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ARTICLE

WBSCR14, a putative transcription factor gene deleted in Williams-Beuren syndrome: complete characterisation of the human gene and the mouse ortholog

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Williams-Beuren syndrome (WBS) is a neurodevelopmental disorder affecting several systems caused by a heterozygous deletion in the chromosomal region 7q11.23. A common interval that includes up to 17 genes reported so far is deleted in the great majority of patients. Elastin haploinsufficiency is responsible for the cardiovascular features, but the specific contribution of other deleted genes to the WBS phenotype remains unknown. We have fully characterised a gene commonly deleted in WBS, *WBSCR14*, previously reported in a truncated form as *WS-bHLH*. The *WBSCR14* cDNA encodes an 852 amino acid protein with a basic helix–loop–helix–leucine–zipper motif (bHLHZip) and a bipartite nuclear localisation signal (BNLS), suggesting a function as a transcription factor. *WBSCR14* is expressed as a 4.2 kb transcript predominantly in adult liver and at late stages of foetal development. The *WBSCR14* locus encompasses 33 kb of genomic DNA with 17 exons. Two intragenic polymorphic dinucleotide repeats have been identified and used to verify hemizygosity in WBS patients. We have also cloned the mouse ortholog and mapped its locus to mouse chromosome 5, in a region of conserved synteny with human 7q11.23. Given that other bHLHZip proteins are dosage sensitive and based on the putative function of *WBSCR14* as a transcription factor, hemizygosity at this locus could be involved in some features of WBS. *European Journal of Human Genetics* (2000) **8**, 215–222.

Keywords: Williams-Beuren syndrome; 7q11.23 deletion; bHLHZip domain; transcription factor; hemizygosity

Introduction

Williams-Beuren syndrome (WBS) is a neurodevelopmental disorder characterised by mental retardation with unique cognitive and personality profiles, distinctive facial features, supravalvular aortic stenosis (SVAS), transient infantile hypercalcaemia, short stature and connective tissue anomalies.[1] Molecular basis of the syndrome is a heterozygous microdeletion about 1.5 Mb at chromosome band 7q11.23, which has been reported for the great majority of patients studied.[2–4] Meiotic mispairing of blocks of duplicated sequences that flank the deleted interval followed by unequal inter- or intrachromosomal crossing-over appear to be the common mechanisms that lead to deletions of similar size in most WBS patients.[5–7] The identification of all genes included in the deleted interval, especially those whose haploinsufficiency causes each of the different aspects of the WBS phenotype, is a primary goal when studying WBS. *Elastin* was the first gene found to be deleted in WBS and its haploinsufficiency has been clearly implicated in SVAS and some of the connective tissue pathology.[2,8] In addition, deletion of the gene encoding LIM-kinase 1 (*LIMK1*) has been proposed as a contributing factor to impaired visuo-spatial constructive cognition in WBS,[9] although this claim was not confirmed by clinical and molecular studies of three different patients with heterozygous *LIMK1* deletions.[10] A total of 15 additional genes has been reported to be part of the common WBS deleted region although none of them has been related to any of the remaining phenotypic features of the WBS phenotype (see Francke[11] for a review). They include the

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single copy genes coding for the immunophilin FK-506 binding protein FKBP6, a *Drosophila frizzled* homolog (*FZD9*), the putative transcription factor WBSCR9 also known as WSTF, the homolog of a gene disrupted by a leukemic translocation breakpoint (*BCL7B*), a β-transducin (*TBL2* also named *WS-βTRP*), the putative transcription factor WS-bHLH, the presynaptic vesicle protein syntaxin 1A (*STX1A*), the *Clostridium perfringens* enterotoxin receptors 1 and 2 (approved symbols *CLDN4* and *CLDN3*), the eukaryotic initiation factor EIF4H also known as WBSCR1, the subunit 2 of the replication factor C (*RFC2*), the neuronal cytoplasmatic linker protein CYLN2, and the GTF2I-related protein GFT2IRD1/WBSCR11. The transcription initiator factor GTF2I and the neutrophilic cytosolic factor 1 NCF1 are part of duplicons that flank the WBS deletion, with the functional copy of both genes being at the telomere edge of the deleted interval.[6,11]

We and others have constructed meiotic and physical maps that totally or partially cover the deleted region.[12–15] Some of the BAC clones have been sequenced as part of the Human Genome Project and a great portion of the region is already sequenced and available in public databases, albeit as unordered pieces. Using this resource, we have fully characterised *WBSCR14* (nomenclature committee approved gene symbol), a gene previously reported as *WS-bHLH* (Genbank acc. no. AF056185). However, we have found significant differences between *WBSCR14* and *WS-bHLH* cDNAs, suggesting that the reported *WS-bHLH* cDNA was a chimeric and truncated form. We have also identified the mouse ortholog and mapped its locus to a region of conserved synteny with human chromosome 7q11.23.

