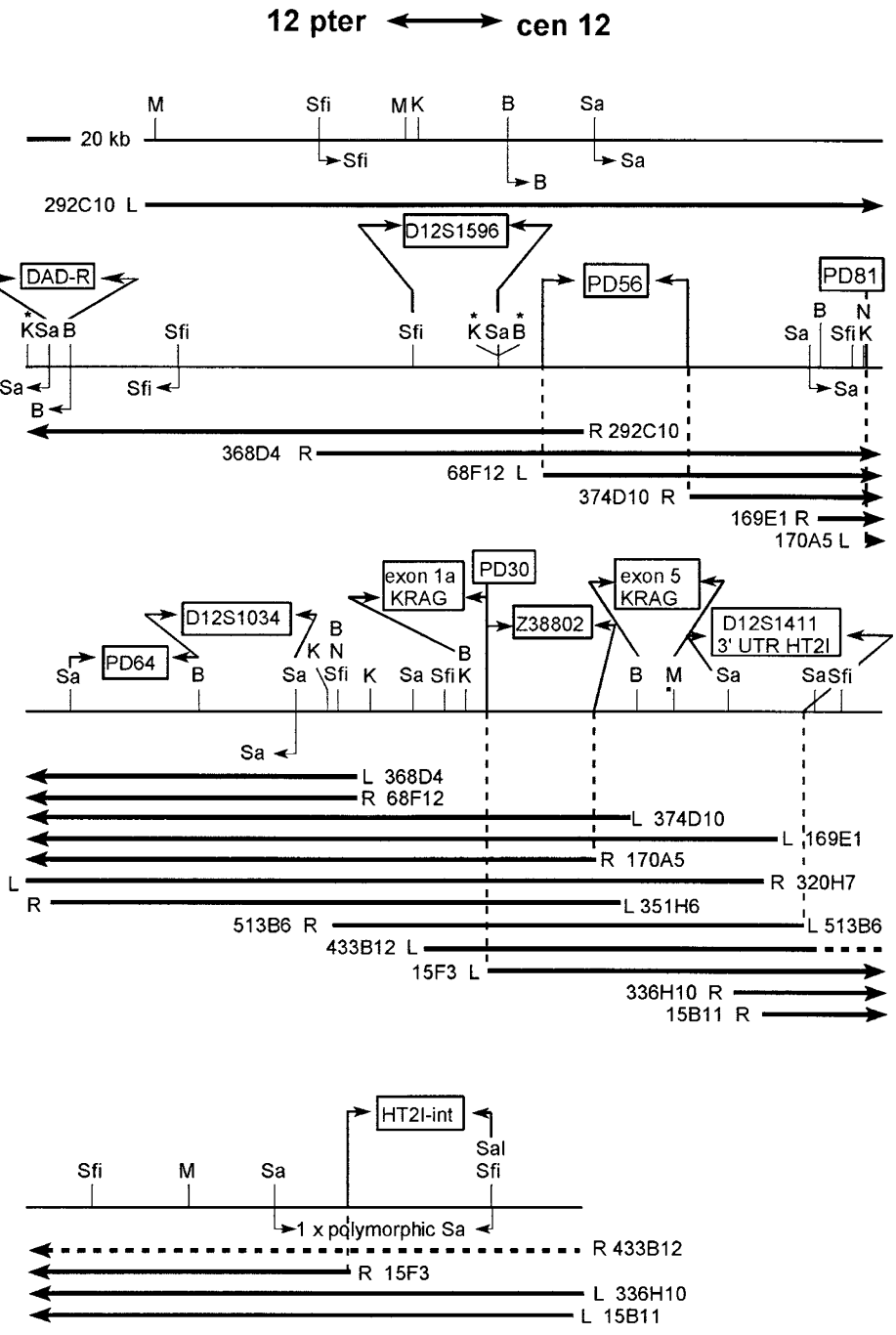

Figure 2 Long-range physical map containing the 12p11.2 breakpoint region. The contig comprises 13 overlapping CEPH YAC clones and extends over approximately 1.5 Mb. The black line at the top indicates the short arm of chromosome 12, with the telomere to the left and the centromere to the right. The relative positions of the restriction sites of rare-cutting enzymes are also indicated (B, *Bss*HII; K, *Ksp*I; M, *Mlu*I; N, *Not*I; Sa, *Sal*I; Sfi, *Sfi*I). Polymorphic restriction sites are marked with an asterisk. DNA intervals predicted to contain additional restriction sites for some of these enzymes are indicated by arrows. DNA markers, shown in green boxes are assigned to particular DNA intervals as indicated by arrows. Primer sets for these markers were either designed as described in Materials and methods (Table 1) or obtained from existing databases. DNA markers located at the ends of particular YACs are indicated by dashed lines. STSs corresponding to exons are shown in red boxes. *DAD-R*, exons 1 and 5 of *KRAG*, the 3' UTR of *HT2I* and another exon of *HT2I* (*HT2I*-int, position 3993–4092). The solid blue lines below the physical map represent the relative position of the overlapping YAC clones. The dashed blue line corresponds to DNA regions of YAC inserts diverging from the consensus long-range restriction map, which may represent co-ligated regions. The left (L) and right (R) arms of the YACs are indicated.

