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Mammalian BUB1 Protein Kinases: Map Positions and *in Vivo* Expression

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The spindle assembly checkpoint modulates the timing of anaphase initiation in mitotic cells containing improperly aligned chromosomes and increases the probability of successful delivery of a euploid chromosome set to each daughter cell. We have characterized cDNA sequences from several organisms with highly significant predicted protein sequence homologies to *Saccharomyces cerevisiae* Bub1p, a protein required for function of the spindle assembly checkpoint in budding yeast. The localization of mouse and human orthologs is in agreement with known conservation of synteny. Mouse backcross mapping data indicate that the murine gene resides on chromosome 2 near IL1A, 73 cM from the mouse centromere. Radiation hybrid mapping data indicate that the human locus exhibits linkage to microsatellite marker D2S176, which is located within 10 cM of human IL1A. Multiple-tissue Northern analysis indicates conservation of expression pattern in mouse and human with markedly high mRNA levels in testis. Northern analysis of two different spindle assembly checkpoint protein gene products from human, BUB1 and MAD2, reveals an expression pattern with common tissue distribution consistent with roles in a common pathway. In addition, we demonstrate that an mRNA found to accumulate in a rat fibroblast cell transformation system encodes rat BUB1, and we find that rat BUB1 mRNA accumulation correlates with the proliferation status of cells in culture.

INTRODUCTION

The spindle assembly checkpoint is a highly conserved function that delays the metaphase-to-anaphase transition in the presence of spindle damage (reviewed in Rudner and Murray, 1996; Nicklas, 1997; Gorbsky, 1997). Treatment of vertebrate cells with microtubule inhibitors induces a preanaphase arrest (Rieder and Palazzo, 1992), as does the presence of chromosomes exhibiting spontaneously late spindle attachment (Rieder *et al.*, 1994). Microbeam ablation of the kinetochore of an unattached sister chromatid pair induces escape from the delay state, strongly implicating chromosomal kinetochores in the generation of a negative *trans*-acting signal (Skibbens *et al.*, 1995). Tension generated at kinetochores by bipolar attachment of microtubules correlates with the disappearance of a kinetochore-associated phosphoepitope widely speculated to reflect activity of the spindle assembly checkpoint (reviewed in Nicklas, 1997). The preanaphase arrest induced by injection of antibodies directed at kinetochore-associated proteins in vertebrate cells is consistent with the hypothesis that abnormally configured kinetochores generate a delay-producing signal (Simerly *et al.*, 1990; Yen *et al.*, 1991; Tomkiel *et al.*, 1994; Campbell and Gorbsky, 1995).

Proteins required for the spindle assembly checkpoint were first identified in budding yeast through mutations in the BUB (Hoyt *et al.*, 1991) and MAD (Li and Murray, 1991) genes that caused a failure in the preanaphase arrest or delay normally induced by microtubule poisons. The early mutageneses were not saturating, and subsequent studies have implicated checkpoint roles for a protein required for spindle pole duplication (MPS1; Weiss and Winey, 1996) as well as for proteins modulating cyclin-dependent kinase control of passage through mitosis (Minshull *et al.*, 1996; Li and Cai, 1997; Wang and Burke, 1997). Mutant yeast cells lacking the spindle assembly checkpoint undergo premature mitosis in the presence of spindle damage and exhibit aneuploidy and cell death (Li and Murray, 1991; Hoyt *et al.*, 1991; Wang and Burke, 1996; Pangilinan and Spencer, 1996; Weiss and Winey, 1996). The yeast spindle assembly checkpoint pathway is activated not only by microtubule poisons, but also by genetic defects in spindle structures, including centromere components (Spencer and Hieter, 1992; Wells and Murray, 1996; Doheny *et al.*, 1993; Goh and Kilmartin, 1993; Strunnikov *et al.*, 1995; Connelly and Hieter, 1996) and components of the microtubule organizing center (Winey and Byers, 1993).

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Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. AF011387 and U89795.

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Vertebrate proteins with structural and functional similarity to budding yeast MAD2p (Li and Benezra, 1996; Chen *et al.*, 1996) and BUB1p (Taylor and McKeon, 1997) have been recently reported. Interference with the normal function of these vertebrate proteins leads to defects in chromosome segregation and cell death, providing further support for the widely held speculation that defects in the spindle assembly checkpoint may play a role in tumorigenesis (Hartwell and Kastan, 1994). While the MAD2p primary amino acid sequence does not suggest a known biochemical function, the BUB1p primary sequence not only exhibits highly significant similarity to known protein kinases but has demonstrated serine–threonine kinase activity *in vitro* (Roberts *et al.*, 1994). The kinase similarity domain occupies the C-terminal one-fourth of yeast BUB1p, which also contains an intriguing region of similarity to another spindle assembly checkpoint protein (MAD3p) near the N-terminus. These two domains are conserved in a mouse BUB1p sequence previously described (Taylor and McKeon, 1997).