Materials and methods

cDNA cloning and genomic structure

The genomic sequence from BAC RG315H11 (Genbank acc. no. AC005089) was analysed using the integrated NIX resource available at the web pages of the UK Human Gene Mapping Project (http://www.hgmp.mrc.ac.uk). ESTs with identity to predicted exons were identified and assembled by computer analysis. Several oligonucleotide primers were designed and used to obtain a cDNA by reverse transcribed (RT)-PCR from human adult liver total RNA. Sequencing of PCR products was performed using an ABI 377 machine (Perkin-Elmer, Roche Molecular Systems Inc, Branchburg, NJ, USA) according to the manufacturer's instructions. Assembling of two non-overlapping sequenced pieces of BAC RG315H11 was done by PCR with primers WS4B: 5'-CAG CCT GGG CAA CAG ATT CA-3'; and 1R: 5'-GTA TTC CCG CAT CAT CAT CT-3'. Comparison of cDNA and genomic sequence permitted the estimation of intron sizes and the identification of intron–exon junctions and boundaries. Transcription initiation was predicted by using the PPNN (http://www.hgc-lbl.gov/projects/promoter.html) and MatInspector (http://www.genomatix.gsf.de) programs.

Mapping of *WBSCR14* and *WS-bHLH* sequences

Somatic cell hybrid lines containing a single human chromosome in a rodent background were obtained from the NIGMS Human Genetic Mutant Cell Repository (Coriell Institute for Medical Research, Camden, NJ, USA). PCR screening of DNAs from the panel of hybrid cells was performed with primers specific for the 5' of the *WS-bHLH* gene: MD: 5'-TCT CAA ACC CTC TCC CTG AC-3'; and MR: 5'-GTC AGT CAT AGC AGC TCA CC-3'. Refined mapping of the *WBSCR14* locus among other genes in the region was performed by STS analysis of several cosmids previously identified with a *TBL2* probe.[16]

Patients and polymorphism genotyping

Criteria for clinical diagnosis of WBS have been previously reported.[3] Fourteen patients with a complete genetic evaluation who fulfil diagnostic criteria were studied. Genomic DNA was obtained from each patient and both parents by using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA). Fluorescent-labelled oligonucleotides were designed for amplification of the polimorfic (CA)n markers D7S2476 and WS4. Sequences of the oligonucleotides designed for WS4 were WS4B, described above, and WS4A: 5'-CCA AAG CAC TGG GAT TAC AG-3'. PCR reactions were performed in a final volume of 10 μl, using standard conditions. PCR products were resolved using an ABI Prism 310 genotyper. Additional molecular diagnosis of patients was made by the analysis of other intradeletion polymorphic markers previously reported along with FISH in some cases.[3]

Expression analysis

Northern blot and dot blot filters containing poly(A)$^+$ mRNA from multiple human and mouse tissues were purchased from Clontech (Clontech Laboratories, Palo Alto, CA, USA) and probed with human and mouse cDNA probes according to the manufacturer's instructions. Probes were generated by RT-PCR from adult liver total RNA or total RNA of whole mouse embryo, respectively, using the same primers in both cases: 6D: 5'-GAG AAC CGA CGT ATC ACA CA-3'; and UR: 5'-CTC TGT GAC TGC CCG TGT GG-3'. The probes were gel purified and radiolabelled using a random priming labelling kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridisation was performed at 65°C for 16 h, and filters were washed at a final stringency of 0.1X SSC, 0.1% SDS at 65°C. As a control for RNA loading and quality, filters were stripped and rehybridised using a β-actin probe.

Cloning and mapping of the mouse *Wbscr14* gene

ESTs with homology to the human cDNA were searched using the BLAST algorithm.[17] The *Wbscr14* cDNA was obtained by RT-PCR combining degenerate primers designed from the human sequence and mouse specific primers designed from ESTs. The yeast artificial chromosome (YAC) WI/MIT library no. 820 was previously screened with several

cDNA probes from the genes deleted in WBS.[18] DNA from the positive YAC clones was analysed by PCR with mouse *Wbscr14* primers 9D: 5'-ACC TCA CTG GCC TGG CTG GA-3' and UR (above). For long-range restriction mapping, high molecular weight mouse genomic DNA was digested with restriction endonucleases *Mlu*I and *Not*I, blotted on to nylon membranes and hybridised with *Wbscr14*, *Stx1a* and *Tbl2* specific probes. DNA radioactive labelling and hybridisation were performed as described for the northern blot assays.

