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Cosmid Contigs from the Tuberous Sclerosis Candidate Region on Chromosome 9q34

Marjon van Slegtenhorst[a]
Bart Janssen[a]
Mark Nellist[a]
Sarvan Ramlakhan[a]
Caroline Hermans[a]
Arjenne Hesseling[a]
Ans van den Ouweland[a]
David Kwiatkowski[b]
Bert Eussen[a]
Julian Sampson[c]
Pieter de Jong[d]
Dicky Halley[a]

[a] MGC Department of Clinical Genetics, Erasmus University Rotterdam and Academic Hospital Rotterdam, The Netherlands;
[b] Experimental Medicine Division, Brigham and Women's Hospital, Boston, Mass., USA;
[c] The Institute of Medical Genetics, University Hospital of Wales, Cardiff, UK;
[d] Roswell Park Cancer Institute, Human Genetics Department, Buffalo, N.Y., USA

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Abstract
Tuberous sclerosis (TSC) is a heterogeneous multisystem disorder with loci on 9q34 (TSC1) and 16p13.3 (TSC2). The TSC2 gene has recently been isolated, while the TSC1 gene has been mapped to a 5-cM region between the markers D9S149 and D9S114. In our effort to localise and clone TSC1, we have obtained three adjacent cosmid contigs that cover the core of the candidate region. The three contigs comprise approximately 600 kb and include 80 cosmids, 2 P1 clones, 1 YAC, 5 anonymous markers and 4 sequence-tagged sites. The ABO blood group locus, the Surfeit gene cluster, the dopamine β-hydroxylase gene (DBH) and VAV2, a homologue of the *vav* oncogene, have all been mapped within the contigs. Exon trapping and mutation screening experiments, aimed at identifying the TSC1 gene, are currently in progress.

Introduction

Tuberous sclerosis (TSC) is an autosomal dominant multisystem disorder. The brain, skin, heart and kidneys are often affected and almost all other tissues and organs may be involved, except muscle syncytia [1]. The disease shows a high penetrance with variable expression and is known for its locus heterogeneity, with one locus mapping to chromosome 9q34 (TSC1) and another to chromosome 16p13.3 (TSC2) [2]. The number of families linked to each locus is approximately equal and there is no significant evidence for a

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M.A van Slegtenhorst
Department of Clinical Genetics
Dr Molewaterplein 50
NL-3015 GE Rotterdam (The Netherlands)

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third locus [3]. The TSC2 gene has been isolated [4] and both genes may act as growth or tumour suppressors, since loss of heterozygosity (LOH) has been demonstrated on 9q34 [5–7] or 16p13 [7] in various hamartomatous tissues from patients with TSC.

The chromosome 9 locus for tuberous sclerosis, TSC1, is tightly linked to the ABO blood group locus [8] and maps in a gene-rich region on chromosome 9q34. Since the initial linkage report by Fryer et al. [8], the TSC1 region has been refined to a region of 5 cM between D9S149 and D9S114 [3, 9–14]. However, there is no consensus on the exact position of TSC1 within this interval, since some groups have found recombinants in favour of a position proximal to ABO and the dopamine β-hydroxylase gene (DBH), while other groups have presented data supporting a location distal to these markers [15]. The conflicting observations have several possible causes, including misclassification of individuals with only minor clinical findings or non-linkage of one or more families.

Several genes have been mapped within the TSC1 candidate region, including ABO, DBH, the Surfeit gene cluster and VAV2 [16–18], while other disorders genetically linked to ABO include torsion dystonia [19] and nail patella syndrome [20, 21].

In this paper we present the results of a contig assembly and gene mapping effort, focused on part of the TSC1 candidate region around ABO and DBH. Our detailed map spans 600 kb, corresponding to more than 2 cM of the TSC1 critical region. Eight genes and several known and novel genetic markers have been precisely positioned on a genomic *Eco*RI map between D9S149 and D9S114.

