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Gene structure and chromosomal localization of mouse *Opa1* : its exclusion from the Bst locus

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Abstract

Background: Autosomal dominant optic atrophy type 1 (DOA) is the most common form of hereditary optic atrophy in human. We have previously identified the *OPA1* gene and shown that it was mutated in patients with DOA. OPA1 is a novel member of the dynamin GTPase family that play a role in the distribution of the mitochondrial network. The Bst (belly spot and tail) mutant mice show atrophy of the optic nerves and previous mapping data raise the possibility that *Bst* and *OPA1* are orthologs. In order to analyse the Bst mouse as a model for DOA, we therefore characterized mouse *Opa1* and evaluated it as a candidate for the Bst mutant mouse.

Results: Comparison of mouse and human OPA1 sequences revealed 88% and 97% identity at the nucleotide and amino acid levels, respectively. Presence of alternatively spliced mRNAs as seen in human was conserved in the mouse. Screening of the whole mRNA coding sequence and of the 31 exons of *Opa1* did not reveal any mutation in Bst. Using a radiation hybrid panel (T31), we mapped *Opa1* to chromosome 16 between genetic markers *D16Mit3* and *D16Mit124*, which is 10 cM centromeric to the *Bst* locus.

Conclusion: On the basis of these results we conclude that *Opa1* and *Bst* are distinct genes and that the Bst mouse is not the mouse model for DOA.

Background

Autosomal dominant optic atrophy (DOA), Kjer type [1], is the most common form of hereditary optic neuropathies with an estimated prevalence of 1:50 000 in most populations [2] and prevalence as high as 1:10 000 in Denmark [3]. The disease appears with an insidious onset of variable visual loss, optic nerve pallor, caecocentral visual field scotoma, and color vision deficit. Histopathological [4,5] and electrophysiological [6,7] studies suggest that the underlying defect is a retinal ganglion cell degeneration. Most families of DOA have been shown to map to 3q28-29 (*OPA1*, MIM 165500) [8–14]. The gene named *OPA1* was subsequently identified [15,16] with more than 70 mutations described today [17–21]. One single family was found to determine a second locus (*OPA4*, MIM 605293) at 18q12.2-q12.3 [22]. *OPA1* codes a mitochondrial dynamin-related GTPase that may play a role in the maintenance of mitochondrial morphology and DNA. Tissue specific expression of *OPA1* alternatively spliced exons may underlie some specific neuronal requirements in OPA1 [18].

In 1977, Southard et al. [23] identified a dominant mutation, belly spot and tail (Bst), which arose spontaneously

in the C57BLKS mouse strain with *in utero* death of homozygotes. Heterozygous mice have a kinky tail, white feet and white spots at the ventral midline. In addition, approximately 50% of the *Bst*/+ mice, show a reduction or a complete absence of the pupillary light reflex in one or both eyes [24]. This neurological phenotype is associated with unilateral or bilateral atrophy of the optic nerves. The severity of the atrophy of the optic nerves is highly variable ranging from a slight reduction in the number of ganglion cell axons in only one optic nerve to a complete elimination of both optic nerves. This is reminiscent of the human situation in which DOA patients show variable expression ranging from asymptomatic carriers to patients with legal blindness. The aspect of the retinal surface and the appearance of the inner and outer nuclear layers in *Bst*/+ mice are qualitatively normal [24]. However, the ganglion cell numbers appear to be reduced because of a failure of the ganglion cell axons to reach the optic nerve head in early development [25].

The *Bst* gene is located on mouse chromosome 16 in a region that is partially conserved on human chromosome 3q28-qter [26–28]. Based on its reported mapping to the syntenic region of *OPA1* and on some phenotypic similarities of the Bst mouse and human DOA, the *Bst* gene could be the murine ortholog of *OPA1*, and therefore the Bst mouse could be proposed as a model for DOA. To address this question we determined the *Opa1* gene sequence including its promoter region, as well as its chromosomal localization and that of the markers of the *Bst* interval. Absence of mutations in the mouse gene and distinct localization of *Opa1* and *Bst* definitely excludes *Opa1* as the gene responsible for the Bst phenotype.