Results and discussion

Computer analysis of genomic sequence from BAC RG315H11 using the integrated NIX resource allowed the identification of several overlapping ESTs with identity to predicted exons.[17] Additional putative coding exons were identified by conserved homology across species with a *Caernorhabditis elegans* open reading frame (ORF) (T20B12.6) predicted from genomic sequencing.[19] A *WBSCR14* cDNA was obtained by assembling overlapping RT-PCR products from adult liver total RNA. The obtained 3270 nt *WBSCR14* cDNA contains a 2559 nt ORF that encodes an 852 amino acid protein, with a predicted molecular weight of 93.1 kDa. The putative translation initiation has a good Kozak consensus.[20] A splicing variant due to the use of an alternative acceptor splice site at the beginning of exon 11 results in a trinucleotide (CAG) deletion in the mRNA (Table 1) and the corresponding deletion of a glutamine residue at position 558 of the protein. No additional splicing variants were identified in the *WBSCR14* cDNA by RT-PCR with combinations of primers.

The predicted WBSCR14 protein contains three known functional motifs: a bipartite nuclear localisation signal (BNLS; aa 158–175),[21] a basic helix–loop–helix (bHLH) region (aa 650–704)[22] and a consensus leucine–zipper (Zip) domain $L(X)_6L(X)_6L$ (aa 704–725) (Figure 1A).[23] Stretches of proline-rich sequences (aa 382–471 and 571–585) as well as additional non-consensus Zip-like motifs (aa 725–738 and 795–835), were also detected. Several regions of the protein including the BNLS and bHLH regions are quite conserved (45–51% similarity) in the *Caenorhabditis elegans* protein T20B12.6 (Figure 1A).

The previously reported 2665 nt *WS-bHLH* cDNA[12] is identical to *WBSCR14* cDNA at the 3' end, in a region corresponding to exons 9, 10 and from 12 to 17 of the *WBSCR14* genomic structure (Figures 1B and 2). However, the initial 918 nt of *WS-bHLH* cDNA were not found in the cloned *WBSCR14* cDNA nor in the genomic sequence of BAC RG315H11. PCR with primers designed from this sequence (MD and MR) amplified the expected 134 bp fragment from control DNA as well as from a cell line containing a single human chromosome 7 with a typical WBS interstitial deletion in a hamster background,[24] indicating the location of at least one copy outside the common deletion (not shown). Using the same primer pair on a somatic cell hybrid panel in which each cell line contains a single human chromosome in a rodent background (Coriell Repositories), specific amplification products were obtained from human chromosomes 3 and 13 along with human genomic DNA; weak amplification was detected from chromosome 21, and no product from any other human chromosome (including chromosome 7), mouse and hamster genomic DNA. In addition, PCR with MD and several *WBSCR14* cDNA reverse primers failed to obtain any amplification product on reverse transcribed adult human liver mRNA. Therefore, we believe that the reported *WS-bHLH* cDNA was probably a chimeric fragment due to a cloning artefact. *WS-bHLH* coded for a putative protein of 231 residues, almost identical to the carboxy-terminus of WBSCR14 except for the absence of two residues in WS-bHLH due to the deletion of 6 nt at the beginning of exon 13 (Figure 1A). The predicted WS-bHLH protein contained the bHLH and Zip domains but lacked the proline-rich segments and the BNLS.