Materials and Methods

Libraries

The ICI YAC library [22] was accessed through the UK Human Genome Mapping Resource Centre and sets of primary, secondary and tertiary pools for PCR screening were provided by R. Elaswarapu. Primary pools from the St. Louis YAC library [23] were supplied by J. den Dunnen from the Department of Human Genetics in Leiden. The P1 library was made from human foreskin fibroblast DNA [24]. The library was gridded into 125 96-well plates with approximately 12 different P1 clones per well and pools were made for PCR screening. The chromosome-9-specific cosmid library LL09NC01'P' was constructed at the Biomedical Sciences Division, LLNL, Livermore, Calif., USA under the auspices of the National Laboratory Gene Library Projects sponsored by the US Department of Energy. The library was replicated on gridded filters as described [25] at the YAC screening core of the Department of Human Genetics in Leiden. Two sets of membranes were used to make pools for PCR screening [26]. The nomenclature of the cosmids in the contigs is the same as the nomenclature of the library source from which they were obtained. Cosmid ABO17 was provided by J. Wolfe.

Cosmid Library Screening

Hybridisation probes were generated by inter-Alu PCR [27] using primers CL1 and CL2 [28] or by isolating end fragments from cosmids in low-melting agarose. Probes were randomly labelled, competed with total human DNA, hybridised to nylon filters and washed using standard procedures [29]. Cosmid library screening by PCR was performed by screening two-dimensional pools of clones as described by Green and Olson [30].

YACs, P1 and Cosmid Clones

Cosmid and P1 DNA was prepared, isolated and fingerprinted using standard techniques [29]. YACs, P1 and cosmid clones were mapped back to 9q34 by fluorescence in situ hybridisation (FISH) [31].

Sequence-Tagged Sites (STSs)

STSs were developed by YAC end rescue inverse PCR or direct sequencing of cosmids. YAC end rescue was performed as described by Silverman et al. [32] and the products were sequenced directly. The Sequence was derived from the cosmid clones by cycle sequencing (Pharmacia) with the appropriate vector primers.

Results and Discussion

Strategy

We aimed to isolate a significant part of the TSC1 critical region between the markers D9S149 and D9S114 on 9q34. Several additional markers are known to map between these two, but have not been convincingly associated with genuine recombination events. The initial strategy was to isolate the region in YACs, P1 and cosmid clones. However, attempts to obtain YACs were hampered by underrepresentation of the region in the available libraries. This prompted us to follow an alternative strategy which consisted of cosmid walking complemented by screening P1 libraries.

YAC Library Screening

Two YACs from the ICI library, 4DD1 (120 kb) and 25DG9 (320 kb), were identified with primers specific for the ABO locus. STS mapping using primer pairs from both ends of the YACs indicated that the left ends of both inserts overlapped; however, inter-Alu PCR in combination with hybridisation experiments suggested that the region of overlap was small. FISH analysis confirmed the localisation of both YACs to chromosome 9q34; however, 25DG9 gave an additional signal on chromosome 18 indicating chimerism. This clone was not investigated further. No additional YACs were identified in the ICI library using the end clone STSs from 4DD1 or 25DG9, or with additional markers from the TSC1 candidate region (D9S10, D9S66, DBH). An STS derived from the left arm of YAC 4DD1 was used to screen the St. Louis YAC library and identified two duplicate clones, 51A7 and 61A10 (200 kb). FISH analysis mapped 51A7 to 9qter; however, STS mapping experiments using primers derived from the right arm of this clone suggested that it contained a large deletion (data not shown) and it was not investigated further. It is interesting to note that the TSC2 locus on chromosome 16 was also found to be underrepresented in YAC libraries [unpubl. results].

Contig Assembly

Starting points for cosmid contig assembly were ABO, DBH and D9S10 (fig. 1). Cosmids were identified with both the left and right end clones of YAC 4DD1 and two contigs were constructed of 110 and 130 kb, respectively (fig. 2, contigs A and B). The orientation of the cosmid contigs was consistent with results from YAC inter-Alu PCR screening of to cosmid library and with the YAC STS mapping experiments. No cosmids could be identified distal to cosmid 255A6 (contig B). Only a single non-rearranged cosmid and a single P1 clone were detected at the ABO locus, and no clone could be detected linking the two contigs. However, from the size of the 4DD1 YAC and direct visual hybridisation (DIRVISH) experiments of streched DNA [33] [unpubl. results], we estimated that the gap is approximately 30 kb.