Here we report the isolation and characterization of several related mammalian cDNA clones encoding proteins with highly significant homology to budding yeast BUB1p, and we define a candidate orthologous family including human, mouse, rat, nematode, and budding yeast proteins. We have determined the map positions of human and mouse genes and have characterized mRNA expression data from multiple tissues in both of these organisms. We find general concordance of BUB1 mRNA accumulation and cytoplasmic thymidine kinase mRNA (a marker for cell proliferation), with the exception of a markedly high level of BUB1 mRNA in testis. Moreover, we present evidence that a BUB1 transcript from rat shows accumulated mRNA levels in a single-step transformation cell culture system, providing a model in which to explore a candidate role for BUB1p in cell proliferation and transformation.

MATERIALS AND METHODS

Clone construction. A PCR product containing the 659-bp cDNA encoding the expressed sequence tag (EST) GenBank L26607 (Kerr *et al.*, 1994) was inserted into the pCRII vector using the TA cloning kit (Invitrogen) to create the clone pA005. Digestion with restriction enzymes *Eco*RI and *Xho*I releases the insert containing the 3′-terminal 659 nucleotides of the murine BUB1 gene.

cDNA isolation and sequencing. Mouse BUB1 was isolated by using the Gene Trapper cDNA Positive Selection System to screen a mouse testis cDNA library (Life Technologies). The oligonucleotide 5′-CGAGGCGAAATCATTCATGGAG-3′, designed from the L26607 EST, was used as the probe, yielding six unique positives. By restriction digest, the three largest clones contained cDNA inserts with a common 3′-terminus. The DNA sequence of the longest of these (MT10, containing a 3.4-kb cDNA insert) was determined for both strands using dye terminator cycle sequencing methods on an ABI machine. The oligonucleotide 5′-GCTACACGGGTAATGACC-3′ was then used to rescreen the testis library, yielding only one larger clone (MT97), which contains an additional 58 bp of 5′ sequence, but is otherwise identical in structure.

Mapping analyses. Mouse BUB1 was mapped through the genome cross-referencing project (which maintains XREFdb) using The Jackson Laboratory BSS backcross panel DNAs. Southern blot analysis of *Eco*RI-digested parental DNAs (C57BL/6 and Spret/Ei) exhibited unambiguous polymorphic bands, with no comigrations. Southern filters containing 94 offspring DNAs and the 2 parental controls were probed with a radiolabeled 659-bp *Eco*RI–*Xho*I fragment from pA005. Two cosegregating C57BL/6 bands (1.5 and 4 kb) indicated a single locus in the mouse genome on chromosome 2. The same hybridization probe was applied to human DNA from the NIGMS somatic cell hybrid panel 2 version 2 (Coriell Cell Institute) digested with *Hin*dIII. A single 4.4-kb human-specific band indicated a human gene locus on chromosome 2. Southern hybridizations were performed in Church–Gilbert buffer at 65°C with moderate stringency washes in 0.2× SSC/0.5% SDS at 55°C. The offspring scorings supporting map results for the mouse backcross and human chromosomal localization can be viewed from links referring to the yeast BUB1 gene within XREFdb (www.ncbi.nlm.nih.gov/XREFdb) and within the Encyclopedia of the Mouse Genome at The Jackson Laboratory Web site (www.informatics.jax.org) by choosing map source JAX BSS within the Mouse Genome Database.

The subchromosomal position of the human locus was directly determined by mapping within the Stanford G3 radiation hybrid mapping panel. The oligonucleotide primers 5′-AAGTGAAAAATGAAGGAGGAG-3′ and 5′-TTTTCGTGAACGCTTACATTC-3′ were designed from sequence near the 3′-terminus of the human BUB1 cDNA. These amplified a unique 1.9-kb product from human genomic DNA, containing sequence predicted by the cDNA interrupted by a single intron (data not shown). Results were submitted to the Stanford Human Genome Center web server, which reported linkage to Généthon microsatellite marker D2S176 at odds of 1000:1.

Multiple-tissue Northern blot analysis. Commercially available filters containing poly(A)$^+$ mRNA from mouse or human adult tissues (Clontech) were probed with radiolabeled cDNA fragments according to the manufacturer's instructions. Except where noted, final wash conditions for blots were at a stringency of 0.1× SSC, 0.1% SDS, 50°C. All reprobings were preceded by exposure to film to ensure complete loss of detectable signal.