Table 1 Exon/intron organization of human *WBSCR14* gene

Exon	(bp) 3′ Intron	5′ Exon	Length (bp)	3′ Exon	5′ Intron (bp)
1		CCCCGC....	340	....CTACAG	**gt**gagggc...(8023)
2	(8023)...ctccccttt gac**ag**	TGGCAA....	106	....TCCAGT	**gt**gagtgg...(8353)
3	(8353)...ccccctt ctct**ag**	ATGTGA....	83	....CGGAGG	**gt**agctgg.....(169)
4	(169)...ggcccctaccc**ag**	CCGTGG....	88	....AAGCGG	**gt**cagtgg.....(311)
5	(311)...gtgtgtccctgc**ag**	CTCCGT....	44	....AAGCAG	**gt**gggtgc.....(864)
6	(864)...ctctgtctccct**ag**	GCGGAA....	201	....AGGATG	**gt**gagggt.....(142)
7	(142)...actgctgcctcc**ag**	CCTACG....	80	....TCTCAG	**gt**ggggcg...(5913)
8	(5913)...tgtctgaatta**cag**	ATTTCT....	169	....CTGCAG	**gt**gagctc...(1813)
9	(1813)...gtccccccaacc**ag**	GCTCGG....	531	....CAGCAG	**gt**gagggc.....(250)
10	(250)...ctgcccgtgcac**ag**	CTAAGC....	67	....TCCCCG	**gt**aagatc..(70/73)
11	(70/73)ctccaccc**cag**(c**ag**)	(CAG)GAG	153/150	....CCAGCG	**gt**aaagag.....(160)
12	(163)...tgtccctaccac**ag**	GCAGTG....	115	....AACAAG	**gt**gggcac.....(92)
13	(92)...ctctgctccccg**ag**	ACCGAG....	119	....CTCAAG	**gt**gagccc.....(184)
14	(184)...ggtggcgtctgc**ag**	GTGAGC....	127	....CATTAA	**gt**aggcag.....(82)
15	(82)...tgccctgcccccc**ag**	CCTGTG....	123	....TGGGTG	**gt**atcctc...(1234)
16	(1234)...tgacctgggccc**ag**	TTCAGC....	129	....GGCCAA	**gt**acgtga.....(291)
17	(291)...tgtctcccaccc**ag**	CTGTCC....	781	....ACTGTC	