Cosmids were identified with the DBH cDNA and probe pMCT136 from the D9S10 locus. DBH and D9S10 map 1 and 2 cM distal to ABO, respectively, and were linked by chromosome walking, covering a physical distance of 150 kb (contig C). This indicates that the genetic versus physical distance ratio in this region is large. The contig was extended proximal of DBH by 125 kb, but could not be extended further towards ABO. We did isolate a P1 clone with an STS from the proximal end of 251C9, but could not bridge the gap. The distance between clone 251C9 (contig B) and 255A6 (contig C) could not be resolved by interphase FISH, indicating that the gap between contigs B and C is small. DIRVISH DNA mapping experiments are in progress to estimate the size of the gap.

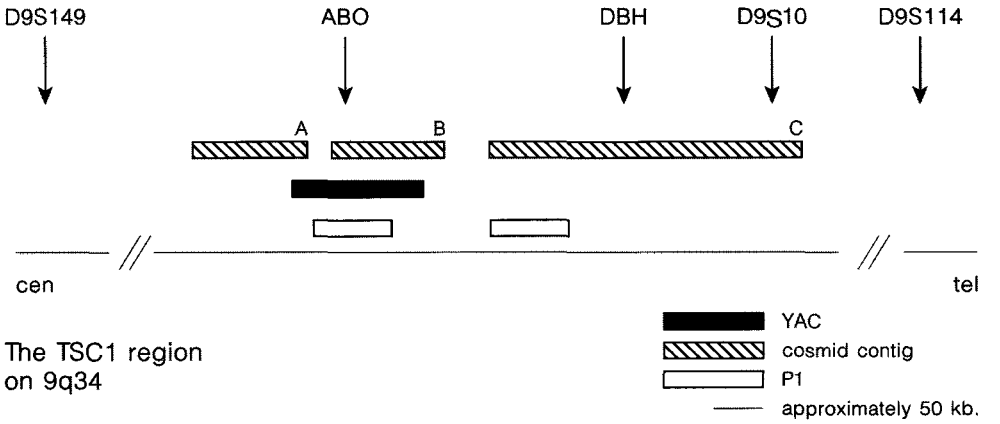


Fig. 1. Schematic representation of the TSC1 region. The starting points for YAC and cosmid walking are indicated, together with the YAC 4DD1, the cosmid contigs and P1 clones.

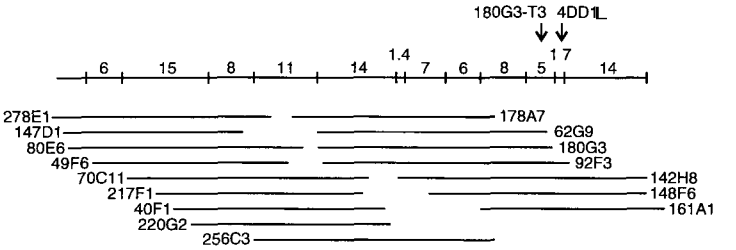
In regions of overlap, the contigs presented here were consistent with the cosmid contigs constructed by *Hin*fI fingerprinting as described by Nahmias et al. [34]. They need at least 50% overlap between cosmids before the clones are joined in a contig. Our data are more detailed and detect smaller overlaps. Additional cosmids have been isolated from the flanking locus D9S149. Chromosome-walking experiments are currently focused on closing the gap between D9S149 and the most proximal ABO contig (contig A).

Mapping of Markers and Genes in the Contigs

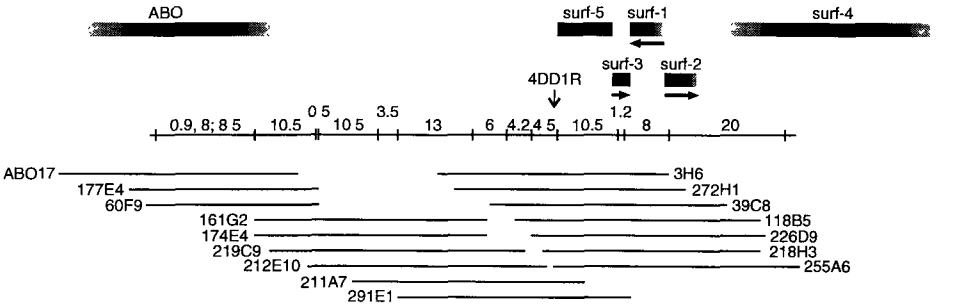
RFLPs and unique STSs are listed in tables 1 and 2. The STSs 180G3-T3 and 4DD1L map to adjacent *Eco*RI fragments in contig A. Two additional STSs, 4DD1R and 251C9-T3 were mapped to contigs B and C, respectively. Existing minisatellite repeats (D9S122 and D9S150) [13] were precisely positioned within this contig (fig. 2, contig C) and a *Hin*dIII polymorphism (D9S968) was detected immediately proximal of DBH.