The mouse blot probed with a 3′ 659-bp *Eco*RI–*Xho*I fragment from pA005 was exposed to film for 3 days. The same blot was stripped and rehybridized with rat 3i3 (U83666) and human actin (Clontech) probes and exposed to film for 18 h and 45 min, respectively. A 5′ 464-bp *Pst*I fragment from MT10 was also hybridized to this blot; the film exposure was 1 day after a final wash in 0.1× SSC, 0.1% SDS, 60°C.

The human multiple-tissue Northern was first probed with a 417-bp *Eco*RI fragment from the 5′ end of the human BUB1 cDNA and exposed to film for 1 day at −70°C. The same blot was stripped and reprobed with a 3′ 885-bp *Bgl*II fragment from the human BUB1 cDNA using identical conditions; the full-length HsMAD2 cDNA (GenBank T48809), a human thymidine kinase cDNA probe consisting of a 1-kb *Xho–Xba* fragment from pGMK28 (Kaufmann and Kelly, 1991); and human actin. Film exposure times were 1 day, 1 day, and 15 min, respectively.

Analysis of the rat cell cultures. Rat1a and Rat1a-myc are previously characterized cell lines (Stone *et al.*, 1987; Hoang *et al.*, 1994). The Rat1a-ras cell line was created (Q. Li and C. V. Dang, unpublished) by cotransformation of Rat1a with a marker vector containing puromycin resistance and a plasmid containing human EJ-Ras (Parada *et al.*, 1982). For Northern analysis cells were cultured on dishes with or without a 0.7% agarose layer for 48 h in Dulbecco's modified Eagle medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). Total RNA was isolated using Trizol (Gibco BRL) according to manufacturer's instructions. Fifteen micrograms of total RNA was loaded per lane onto a 1.0% agarose/formaldehyde gel. After transfer to nylon membrane, the blot was hybridized with radiolabeled 3i3 cDNA (431 bp). Final wash conditions were 0.2× SSC, 0.1% SDS, 50°C. Radiolabeled hybridization signals were quantitated by a phosphorimager (Molecular Dynamics), and ethidium bromide staining of the 18S ribosome was quantified using NIH Image software. The proliferative status of cell cultures was determined by relative BrdU incorporation and flow cytometry as previously described (Lewis *et al.*, 1997).

Sequence analysis. DNA sequencing was performed using dye-labeled terminator chemistry and automated analysis (ABI). All re-

gions were subjected to sequence analysis on both strands. Mouse BUB1 and human BUB1 partial cDNA and protein sequences have been deposited with GenBank, with Accession Nos. U89795 and AF011387, respectively. All BLAST searches were done using GenBank release 100.0 with default parameters. Multiple protein alignments were carried out by MACAW (Schuler *et al.*, 1991) using the BLOSUM80 matrix to define the blocks shown in Fig. 1.

RESULTS

Identification and characterization of candidate yeast BUB1 orthologues. A 1058-amino-acid mouse protein (GenBank AF002823) predicted from a cDNA clone isolated from a mouse teratocarcinoma cell line has recently been reported (Taylor and McKeon, 1997). In independent concurrent studies we obtained and characterized cDNA clones from a normal mouse testis library (Life Technologies) and have determined a 3444-nucleotide cDNA sequence (GenBank U89795) encoding an 1102-amino-acid open reading frame. Direct comparison between the two independently derived murine cDNA sequences reveals only four single nucleotide discrepancies within the open reading frame segment held in common. Three differences are in third-codon positions that do not alter the predicted protein sequence. The fourth disparity predicts an arginine to lysine change at amino acid 230, which represents a conservative substitution that does not alter either of the prominent conserved protein motifs (see below). Thus, our comparison of teratocarcinoma to normal testis cDNA sequence reveals no obvious functional consequences at the protein level.