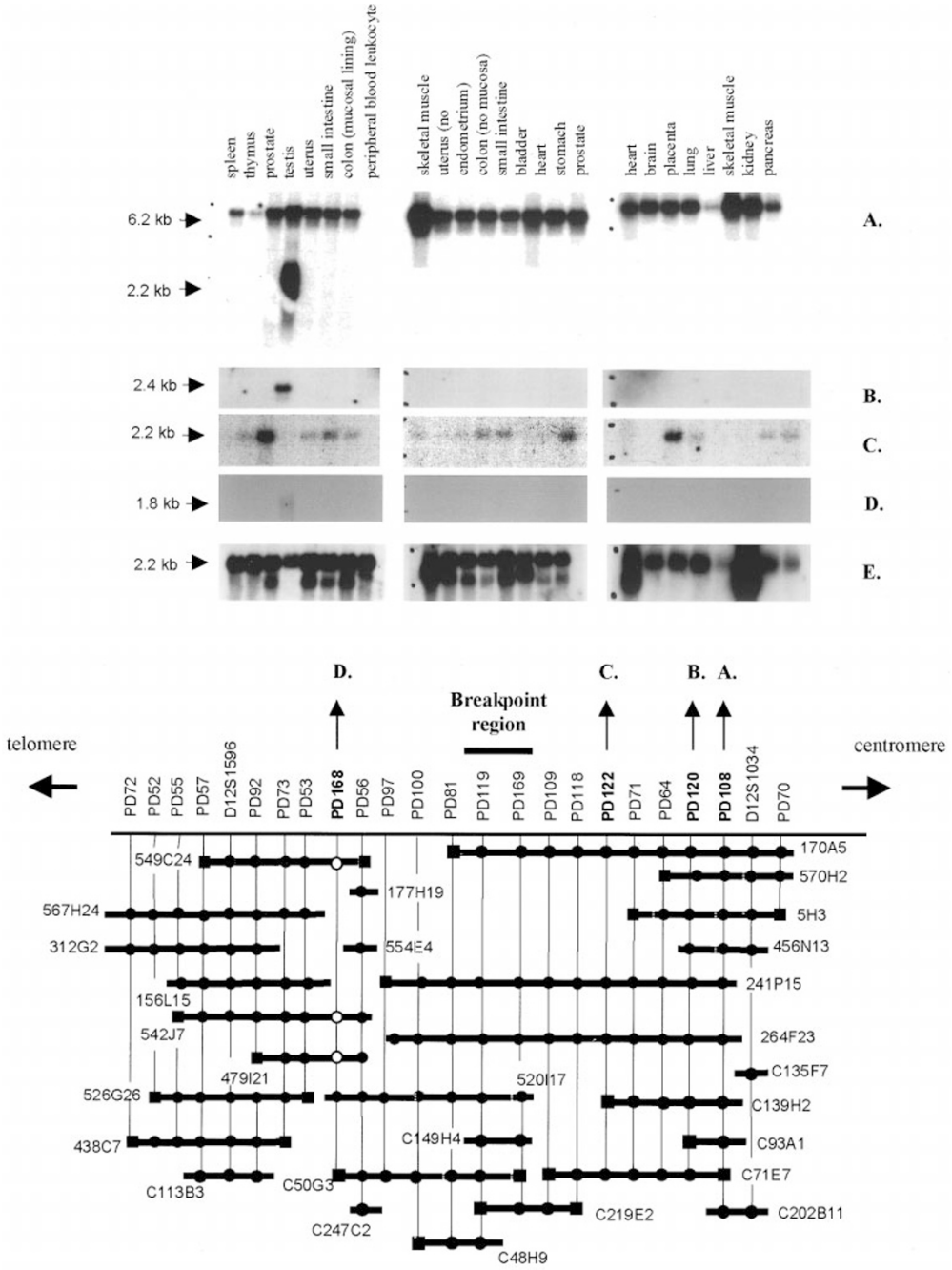

Figure 3 STC/STS content map (not to scale) of the breakpoint region consisting of 16 overlapping BAC and 11 overlapping cosmid clones. The left end of CEPH YAC 170A5 (corresponding to DNA marker PD81) is also included to allow comparison with Figure 2. The line at the top corresponds to the short arm of chromosome 12 with the telomere to the left and the centromere to the right. DNA markers derived from clone insert ends are represented by solid squares, whilst the positions of markers within individual clones are indicated by solid circles. An open circle indicates that the STC/STS could not be mapped to that particular clone. STCs in bold type (PD108, PD120, PD122 and PD168) correspond to anonymous ESTs. The expression pattern of these four ESTs on northern blot analysis of multiple adult human tissues (Clontech) is shown in the panels at the top. **A** EST R72964, corresponding to STC PD108; **B** EST AA190175, corresponding to STC PD120; **C** EST T48235, corresponding to STC PD122; **D** overlapping ESTs AA701549, AA308212, AA782961 and AA532445, all corresponding to STC PD168; **E** Human β actin cDNA, used as a control probe. The sizes of the transcripts detected are indicated on the left.

breakpoint, narrowing the relevant region to approximately 40 kb. As shown in Figure 4, signals on the normal chromosome 12, the der(12), and the der(22) were clearly visible, confirming that this cosmid spans the breakpoint. We therefore constructed several subclones of LL12NCO1–149H4 for use in Southern blot experiments. Probe 149H4–B5–SB, derived from a 11 kb *Bam*HI subclone (p149H4–B5) of L12NCO1–149H4, hybridised to a 2.6 kb *Bam*HI fragment in