The position and orientation, where known, of genes identified within the contigs are indicated in figure 2. The role and expression pattern of the ABO blood group transferase indicate that it is not a good candidate for TSC1. The Surfeit gene cluster had been previously mapped by in situ hybridisation telomeric to the c-*abl* and *can* genes on 9q34 [35]. A oligonucleotide derived from the Surf-3 cDNA sequence detected a 1.2-kb *Eco*RI fragment in several cosmids, slightly distal to ABO in contig B. Cosmid 255A6 was digested with *Xba*I to orientate the cluster in the map. In the mouse this cluster consists of 6 housekeeping genes, which are unrelated by sequence homology [35]. To date, the Surfeit genes form the tightest gene cluster known in mammals. Since these genes are in the critical region of TSC1 and not much is known about their function, mutation analysis in TSC patients must be considered.

Contig A



Contig B



Contig C

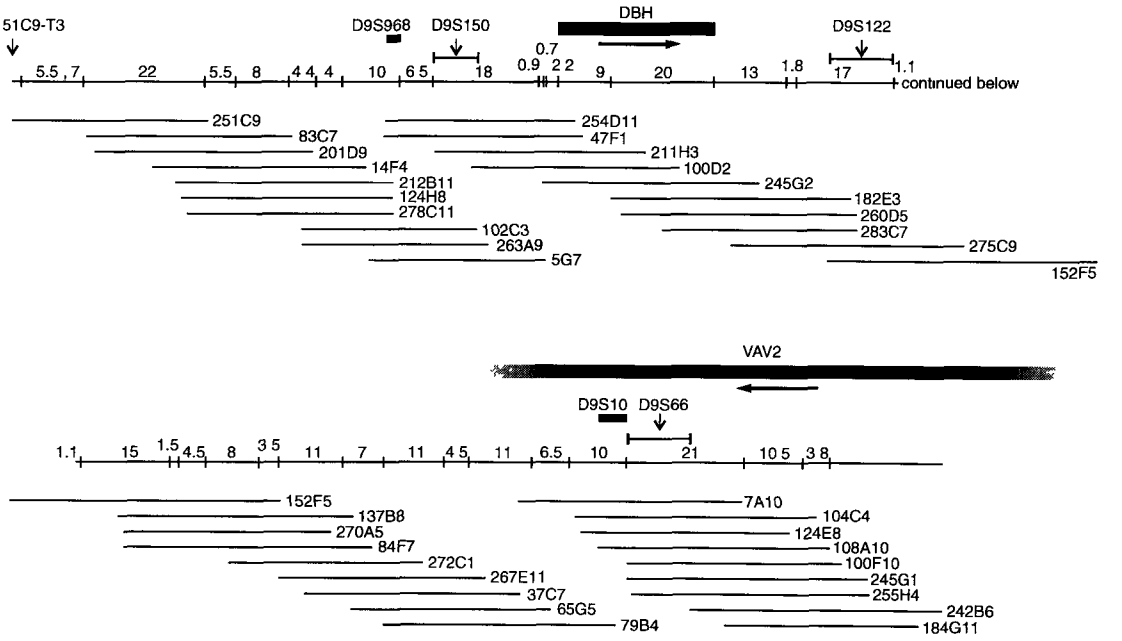


Table 1. List of RFLPs in the region

Locus	Enzyme	Probe	Fragment sizes, kb	Heterozygosity, %
D9S10	*Pst*I*	MCT136	2.5 and 2.3	50
	*Hin*dIII	MCT136	2.2 and 2.0	50 (200 chromosomes)
	(RFLPs show linkage disequilibrium)			
D9S968	*Hin*dIII	RD560	4.5 and 2.6	14 (115 chromosomes)
DBH	(several RFLPs* + $(CA)_n$, all listed in GDB)			
VAV2	*Pst*I	5′ VAV2 (bases 1–865)	5, 4.2 and 2.2	48 (>100 chromosomes)

All RFLPs marked with an asterisk are already listed in GDB. The heterozygosity percentages of the new RFLPs (without asterisk) have been determined in at least 100 chromosomes from Causasians. The map position of each locus is indicated in figures 1 and 2. The VAV2 RFLP maps within the VAV2 gene, distal to the end of the cosmid contig.