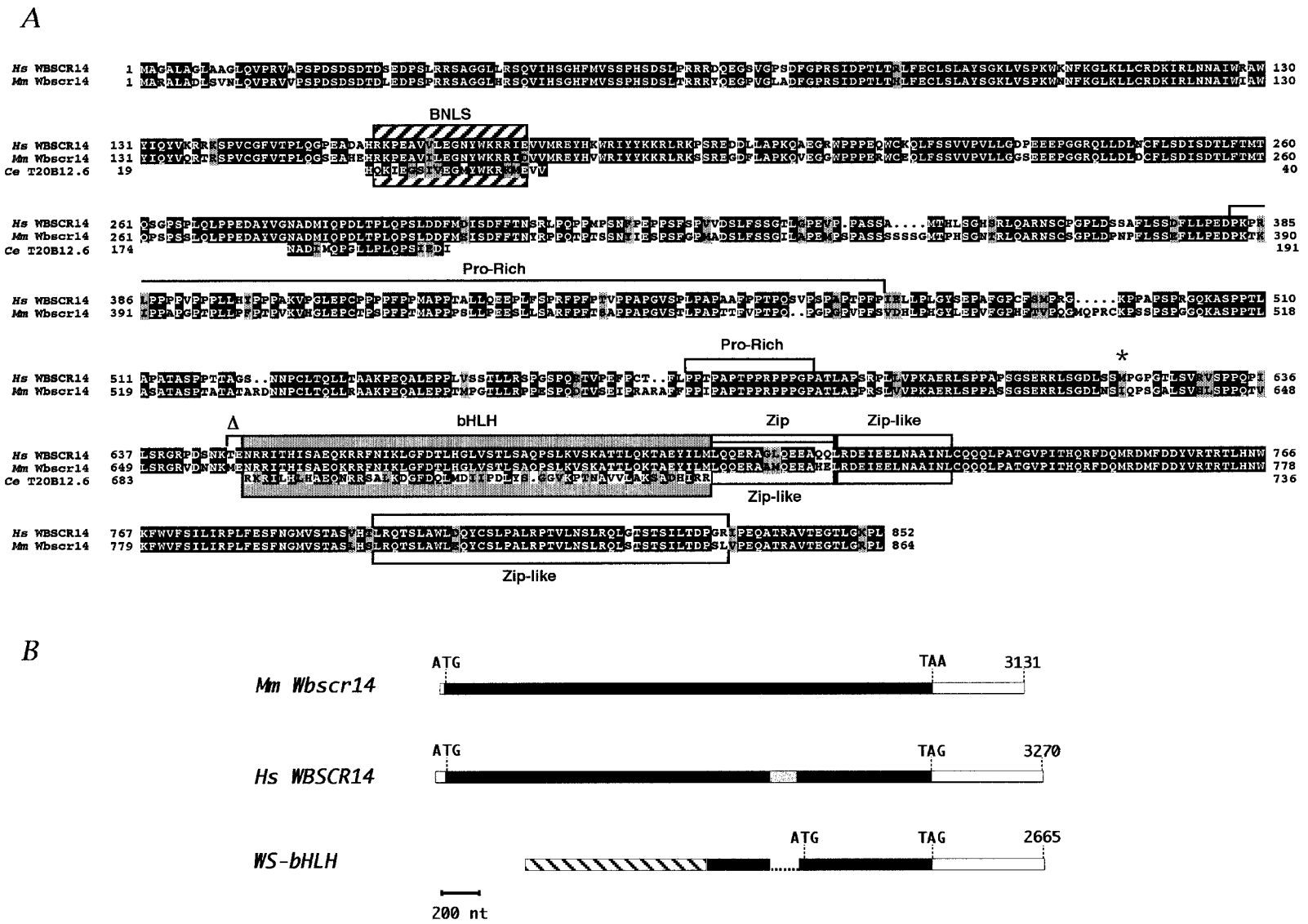

Figure 1 **A** Aminoacid sequence alignment of human (*Hs* WBSCR14) and mouse (*Mm* Wbscr14) proteins along with partial sequences of a related protein from *Caenorhabditis elegans* (*Ce* T20B12.6). Identical aminoacids are included in black boxes while conservative changes are marked with grey boxes. The location of the most relevant protein motifs is shown: bipartite nuclear location signal (BNLS), proline-rich stretch (Pro-rich), basic helix–loop–helix (bHLH), leucine–zipper (Zip) and leucine zipper-like (Zip-like) domains. An asterisk indicates the initial methionine predicted for WS-bHLH and a Δ symbol indicates the two aminoacids deleted in WS-bHLH with respect to WBSCR14.[12] **B** Schematic representation of mouse and human *WBSCR14* cDNAs along with the reported *WS-bHLH* cDNA.[12] Open boxes represent the 5' and 3' UTRs while dark boxes represent exons containing the ORF. Exon 11 is represented as a grey box, missing in *WS-bHLH* (dotted line). The hatched box of *WS-bHLH* corresponds to sequences not found in BAC RG315H11, and the dark box upstream of the ATG contains a region homologous to *WBSCR14* ORF but described as 5' UTR[12].