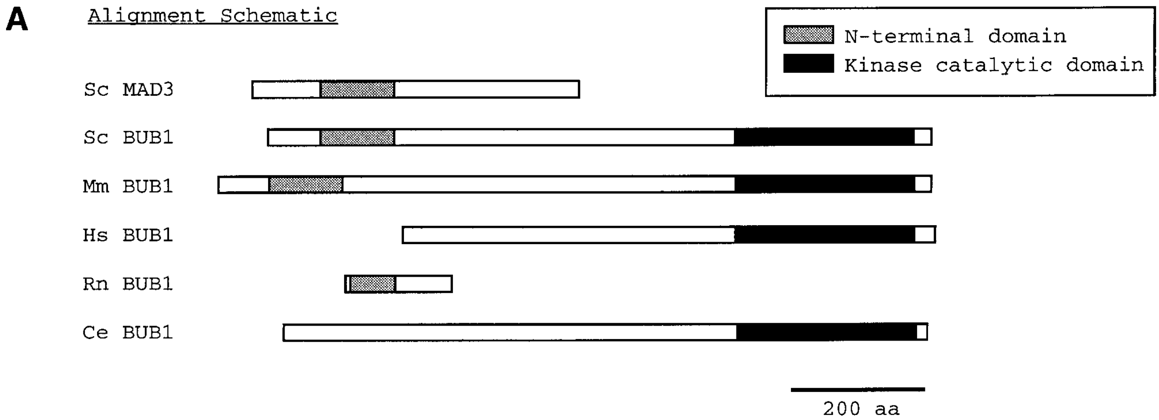
As also found by Taylor and McKeon (1997), our analysis of sequence alignment between mouse and yeast BUB1 predicted proteins revealed two regions of highly significant conservation (Fig. 1A). Near the N-terminus, mouse BUB1p and Sc BUB1p shared a common motif also found in another budding yeast protein, MAD3p (Fig. 1B). The protein kinase catalytic domain near the C-terminus contains many residues outside of the dispersed conserved amino acids constituting the signature-defining protein kinase homology (Fig. 1C). In FASTA alignment (BLOSOM 80) (Altschul *et al.*, 1990), the yeast and mouse BUB1 proteins share 24.9% identity and 55.7% similarity. When the mouse predicted protein is used to search the GenBank nonredundant protein database (NRDBp) using the BLAST algorithm (Pearson and Lipman, 1988), the best match obtained is to yeast Bub1p with a P value of 4.2×10^{-40}.

It is worth noting that the predicted 1058-amino-acid sequence for mouse BUB1p (Taylor and McKeon, 1997) may not represent the full-length protein. The 3.8-kb mRNA predicted by Northern blot (see below) is an estimated 360 nucleotides larger than the longest cDNA sequence recovered to date (this work). The open reading frame of cDNA sequence we have determined is continuous to the N-terminus, with the potential of contributing an additional 44 amino acids. On the other hand, the extent of the mouse protein revealed by extant cDNA analysis does clearly cover the majority of amino acids that can be encoded by the mRNA and does contain similarity to the two recognized domains in yeast BUB1p.

In additional studies, clones containing human BUB1 cDNAs were identified through comparison of the murine BUB1p to a six-frame translation of the database of expressed sequence tags (dbEST; Boguski *et al.*, 1993), which revealed seven highly similar human sequences. We obtained 2.6 kb of cDNA sequence from both strands of the human insert in the clone exhibiting the most 5′ alignment (GenBank R94348) to yeast BUB1p. Comparison of the 810-amino-acid sequence predicted by the entire human insert revealed striking conservation with murine BUB1p along the entire length (BLAST P value = 0.0) and similarity to budding yeast Bub1p (BLAST P value = 2.2×10^{-31}), as depicted in Fig. 1. We therefore refer to the gene encoding the partial human cDNA as human BUB1 and to its predicted protein sequence as human BUB1p.

A protein predicted from the *Caenorhabditis elegans* sequencing project was noted in results from searches of GenBank NRDBp using mammalian BUB1p queries, which revealed that the second best match after yeast BUB1p was to the worm protein R06C7.8 (GenBank Z71266) with a BLAST P value of 5.0×10^{-27} for the worm to mouse match. This predicted protein is highly homologous to the carboxy-terminal kinase domain shared by budding yeast, mouse, and human proteins, but does not contain the amino-terminal domain (Fig. 1A). Further inspection of the worm genomic sequence 5′ to the R06C7.8 open reading frame indicates the presence of a divergently transcribed gene with translational initiation just 675 nucleotides upstream of the R06C7.8 translational start. We therefore consider it unlikely that an N-terminal extension including the other homology domain has been overlooked in analysis of the worm genomic sequence.

Mapping of mammalian BUB1 genes in mouse and human genomes. To determine the position of the murine BUB1 gene, a 659-bp cDNA insert encoding the 3′-terminus of the mouse cDNA was used as a Southern hybridization probe against The Jackson Laboratory BSS backcross mapping panel (Rowe *et al.*, 1994). The probe unambiguously identified a single mouse locus (D2Xrf87), mapping approximately 73 cM from the centromere of mouse chromosome 2 in the same genetic interval as the IL1A gene (Fig. 2). Analysis of markers in this region of mouse chromosome 2 that have been mapped in both mouse and human genomes predicts the existence of a human gene in the human chromosomal region 2q11–q21 by conservation of synteny. Evidence for localization to human chromosome 2 was obtained directly by Southern blot hybridization of the mouse probe to cell line DNAs from the NIGMS somatic cell hybrid panel 2 (version 2), which consists of a collection of rodent cell lines each containing a single human chromosome. A unique human-specific 4.4-kb *Hin*dIII band confirmed the presence of a human gene on chromosome 2.