Results and Discussion

cDNA sequence and gene structure of mouse Opa1

In a previous study we isolated OPA1 [15], a human dynamin-related protein mutated in DOA. To isolate and characterise the mouse ortholog of the human *OPA1* gene, we amplified an adult mouse brain cDNA using primers designed from the human cDNA sequence. 5' RACE-PCR experiments extended the sequence 238 bp upstream from the ATG triplet. The comparison of the RACE-PCR product with the genomic sequence upstream from the start codon revealed that the newly identified 5'cDNA sequence is contiguous with the previously defined exon 1 in the *Opa1* genomic DNA, thereby only extending exon 1 without forming a new exon. The open reading frame was found to be 2883 bp in size encoding a mouse Opa1 protein of 960 amino acids. As in human OPA1, mouse Opa1 contains a putative mitochondrial targeting signal, GTPase and dynamin central region domains and two predicted coiled-coil structures (upstream the GTPase domain and in the C-terminal domain) with 97 % overall identity with the human sequence. Human and mouse sequences are somewhat divergent in the first 200 N-terminal amino acids (83 % identity) that mostly contains the mitochondrial leading sequence.

To examine the genomic structure of mouse *Opa1* we performed a Blast search against the mouse genomic database with the full-length mouse *Opa1* cDNA sequence. A mouse "working draft" BAC clone was identified that contained the entire mouse *Opa1* genomic sequence. By rearranging the sequences in this BAC, the intron/exon boundaries and most intron sequences of mouse *Opa1* were determined. We established that *Opa1* consists of 31 exons and 30 introns extending over 68 kb (Fig. 1). We found that exon/intron boundaries of human and mouse OPA1 were preserved and followed the GT/AG rule. The sizes of all exons are remarkably conserved between the murine and human gene but 16 mouse *Opa1* introns are smaller than human ones. As for the human gene, mouse *Opa1* exhibits 8 transcripts that result from the alternative splicing of exons 4, 4b and 5b with a predominance of the transcript without exons 4b and 5b in neuronal tissues (retina and brain) (Fig. 2).

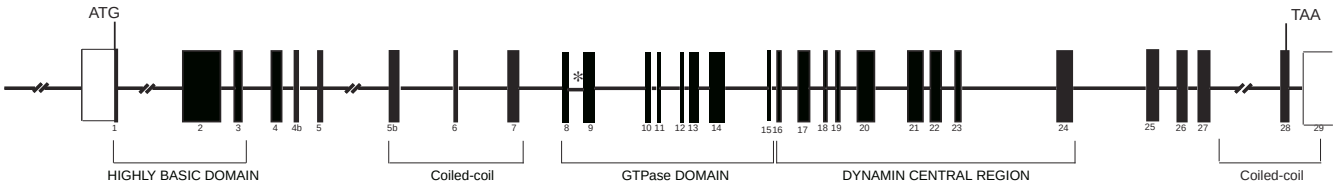

Figure 1

Genomic structure of mouse *Opa1*. The bars and numbers represent coding exons and the lines represent introns. Exons encoding the different domain of the protein are in brackets. The 5' UTR and the 3' UTR (white bar) are indicated. The conserved region in intron 8 is indicated with an asterisk.

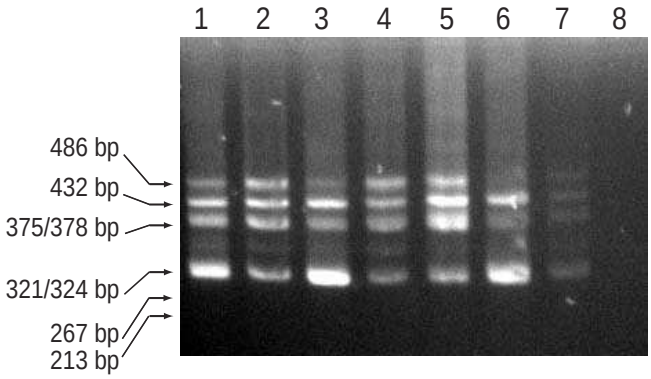

Figure 2
RT-PCR analysis demonstrating multiple *Opa1* transcripts in mouse tissues. Use of ME3S and MK5bAS primers resulted in the amplification of 6 distinct fragments, the size of which is indicated on the left. RT-PCR was performed on mouse lung (lane 1), kidney (lane 2), brain (lane 3), liver (lane 4), testis (lane 5), retina (lane 6) spleen (lane 7) and H_2O (lane 8).

In search for conserved putative regulatory sequences, we performed a comparative analysis of the *Opa1* introns. We found small conserved regions (ranging from 50 to 155 bp in length) in almost all introns but the largest homologous fragment was found in intron 8. This 155 bp-long fragment is 79% identical in both species and covers 1/3 of the intron length. Polymorphisms IVS8 + 4 C/T and IVS8 + 32 T/C putatively involved in normal tension glaucoma [29] are located upstream of this fragment. We sequenced this fragment in DOA patients (n = 3) who had no mutations in the exons and splice junctions of OPA1 and found no mutations.