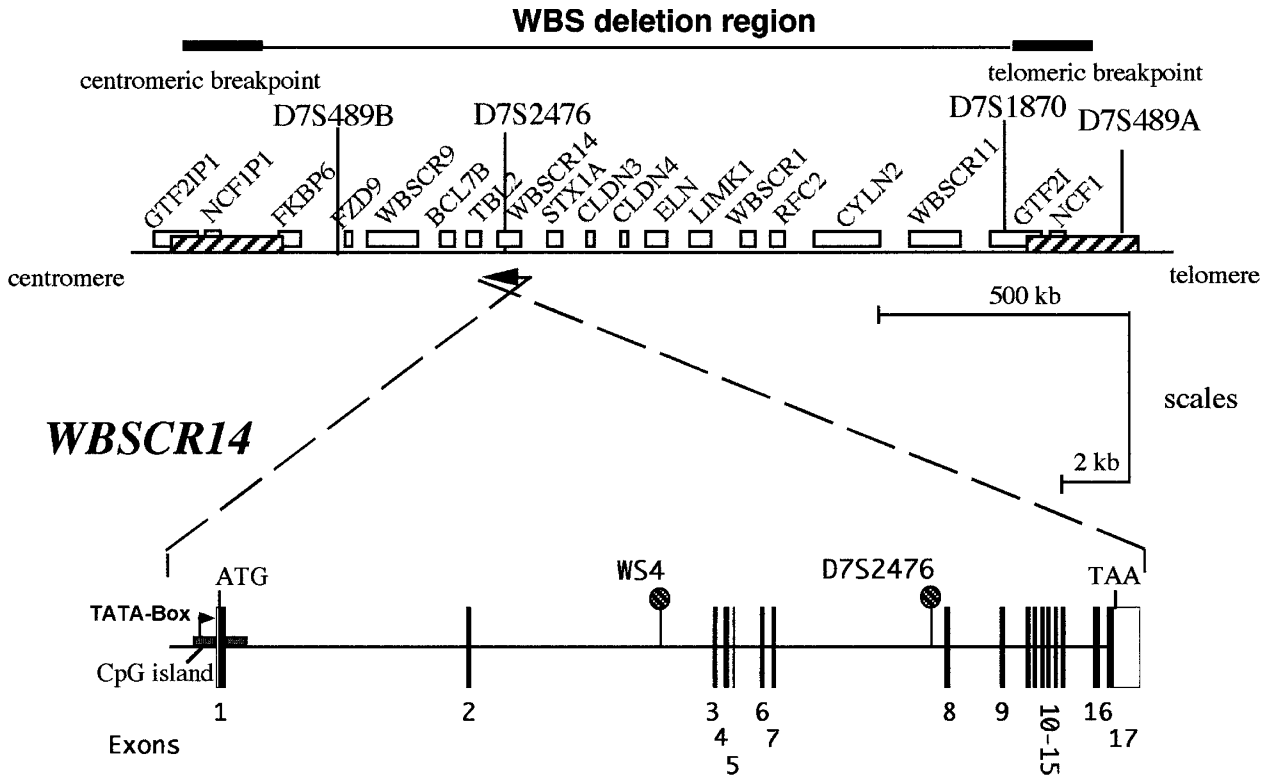

Figure 2 Mapping and genomic structure of the *WBSCR14* gene. **Top**: Schematic representation of the transcription map of the WBS commonly deleted region in 7q11.23,[11] with the location and transcriptional orientation (indicated by an arrow) of the *WBSCR14* gene. **Bottom**: Genomic structure of the *WBSCR14* gene with the 17 exons represented by boxes numbered below. Open box contains 3' untranslated region (UTR). The locations of the CpG island with the predicted TATA box and the two intragenic CA repeats, WS4 and D7S2476, are indicated.

PCR with primers WS4B and 1R on genomic DNA obtained the expected 2.2 kb fragment allowing the joining of two non-overlapping sequenced pieces of BAC RG315H11 and the completion of the *WBSCR14* genomic sequence (Genbank acc. no. AF156673). The exon–intron structure of *WBSCR14* was determined by comparison of *WBSCR14* cDNA and genomic sequences. The gene encompasses 33 Kb of genomic DNA, including 17 exons with 16 introns (Figure 2). All intron–exon junctions conform to the AG–GT rules (Table 1). A CpG island surrounds the first exon and a good putative transcription initiation site with a TATA box 23 bp upstream was predicted. BAC RG315H11 also contains other genes included in the WBS critical region such as *TBL2/WS-βTRP* and *BCL7B*.[12,16] To determine the transcriptional orientation of the *WBSCR14* gene and its relative order with respect to the other genes in the region, PCR analysis with several STSs developed from the genomic sequence was performed using overlapping cosmids previously obtained with a *TBL2* probe.[16] The results were consistent with the order depicted in Figure 2, all three genes being in the same transcriptional direction. Our results agree with two recent reports[13,14] but differ from others.[12,15]

The location of *WBSCR14* within the 7q11.23 WBS deletion is strongly supported by our mapping studies along with the previous definition of the commonly deleted interval (Figure 2).[3,4] However, for additional confirmation, we used two *WBSCR14* intragenic polymorphic $(CA)_n$ repeats to determine allelic inheritance in WBS individuals. One of them corresponds to the previously described locus D7S2476, which was found in intron 7 of the gene and has a heterozygosity of 70%. The second marker, named WS4, was identified in intron 2 of the gene and typed using specific primers WS4A and WS4B. The observed heterozygosity of WS4 in 28 unrelated individuals was 40%, with four different alleles found. Lack of parental inheritance at both loci was observed in all informative WBS patients genotyped, corroborating the location of *WBSCR14* within the 7q11.23 common deletion (data not shown).

To obtain the mouse orthologous gene, several mouse ESTs with homology to the human cDNA were identified through a BLAST search.[17] A 3131 nt mouse *Wbscr14* cDNA was obtained by RT-PCR combining degenerate primers designed from the human sequence and mouse specific primers designed from ESTs. It contains a 2595 nt ORF that codes for a 864 amino acid protein with a predicted molecular weight of 94.8 kDa. No alternative splicing was found in the mouse cDNA sequence. Human and mouse protein sequences display 81% identity and 85% similarity, the homology being