digested genomic DNA from patient II.2 which was not present in similarly digested genomic DNA from two control individuals (Figure 4). Probe 149H4–B7–SB also detected rearranged *Bam*HI, *Eco*RI, *Hind*III and *Pst*I fragments in digests of genomic DNA from patient II.2 (results not shown). On the basis of this data, we predict that the breakpoint lies close to the first *Hind*III site shown in Figure 4. Since the length of the rearranged *Bam*HI fragment was only 2.6 kb and since probe 149H4–B5–SB did not detect a rearranged *Hind*III fragment, we conclude that the breakpoint must lie immediately adjacent to the first *Hind*III site in p149H4–B5, as was confirmed by Southern blot analysis with probe 149H4–B7–SB.

Isolation of the CpG island

In order to isolate sequences mapping near the CpG island located in the immediate vicinity of the breakpoint, three flanking cosmids were analysed for the presence of a *Not*I restriction site. Cosmids LL12NCO1–50G3, LL12NCO1–71E7 and LL12NCO1–219E2 were digested with *Bam*HI, *Bam*HI/*Not*I, *Eco*RI, *Eco*RI/*Not*I, *Xba*I, and *Xba*I/*Not*I and analysed on a 0.8% agarose gel. Only in cosmid LL12NCO1–50G3, which overlaps with the breakpoint-spanning cosmid LL12NCO1–149H4 (Figure 3), was a *Not*I restriction site identified. Subsequent sequencing around this *Not*I site yielded genomic sequences showing extensive homology to ESTs AA308212, N30918 and N36628, which all reside within the PD168 cDNA contig, as well as to EST R72964, belonging to the PD108 contig.

Discussion

Mutations in the *HOXD13* gene cause both classical SPD[1–3] and an atypical form of SPD.[4] No mutations were identified in the *HOXD13* gene of affected individual II.2, supporting