Promoter regions of mouse and human Opa1

Comparison of the 5'genomic sequences of mouse and human genes revealed a 26 bp-long stretch that exhibits 80 % homology (as found for the rest of exon 1) and lies next to the 5' limit of our RACE-PCR sequence (Fig. 3). It is possible that this stretch corresponds to the 5' end of the mRNA that could have been missed by the RACE-PCR experiment. Mouse and human upstream regions did not contain any binding sites for RNA polymerase II core promoter elements such as CAAT and TATA boxes. This was not unexpected since TATA boxes are found in only 32 % of the promoters [30]. However, we did found several conserved GC boxes, 3 in mouse and 2 in human sequences, that are binding sites for the SP1 transcription activator (Fig. 3). The presence of these GC boxes is typical of housekeeping gene promoters, corroborating previous findings showing ubiquitous expression of OPA1 [15,16]. A sequence matching Nuclear Factor 1 (NF1) binding site was also identified and conserved between human and mouse. As for GC boxes, genes regulated by NF1 proteins are widely expressed.

Mutation screening of Opa1 in Bst mouse

Because *OPA1* mutations presumably cause the degeneration of retinal ganglion cells in DOA, we evaluated it as a candidate gene in the mutant mouse Bst which is phenotypically comparable to the DOA in man [24,25]. Indeed, both *OPA1* and *Bst* mutations are inherited as dominant phenotypes with variable expressivity, and both target the retinal ganglion cells. We therefore sequenced the entire cDNA, the genomic DNA (exons and flanking sequences) and the putative promoter region of *Opa1* in Bst/+ heterozygous mice and in the +/+ background strain. No mutation was detected in the coding region or intron/exon boundaries. No deletion was detected by amplification of the full length cDNA. This virtually excluded point mutations in amino acid coding sequences and in splice site regions but large deletions or mutations present in unexplored regions of the gene were still possible.

Mapping of mouse Opa1 to chromosome 16 in a region distinct from the Bst locus

Using radiation hybrid mapping, we found that *Opa1* is located on chromosome 16 between genetic markers *D16Mit3* and *D16Mit124*, 10.09 cR distal to the marker *D16Mit3* (lod>3) which correspond to 20.9 cM from the centromere (Fig. 4). Previous studies have mapped the *Bst* locus to mouse chromosome 16, 1.9+/- 1.1 cM from *D16Mit168* [25,27]. In order to compare the location of *D16Mit168* on genetic map with the physical map developed with the T31 radiation hybrid panel, we mapped the marker *D16Mit168* with the T31 RH panel and found it at 2.12 cR from *D16Mit113*, which is approximately at 33 cM from the centromere. This position fits with the location of this marker on the consensus map for mouse Chromosome 16 at 30 cM from the centromere [28]. According to the placement of *D16Mit168*, Bst lies approximately 31.5 cM from the centromere, in a region syntenic with human 3q13. In the integrated genetic map, Opa1 locus is approximately 10 cM centromeric to the Bst locus, indicating that Opa1 and Bst are not allelic. This excludes O*pa1* as the candidate gene for the Bst mutant mouse and shows that the Bst mouse is not the corresponding animal model for DOA.

The exclusion of *Opa1* from the Bst locus is in accordance with some phenotypic discrepancies between the DOA patients and Bst mice. Indeed, there are a variety of organs consistently affected by the Bst mutation. The Bst mouse is smaller than its normal counterpart, shows depigmentation of hair and exhibits numerous skeletal

Figure 3
Comparison of the genomic sequence upstream of the human and mouse *OPA1* gene. The transcribed sequence is shown in capital letters. Asterisk indicates the 5' limit found by RACE-PCR and highlighted in grey is the conserved sequence that may represent the actual 5' end of the mRNA. The initiation codon for translation (ATG) is in bold. Putative binding sites conserved between human and mouse as identified by computer searches are shown with horizontal bars on top of the sequence line.

abnormalities attributed to developmental delay: polydactyly of fingers and toes and a short kinked tail [26]. In addition, some Bst mice have colobomas of the optic nerve and retina with subretinal neovascularisation [31], which is never seen in DOA patients. These defects suggest that the normal *Bst* gene is essential to development and may regulate cellular differentiation during organogenesis. Nevertheless the mitochondrial origin of the Leber Hereditary optic atrophy, DOA type OPA1 and Type III 3-Methylglutaconic Aciduria (Costeff optic atrophy syndrome) suggests that the Bst gene could encode a mitochondrial protein [32]. Moreover, a mutation in a mitochondrial transmembrane protein has been described in a mouse with flexed tail, white belly spots and skeletal abnormalities resembling the Bst phenotype [33]. We found a human gene, *TOMM70A* (OMIM#606081) that encode a translocase of the outer mitochondrial membrane and that map to 3q12.3 in the syntenic region of the Bst locus. We sequenced the full genomic DNA of the Tom70 mouse homolog of Bst mice and found no changes, thereby excluding also this gene as the Bst gene.