B N-terminal domain

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Sc MAD3    GNSKQSGMLTLLERCLSHLKDLERYRNDVRFLKIWFWYIE      135
Sc BUB1    SESGQEVLRSTMERCLIYIQDMETYRNDPRFLKIWIWYIN      116
Mm BUB1    FPDNKEYLMTTLLEHLMKEFLHKKNYHNDSRFINYCLKFAE     121
Consensus   * ** * ***E***    * * Y*ND RF**** ****

Sc MAD3    MESRDIFMYMLRNGIGSELASFYEEFTNLLIQKEKFQYAVKILQLGIKNKARPNKVLEDRLNHLLREL  210
Sc BUB1    HESENTFKYMFNKGIGTKLSLFYEEFSKLLENAQFFLEAKVLLELGAENNCRPYNRLLRSLSNYEDRL  191
Mm BUB1    SDRHQFFEFLYNQGIGTKSSYIYMSWAGHLEAQGELQHASAIFQTGIHNEAEPKELLQQQYRLFQARL  191
Rn BUB1    NEPLDMYSYLHSQGIGISLAQFYISWAEEYEARGNFKKADIVFQQGIECKAEPLDRLQSQHRQFQARV   74
Consensus  *    * *    GIG  *  *Y       **    *  A    *G* *    P   L           **
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C Kinase catalytic domain

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            * *    *                    * * *                   *
Sc BUB1    LGEGGYATVYLAESS------QGHLRALKVEKPASVWEYYIMSQVEFRLRKSTILKSIIN   764
Mm BUB1    LGEGAFAQVFEAIHGDVRNAKSEQKCILKVQRPANSWEFYIGMQLMERL-KPEVHHMF--   867
Hs BUB1    LGEGAFAQVYEATQGDLNDAKNKQKFVLKVQKPANPWEFYIGTQLMERL-KPSMQHMF--   574
Ce BUB1    IGQGGYAKVYRAVTDDQR------TVAVKYEVPSCSWEVYICDQMRNRL-KDRGLELMAK   748
              I                       II                III

Sc BUB1    ASAL-----HLFLDESYLVLNYASQGTVLDLINLQREKAIDGNGIMDEYLCMFITVELMKV   820
Mm BUB1    ---IKFYSAHLFKNGSILVGELYSYGTLLNVINLYKNTSEK---VMPQALVLTFAIRMLYM   922
Hs BUB1    ---MKFYSAHLFQNGSVLVGELYSYGTLLNAINLYKNTPEK---VMPQGLVISFAMRMLYM   629
Ce BUB1    CCIMEVMDAYVYSTASLLVNQYHEYGTLLEYANNMKDPNW--------HITCFLITQMARV   801

                       *     *                                   ***
Sc BUB1    LEKIHEVGIIHGDLKPDNCMIRLEKPGEPLGAHYMRNGEDGWENKGIYLIDFGRSFDMTL   880
Mm BUB1    VEQVHSCEIIHGDIKPDNFILGHRFLEQADED----------LATGLALIDLGQSIDMKL   972
Hs BUB1    IEQVHDCEIIHGDIKPDNFILGNGFLEQDDED---------DLSAGLALIDLGQSIDMKL   680
Ce BUB1    VKEVHASKIIHGDIKPDNFMITRKIDDKWGKD-----ALMSNDSFVIKIIDWGRAIDMMP   856
                          VI                                VII

                             *             *   *
Sc BUB1    LPPGTKFKSNWKADQQDCWEMRAGKPWSYEADYYGLAGVIHSMLFGKFIETIQLQNGRCK   940
Mm BUB1    FPKGTVFTGKCETSGFQCPEMLSNKPWNYQIDYFGVAATIYCMLFGSYMKVKNEGGVWKP  1032
Hs BUB1    FPKGTIFTAKCETSGFQCVEMLSNKPWNYQIDYFGVAATVYCMLFGTYMKVKNEGGECKP   740
Ce BUB1    L-KNQRFKGRAGTEAFDCPEMVDGRSWTYQADYFGFAATMAVVVAGKYAQLTGASVGDYS   915
                              VIII        IX