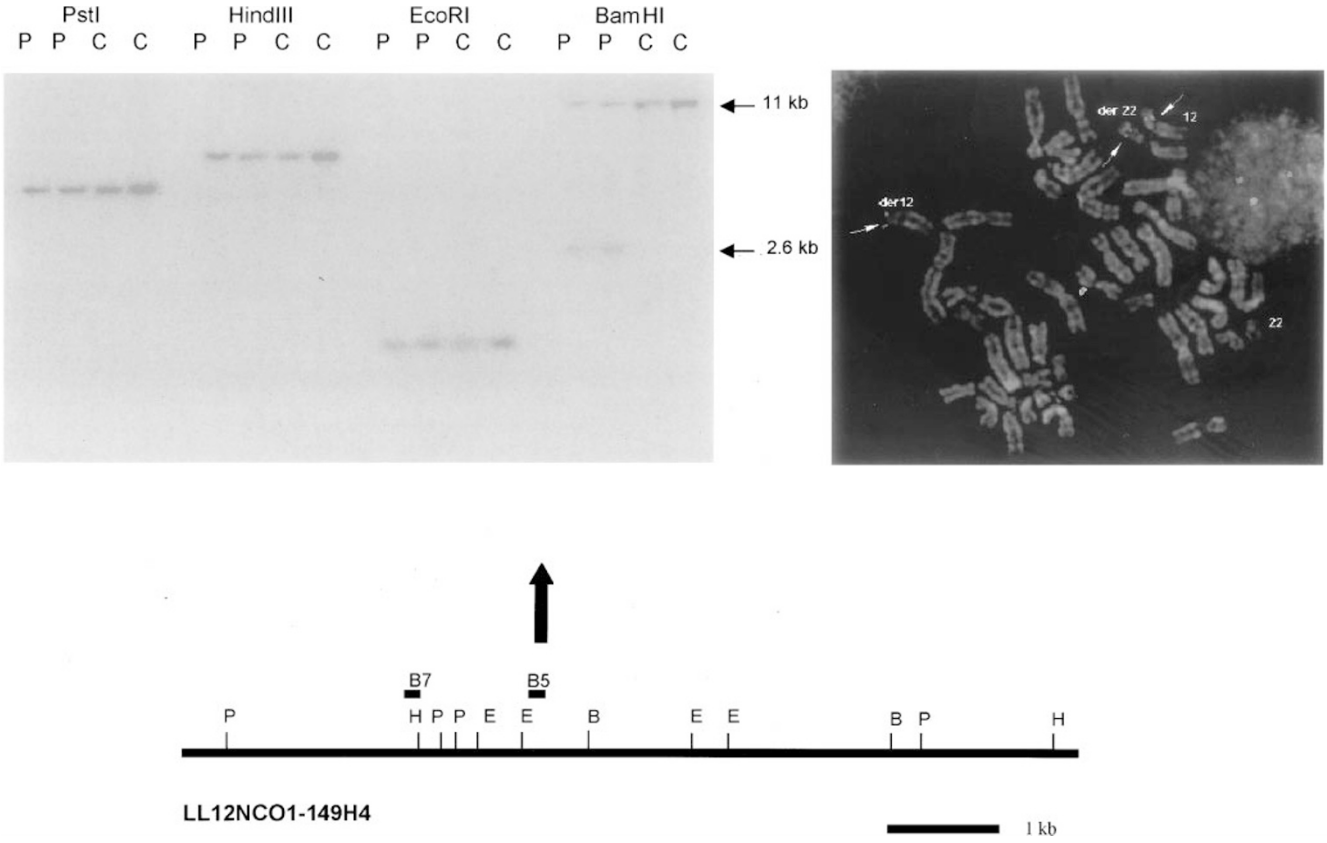

Figure 4 Cosmid LL12NCO1–149H4 maps across the breakpoint on chromosome 12p11.2 on FISH analysis, as shown in the upper right panel. A schematic restriction map of the relevant portion of cosmid LL12NCO1–149H4 is given in the lower panel. The positions of various restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I), as are the positions of the probes used in Southern blot experiments (B5, probe 149H4–B5–SB; B7, probe 149H4–B7–SB). As shown in the upper left panel, hybridisation of digested genomic DNA with probe 149H4–B5–SB revealed an additional rearranged 2.6 kb *Bam*HI fragment in two affected patients (P: individuals II.2 and III.1), whereas in two control individuals (C) only the 11 kb wild-type fragment was detected.

our hypothesis that the limb malformation in this family represents a unique clinical entity involving a novel gene (or genes). As the phenotype in our patients is nevertheless related to classical SPD, the product of the underlying gene could well act in the same pathway as HOXD13 during autopod development.