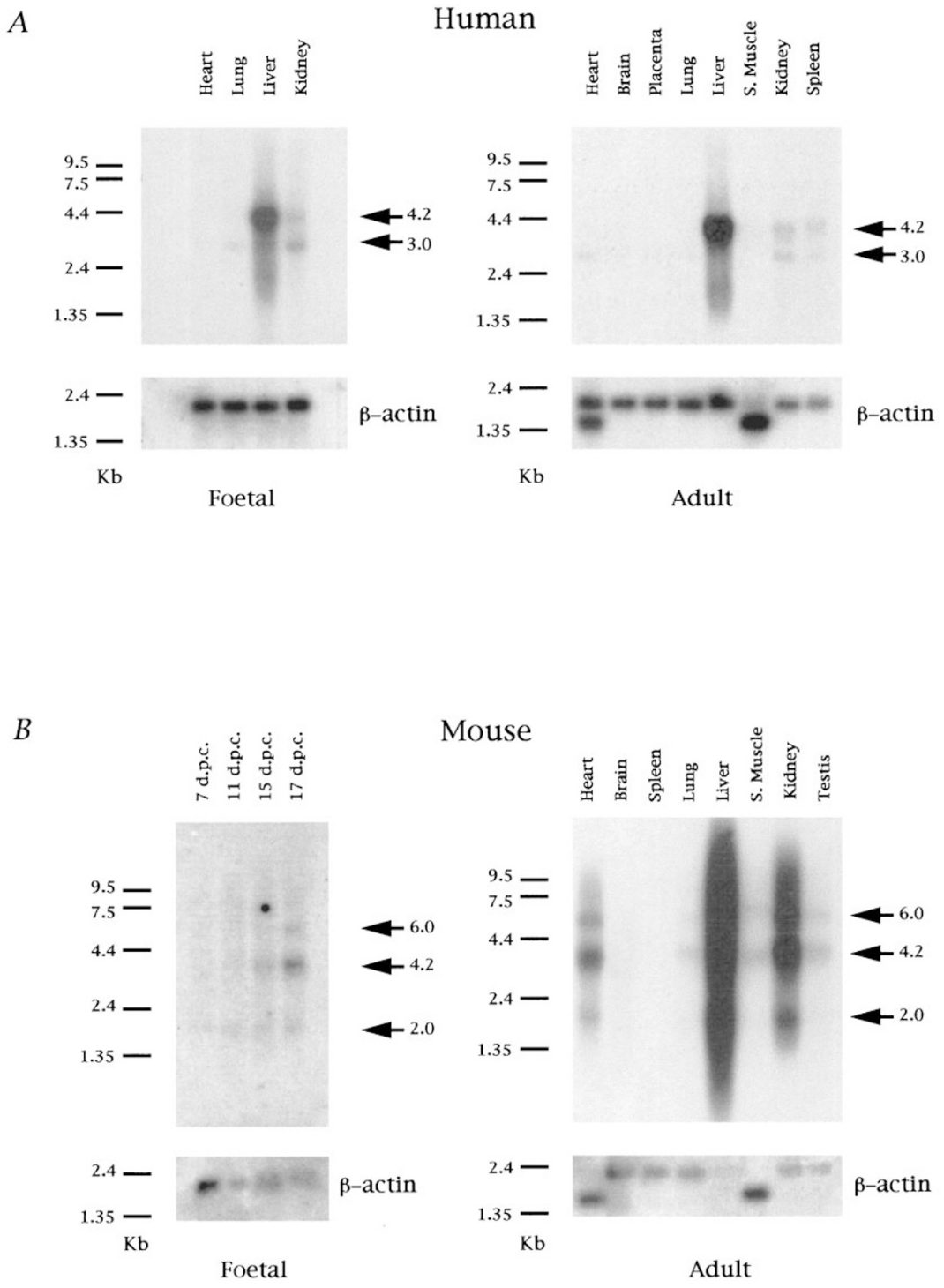

Figure 3 Expression analyses of human **A** and mouse **B** *WBSCR14* genes in foetal and adult tissues. Tissues used are described on top of each lane. dpc: days of *postcoitum* mouse foetal development. **A** A major transcript of 4.2 kb is observed in adult and foetal liver in humans with almost no signal in other tissues. **B** In mouse, a similar band of 4.2 kb is found in adult liver with weaker signal in kidney and heart. During development, the 4.2 kb *Wbscr14* transcript is barely detected at 15 dpc and increases thereafter. The hybridisation of the β-actin probe used as control for RNA loading and quality is seen below each blot.

higher at the amino- and carboxy-terminal regions where the main putative functional domains are located (Figure 1A).