                                              *
Sc BUB1    LKNPFKRYWKKEIWGVIFDLLLNSGQASNQALPMTEKIVEIRNLIESHLEQHAENHLRNV  1000
Mm BUB1    EGLFRRLPHLDMWEEFFHIMLNIPDCHNLPSLDFLRQNMKKLLEQQYSNKIKTLRNRLIV  1042
Hs BUB1    EGLFRRLPHLDMWNEFFHVMLNIPDCHHLPSLDLLRQKLKKVFQQHYTNKIRALRNRLIV   800
Ce BUB1    LNVDIKRRNILRDACYDVINRFLNIPSCDSLPDWNILIKSFSEIWNEKFEASGWRQAVSK   975
                 X                                     XI
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FIG. 1. Predicted protein sequence alignments. (**A**) Diagram showing the position and extent of the carboxy-terminal kinase domains and the N-terminal motif with homology to Sc MAD3. Sc BUB1p, Sc MAD3p, and Ce BUB1p predicted proteins are full length; Mm BUB1p, Hs BUB1p, and Rn BUB1 are partial sequences, as described in the text. (**B**) The N-terminal motif blocks aligned by MACAW (BLOSUM80). The consensus displays the invariant amino acids in 14 of the 108 positions, with amino acids that exhibit a single mismatch indicated by an asterisk. Identical amino acids are highlighted in boldface. The two homology blocks shown are separated by 2–7 amino acids. The N-terminal limit of known sequence from the rat protein prevents its alignment in the first homology block. (**C**) The kinase domain blocks aligned by BLAST search results, with extension by visual inspection of surrounding sequence. Conserved kinase signature residues (Hanks *et al.*, 1988) are indicated by asterisks, with roman numerals indicating the positions of recognized subdomains. Identical amino acids are highlighted in boldface.