Conclusions

In this work, we report the genomic structure and the position of the mouse *Opa1* gene by radiation hybrid mapping. The exclusion of the *Opa1* candidate gene for the Bst phenotype suggests that the Bst mouse was not the mouse model of DOA. Considering these data, the development

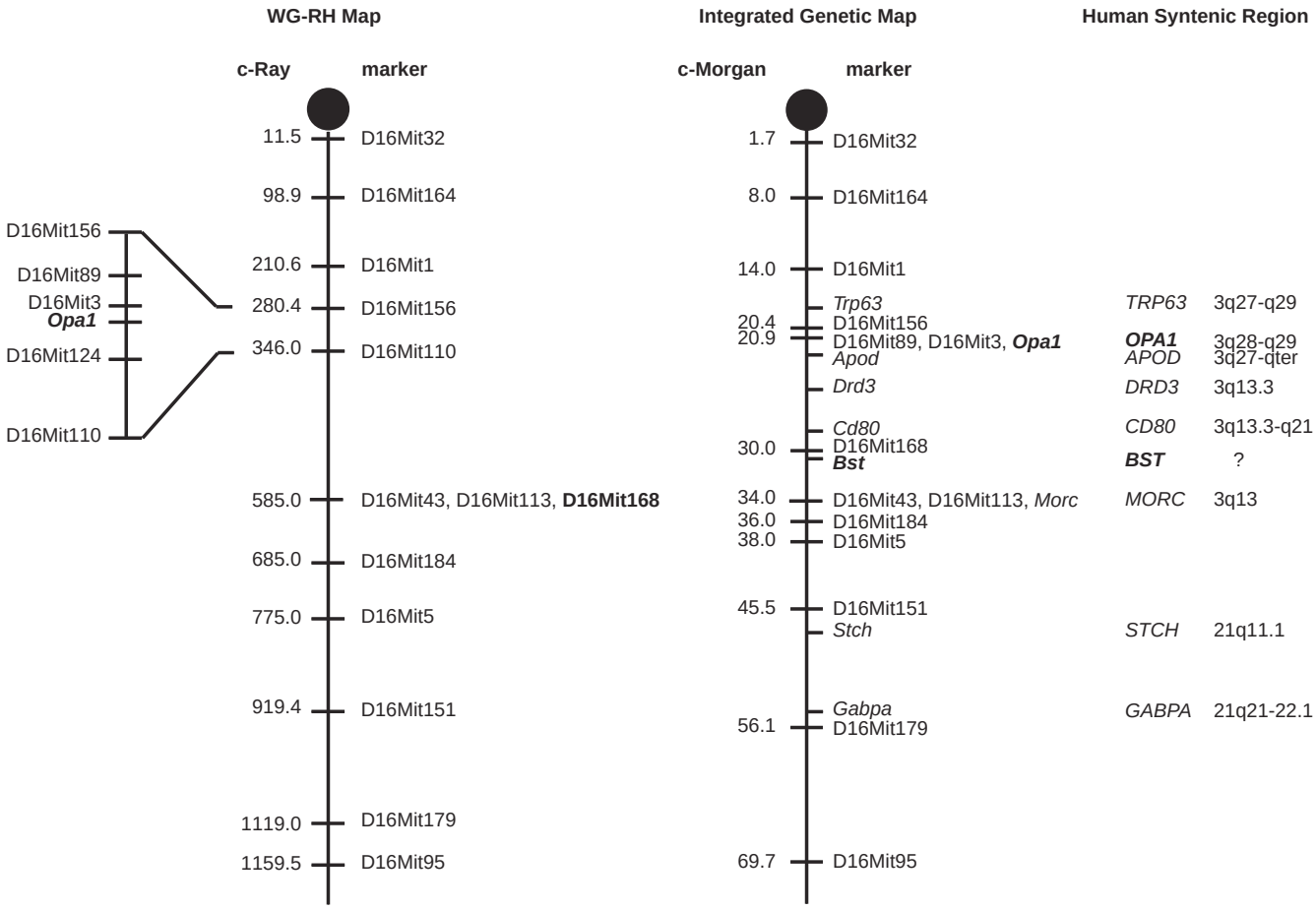

Figure 4

Mouse *Opa1* mapped to Chromosome 16 outside the *Bst* interval. On the WG-RH mapping panel T31 (left diagram), mouse *Opa1* locus maps to Chromosome 16 between genetic markers *D16Mit3* and *D16Mit124*. On the integrated genetic map (middle diagram) prepared from the mouse gene database (Mouse Chromosome 16 Linkage Map: http://informatics.jax.org/searches/linkmap_form.shtml), *Opa1* mapped 10 cM centromeric to *Bst* locus. Analysis of human syntenic regions of *Opa1* and of the *Bst* locus (right diagram) indicates that the Bst locus (the synteny of which being unknown, question mark) is flanked by human 3q13 syntenic regions.

of a mouse model will permit further understanding of Opa1 function and the study of the pathophysiological mechanisms of DOA, as well as the future evaluation of treatment strategies for the disease.

Methods

RNA and DNA purification

The mouse strains C57BLKS (control) and C57BLKS *Bst*/+ (heterozygous *belly spot and tail*) were purchased from The Jackson Laboratory (Bar Harbor, ME). Adult *Bst*/+ mice exhibit a kinked tail and a white belly spot. Total RNA of various tissues was extracted with the Rneasy kit (Qiagen, Germany) following the manufacturer's instructions. First strand cDNA synthesis was performed with Super Script II reverse transcriptase (Invitrogen, The Netherlands) using 1 μg of RNA and 50 pmol of random primers (Promega, USA). DNA was isolated from tail snips by standard methods.

DNA amplification

Genomic DNA and overlapping RT-PCR products were amplified under standardised conditions as follows: denaturation at 94°C for 3 min followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C, 1 min at 72°C and a final elongation at 72°C for 10 min. Each PCR fragment was purified using the Qiaquick purification kit (Qiagen, Germany) and sequenced on an ABI 310 capillary sequencer (Applied Biosystems, USA). The alternatively spliced tran-