Table 2. List of STSs in the region

STS	Primer sequences	Product length	Map position
180G3-T3	5′ GGTGT GGTTC TCCCA AGGG 3′ GAGAG AGGCT TCCTG CTTGC	128 bp	distal part of contig A
4DD1L	5′ CCAAG GGAAG CTGGA GAAGT 3′ CCAGA CCCAG CCTAC ATTTC	97 bp	left arm of YAC 4DD1
4DD1R	5′ CATGC TGTTG GCACT GTTGTA 3′ TTTCT CTTTG GCTTC CCTCTT	135 bp	right arm of YAC 4DD1
251C9-T3	5′ GGAAA GAGGA GCGAG GAAG 3′ CACAA TCTCA CAGTG AATGCC	152 bp	approx. end of contig C

A number of polymorphic STSs at ABO, DBH, VAV2, D9S149, D9S150, D9S122, D9S66 and D9S114 have been described previously and are therefore not included in the list.

Fig. 2. Detailed *Eco*RI restriction map of the three contigs described in this paper. Cosmids are shown below the the *Eco*RI map. Thin bars represent RFLP markers and vertical arrows indicate STSs and microsatellites. Genes are shown above the restriction map as thick bars. The size of the bars indicates the maximal genomic extent. The direction of transcription is indicated by arrowheads. For DBH, *surf-1, surf-2, surf-3* and VAV2, the gene structure was studied by Nahmias et al. [34], Yon et al. [35] and Kwiatkowski et al. [16]. The position and orientation of the genes in the cosmid contigs were deduced from our experimients and previously published restriction maps [34, 35].

Our *Eco*RI mapping data from the DBH locus is consistent with that of Kobayashi et al. [36]. The direction of transcription is towards the telomere. The role of DBH in the conversion of dopamine to noradrenaline and the neurological manifestations of TSC led to the proposal that DBH could be a candidate for the TSC1 gene [37]. However, more recent results suggest that TSC1 maps either distal or proximal to DBH and consequently DBH is not such an attractive candidate.

Exon trapping [38] efforts using our cosmids from the D9S10 locus identified a gene homologous to the *vav* oncogene [16]. This gene, designated VAV2, was considered a good candidate for the TSC1 gene. However, intensive screening failed to identify any mutations, and VAV2 was consequently excluded as a candidate gene for TSC1 [16, 17].

Eight different genes could be placed on the map. The region is gene dense and although some genes map extremely close to each other, we cannot exclude the presence of other, as yet unidentified, expressed sequences in the same region. Experiments to identify and characterise additional genes from the TSC1 candidate region are in progress.

Further efforts are being directed towards extending the contigs and screening TSC patients for mutations by pulsed-field gel electrophoresis using novel probes derived from our cloned material. The identification of large deletions at the TSC2 locus made a significant contribution to the rapid isolation of the TSC2 gene [4].

In conclusion, we have identified 80 cosmids, 2 P1 clones and a single non-rearranged YAC from the TSC1 candidate region on 9q34. We have constructed a detailed restriction map of three adjacent cosmid contigs and oriented the maps with respect to known and previously unidentified genes and DNA markers. We have shown that DBH and D9S10, previously estimated to be 1 cM apart, are separated by less than 300 kb, and estimate that the physical distance between ABO and DBH is less than 300 kb.

In conjunction with the accompanying article [34], we have shown that cosmid walking, using a large chromosome-specific cosmid library can provide almost complete coverage of a large genomic region. This minimises the need to search for non-chimeric non-rearranged YAC clones, which have been difficult to obtain from the TSC1 region. Moreover, our contigs and the associated maps provide a good tool for generating novel markers and cloning additional genes from this region. It would be of great help to get more excluding data on the recombinants within the region, so that the search for TSC1 can be restricted to a smaller area. LOH studies in tumours of patients and the development of new polymorphic CA repeats in the area, especially between ABO and D9S149, could help reduce the critical region. Ultimately, it is hoped that this work will lead to the identification of the TSC1 gene.

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