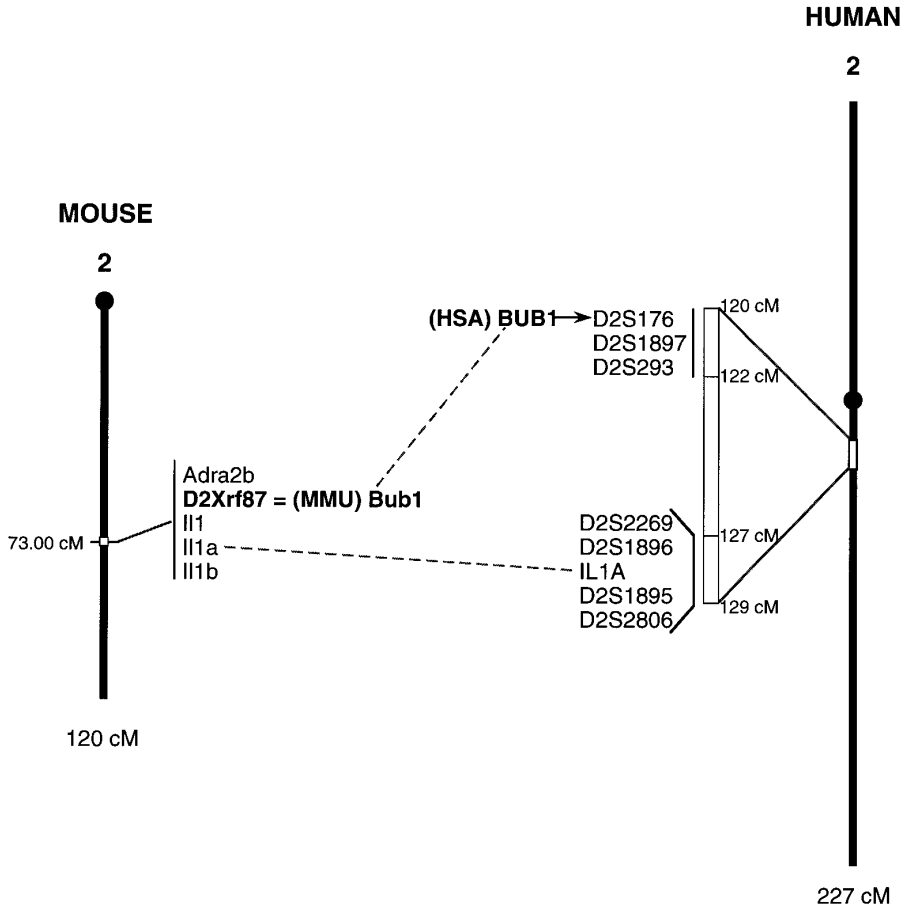


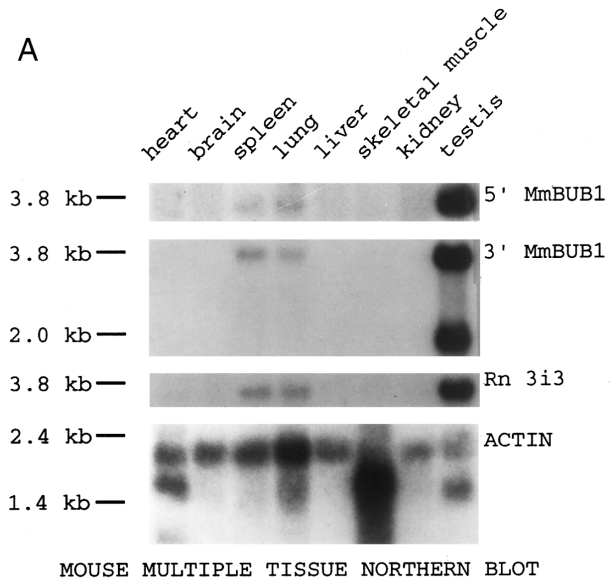
FIG. 2. Relative map positions of BUB1 in the mouse and human genomes. Mouse BUB1, originally mapped as D2Xrf87, is found at a distance of 73.0 cM from the centromere in the Mouse Genome Database chromosome 2 map (www.informatics.jax.org/encyclo.html). Human BUB1 is linked to D2S176 at a distance of 59.0 $cR_{10,000}$ (lod score 3.6) on the Stanford Human Genome Center radiation hybrid human map (www-shgc.stanford.edu/Mapping/). The mammalian BUB1 genes map to an interval of known synteny that includes IL1A and falls within the human cytological region 2q11–2q21. Centimorgan positions on human chromosome 2 were extrapolated from the human gene map at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/SCIENCE96/).

The Stanford G3 radiation hybrid mapping panel was used to determine a human subchromosomal location directly, using PCR primers amplifying a 1.9-kb product from the extreme 3′ end of human BUB1. Resolution of the Stanford G3 panel places markers within "bins" of approximately 500-kb average size (Schuler *et al.*, 1996). Analysis of the results using the Stanford Human Genome Center Web server indicated a position for human BUB1 on human chromosome 2 in the bin containing the Généthon framework marker D2S176 at a distance of 59 $cR_{10,000}$ (lod score 3.6). This localization is at the "second-tier" level provided by the Web server and therefore does not provide unambiguous ordering with respect to nearby markers. However, it is noteworthy that these results are consistent with the cytological location indicated by the analysis of synteny with mouse, as depicted in Fig. 2.

Expression of mammalian BUB1 mRNA in adult tissues. The distribution of accumulated murine BUB1 mRNA in adult tissues was determined by Northern blot analysis. Using probe containing the terminal 659 nucleotides of the mouse cDNA, we detected a single 3.8-kb mRNA species in spleen, lung, and testis, with an additional 2-kb species found only in testis (Fig. 3A). The representation of both testis transcripts was clearly much higher than that of spleen and lung. A significant accumulation of mRNA in mouse testis is consistent with the original report describing isolation of the A005 cDNA PCR fragment in a screen for testis-enriched gene products (Kerr *et al.*, 1994).

The nature of the 2-kb testis transcript was investigated further. A 5′ probe from the mouse BUB1 cDNA (nucleotides 1 to 421 of MT10) detected only the 3.8-kb species, indicating that the 2-kb transcript in testis does not contain the same 5′ end as the larger product. Because the 3′ probe clearly identified a single genetic locus in mouse, the 2-kb band must represent a transcript derived from an alternative start site or alternative splicing event.

We found a similar tissue distribution of expression of human BUB1 mRNA (Fig. 3B). The highest levels observed were in testis and thymus, and weaker hybridization was visible in colon and small intestine. Very faint signals from spleen and ovary were some-