Since the phenotype in this family appeared to co-segregate with a balanced t(12;22)(p11.2;q13.3), a positional cloning project was undertaken to identify the gene (or genes) affected by the translocation. This resulted in the establishment of a 1.5 Mb long-range restriction map of the chromosome 12p11.2 breakpoint region, together with a contig containing 13 YACs, 16 BACs and 11 cosmids. By determining the sequence of the ends of these clones, we have also generated 23 new STCs. These 23 STCs have been placed on the physical map and the positions of the most relevant STCs have been indirectly confirmed by FISH studies. Further chromosome walking combined with FISH and Southern blotting experiments revealed that the chromosome 12p11 breakpoint lies within an 11 kb *Bam*HI fragment (p149H4–B5) derived from cosmid LL12NCO1–149H4, which includes the entire overlap region and maps across the breakpoint (Figure 4).

Of the seven ESTs we have thus far identified within the contig, EST AA190175 and the overlapping ESTs, AA701549, AA308212, AA782961 and AA532445 appear to be expressed in testis only, making them unlikely candidates for the phenotype observed in our patients. Both EST T48235 and EST R72964 are widely expressed. Whilst the 5' end of T48235 lies close to the breakpoint, its 3' end is more distant, and the corresponding gene is unlikely to be disrupted by the translocation, although a position effect cannot be ruled out. Interestingly, extended genomic sequencing of the *Not*I-containing CpG island led to the identification of a downstream (but not terminal!) exon contained within the composite cDNA contig anchoring to R72964. Since this *Not*I site and the flanking exon are present in cosmid LL12NCO1–50G3 [situated telomeric/distal (ie to the left in Figure 3) to the breakpoint] but absent in cosmid LL12NCO1–149H4 (containing the breakpoint), this R72964 exon is situated on 'the other side' of the breakpoint. This implies that the gene represented by EST R72964 is indeed disrupted by the translocation. Further experiments aiming at the elucidation of the functional consequences of this disruption are currently under way. Northern blot analysis of patient-derived RNA has so far not revealed any aberrant-sized transcripts. Northern blot analysis of a commercially available northern blot (Clontech) containing four foetal tissues (liver, kidney, lung, and brain) confirmed expression of R72964 (as a single, 6.2 kb transcript) during foetal development (results not shown).

Our contig also contains three previously reported genes, *DAD-R*, *KRAG*, and *HT2I*. The one-exon gene *DAD-R* was recently isolated by Kuittinen *et al* (personal communication, 1999). Its homolog *DAD1* was first isolated in 1993 by Nakashima and co-workers as a defender against apoptotic cell death,[19] and was subsequently shown to be a subunit of oligosaccharyltransferase.[20] Interestingly, some heterozygous Dad1-deficient mice have been reported to display soft-tissue syndactyly, making its homolog *DAD-R* a likely positional as well as functional candidate gene.[21] *KRAG* consists of five exons spanning about 20 kb of genomic DNA and encodes a protein which is probably involved in the regulation of cell proliferation.[22] *HT2I* encodes the type 2 IP_3R, which plays an important role in signal transduction.[23] All three of these genes, however, lie too far from the breakpoint to be directly disrupted by the translocation, although a positional effect cannot be entirely ruled out.

As human IP_3R is involved in signal transduction and regulation of intracellular Ca^{2+} levels, *HT2I* is nevertheless an excellent positional as well as functional candidate gene for the dominantly inherited hypertension syndrome mapped to chromosome 12p11 by Schuster *et al*.[8] Lack of the HT2I receptor would reduce endothelial release of intracellular Ca^{2+}, which in turn would reduce prostacyclin release, thus producing vasoconstriction and elevation of the blood pressure. In the family described by Schuster *et al*, the hypertension co-segregated completely with the form of brachydactyly, suggesting that the two traits were caused either by two different mutations in two closely situated genes or by one mutation in a single pleiotropic gene. A mutation in *HT2I* might also product brachydactyly, as the inositol-triphosphate/Ca^{2+} signalling pathway affects cell growth and proliferation, although it is unclear why the effects of such a mutation should be confined to the developing distal limb.

In summary, we have shown that the complex SPD in our patients is not part of the phenotypic spectrum caused by *HOXD13* mutations. To isolate the gene responsible, we have constructed a long range physical map of YACs, BACs and cosmids encompassing the 12p11.2 translocation breakpoint region in this family. This map should also aid in a structured approach to sequencing the region in question. Since EST R72964 seems to be directly involved in this translocation, our research is currently directed in further elucidating the functional consequences of this disruption.

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