scripts of mouse *Opa1* were amplified in several tissues (lung, kidney, brain, liver, testis, retina and spleen) using primers located in exon 3 (ME3S: 5'-GTGACTATAAGTGGATTGTGCCT-3') and in exon 7 (MK5BAS: 5'-CGCTCCAAGATCCTCTGATAC-3'). The obtained RT-PCR products were cloned into the pGEM-T vector (Promega, USA) and sequenced using primers M13F and M13R. The 5'-untranslated region of *Opa1* was obtained by RACE-PCR amplification of cDNA sequences generated from brain mRNA (GeneRacer Kit, Invitrogen, The Netherlands) according to the manufacturer's instructions. The gene-specific primer B9AS (5'-AAGAGTCTTGCAGCTAACCTTGC-3') located in exon 2 was used for the initial reverse transcription. The second PCR was performed with the provided GeneRacer 5' nested primer and a nested gene-specific primer located in the 5' region of exon 2 (B8AS: 5'-TTGAAGCTTGAGGGCAGGATGAT-3'). PCR products were then subcloned in the pPCR4Blunt-TOPO vector (Invitrogen, The Netherlands) and sequenced using the nested gene-specific primer.

Radiation hybrid mapping

The 100 radiation hybrid (RH) DNAs of the T31 mouse/hamster RH panel [34] (Research Genetics, USA) were used as templates for PCR amplification of *Opa1* and *D16Mit168*, this marker being close to the *Bst* locus [26,27]. For *Opa1*, we designed forward (MK5S: 5'-TTGCCAGTTTAGCTCCCGACTT-3') and reverse (MK6AS: 5'-CAGATCCATGATCTGTTGCTCG-3') primers to amplify a 1438-bp PCR fragment from mouse genomic DNA. No fragment was obtained when hamster genomic DNA was subjected to amplification. For the *D16Mit168* marker, we used forward (D16Mit168F: 5'-TGTGGTAGATGGATAAAAGAATGTG-3') and reverse (D16Mit168F: 5'-CATGGAGAAGTTCCTGTAAGCA-3') primers to amplify a 152-bp amplicon. No signal was detected from hamster DNA amplification. The results were submitted to the linkage database at the Whitehead Institute/MIT Center for Genome Research http://www.genome.wi.mit.edu.

Sequence analysis

The human and mouse putative promoter regions were compared by BLAST2 sequences [35]. Potential transcription regulatory sites were detected using the MatInspector program http://transfac.gbf.de/cgi-bin/matSearch/matsearch.pl as implemented by the Baylor College of Medecine Search Launcher. This software accesses the TRANSFAC MATRIX database which contains the consensus binding sites for a variety of transcription factors.

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