The *Wbscr14* locus was mapped to mouse chromosome 5 by physical methods. We had previously obtained overlapping yeast artificial chromosomes (YACs) covering the mouse region that contains the orthologs to genes deleted in WBS, from *Fkbp6* to *Ncf1*.[18] By PCR and hybridisation, *Wbscr14* was found in two overlapping YACs of the region (WI/MIT library YAC addresses 446D3 and 403H9). In addition, long range restriction mapping by pulse-field gel electrophoresis revealed that the *Wbscr14* locus is located between *Tbl2* and *Stx1a* (not shown). These mapping results provide an additional proof that we have identified the true ortholog of *WBSCR14*, since they are consistent with the conservation of synteny between mouse chromosome 5 band G1 and human chromosome 7q11.23.[18]

Northern blots from multiple tissues hybridised with a *WBSCR14* specific probe revealed a 4.2 kb signal detected very predominantly in liver and significantly weaker in kidney (Figure 3A). With longer exposure, the 4.2 kb band as well as an additional transcript of 3.0 kb was detected in most tissues. The same pattern was obtained in foetal tissues with strong signal in liver, much weaker in kidney and barely detectable in heart and lung (Figure 3A). Our results are quite similar to those previously reported with a *WS-bHLH* probe. A dot-blot was also used to obtain a broader and quantitative spectrum of *WBSCR14* expression. A strong signal was evident in adult liver and much weaker in foetal liver, with almost no detectable signal in any other tissue (not shown). Expression pattern of the mouse gene was obtained with a mouse cDNA probe. In adult animal tissues, a main transcript of 4.2 kb was observed in liver, weaker in kidney and heart, with additional weak bands of 2.0 and 6.0 kb with proportional intensities (Figure 3B). A strong smear was also seen in adult liver and we believe that this was likely due to the presence of RNA aggregates and/or RNA degradation in the sample of that specific lane. On a developmental northern blot, *Wbscr14* expression was barely detectable at 15 dpc of foetal development, increasing significantly by 17 dpc (Figure 3B).

Basic helix–loop–helix (bHLH) and basic leucine–zipper (bZip) transcription factors have highly conserved bipartite domains for protein–protein (HLH or Zip motifs) and DNA–protein (basic region) interaction; they act as important regulators during processes of cell proliferation and differentiation.[22,23,25] Most of them are expressed during development in a tissue- and stage-specific pattern. Examples of bHLH transcription factors include MyoD and NeuroD, which regulate the tissue-specific gene expression that controls cell determination and differentiation in myogenesis and neurogenesis, respectively.[26,27] The presence of both, bHLH and Zip regions in some proteins, is thought to enhance protein dimerisation capabilities. The bHLHZip domain specifies dimerisation and determines sequence specific DNA binding at the E box (CANNTG) consensus on the promoter of target genes.[12] Haploinsufficiency causing human genetic diseases has been shown at genes coding for bHLH proteins. The *MITF* gene encodes a bHLHZip transcription factor that plays a key role in melanocyte differentiation.[28] Null mutations at one allele of the *MIFT* locus result in the Waadenburg syndrome type 2A, a disease that associates neurosensorial deafness with pigmentary disturbances.[29] The *TWIST* gene encodes another bHLH transcription factor proposed to function as an upstream regulator of fibroblast growth factor receptor genes. Haploinsufficiency for *TWIST* causes the autosomal dominant disorder Saethre-Chotzen syndrome, a relatively common form of craniosynostosis.[30]

The task of determining whether any of the deleted genes in WBS contributes to particular features of the phenotype is difficult. To date, clinical–molecular correlations in some exceptional patients with partial deletions have suggested that the genes mainly responsible for abnormal cognition map to the telomeric interval of the deletion, whilst genes from *FKBP6* to *WBSCR14*, at the centromeric edge of the deletion, might contribute to other phenotypic aspects including mental retardation.[10] However, these conclusions lack strong supportive evidence and additional clinical and molecular studies are required. *WBSCR14* is included in the common interval deleted in WBS patients. Like other genes encoding bHLH-type transcription factors, *WBSCR14* may be dosage sensitive and its heterozygous deletion could have phenotypic consequences.

Participation of bHLH and bZip transcription factors in vertebrate development suggests that other family members such as WBSCR14 can also be implicated in developmental patterns that, when altered by insufficient dosage, may lead to morphological and/or functional abnormalities. The expression pattern of *WBSCR14* suggests a main involvement in liver regulatory functions and during late embryonic development although mRNA expression is not always a suitable parameter to predict the function of a gene. A definitive proof of involvement of *WBSCR14* in any aspect of the WBS phenotype awaits the identification of patients with specific manifestations of the syndrome and intragenic mutations. Elucidation of the specific cellular function of WBSCR14, identification of its target genes and interacting proteins as well as the inactivation of the gene in mouse, will be helpful to further define its putative implication in WBS.

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