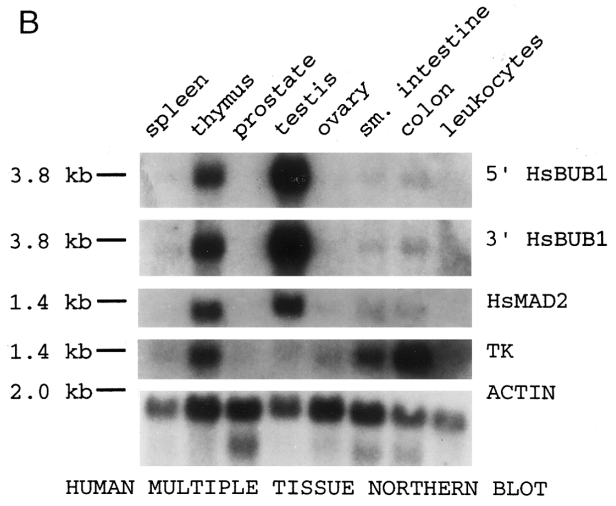


FIG. 3. Multiple-tissue Northern blot analysis. (**A**) A Northern blot filter containing 2 μg poly(A)-selected mRNA from the mouse tissues indicated was sequentially hybridized with the random-primed cDNA probes indicated. Hybridization, wash, and exposure conditions are described in detail under Materials and Methods. (**B**) A Northern blot filter containing 2 μg poly(A)-selected mRNA from the human tissues indicated was similarly hybridized sequentially with multiple probes as indicated. For both filters, a human actin probe provided by the manufacturer provided a positive control for the presence of mRNA in each lane.

times detected. Unlike the case in mouse, both 3′ and 5′ cDNA probes generated from human BUB1 hybridized to a single species in testis.

To address whether the observed tissue distribution is a property specific to mammalian BUB1 or a more general property of spindle assembly checkpoint gene products, we hybridized the same human Northern blot with a full-length cDNA encoding human MAD2. This analysis revealed that human BUB1 and MAD2 genes share a strikingly similar pattern of expression (Fig. 3B). While detectable in colon, small intestine, spleen, and ovary at the film exposure shown, human MAD2 was again most strongly expressed in human thymus and testis.

An mRNA encoding a mitotic surveillance function might be expected on theoretical grounds to exhibit broad representation in tissue samples containing mitotically active cells. To address the question of whether the observed tissue distribution could result simply from an abundant representation of cycling cells in the thymus and testis sampled on the commercial blot, we probed the human Northern filter with human cytoplasmic thymidine kinase (TK1) cDNA (Kaufmann and Kelly, 1991). The cytoplasmic protein is representative of a class of enzymes involved in DNA precursor biosynthesis, the activity of which is observed to decline rapidly as cells from several vertebrate systems withdraw from the cell cycle in response to serum starvation or contact inhibition (Johnson *et al.*, 1982; Travali *et al.*, 1988; Ito and Conrad, 1990) or during embryonic differentiation (Gross *et al.*, 1987). Although cytoplasmic thymidine kinase mRNA levels fluctuate within an approximately twofold range in each mitotic cell cycle (Sherley and Kelly, 1988), the message is dramatically decreased as cells exit proliferation to enter quiescent or differentiated states (Gross *et al.*, 1987; Ito and Conrad, 1990; Travali *et al.*, 1988). With the simple assumption that the cellular requirement for TK activity in support of DNA replication does not vary greatly by cell type, the clear prediction is that the level of TK mRNA in a given tissue should reflect the representation of proliferating cells.

The human multiple-tissue Northern blot filter was hybridized with a TK1 probe to determine tissue distribution of positive signal. A qualitative evaluation of relative band intensities (Fig. 3B), comparing human BUB1 and TK1 mRNA levels at similar film exposures, strongly supports the conclusion that tissue distribution is generally coincident on these filters, with the interesting exceptions of testis (in which the human BUB1 mRNA level is high) and colon (in which the human BUB1 mRNA level is low). These results suggest two possibilities. If BUB1 mRNA levels fluctuate appreciably in the cell cycle, then differential band signal might indicate tissues that contain greater or smaller numbers of cells at specific cell cycle positions. Alternatively, the pattern of human BUB1 expression in different tissues may be indicative of a function distinct from a simple role in proliferating cells.

Rat BUB1 transcription is increased in Rat1a lines overexpressing human MYC. c-Myc is an oncogenic transcription factor involved in the processes of cellular proliferation, differentiation, and apoptosis. Abnormal regulation of c-Myc has been observed in Burkitt's lymphoma and other neoplasias (Dang and Lee, 1996). Rat1a fibroblasts exhibit c-Myc-dependent cell transformation, gaining the ability to grow as unattached colonies in soft agar (Stone *et al.*, 1987). To identify genes contributing to this c-Myc-mediated transformation phenotype, a cDNA representational difference analysis (RDA) screen was performed to compare the

parental (Rat1a) and the transformed (Rat1a-myc) rat fibroblast lines when grown in an unattached state on soft agar (Lewis *et al.*, 1997). While cDNAs with differential representation should be enriched for genes contributing to the transformed phenotype, gene products supporting the more permissive proliferation state of Rat1a-myc cells are also expected.

One of the novel sequences identified from this screen (3i3; GenBank U83666) shared highly significant homology with the N-terminal conserved motif of the mammalian BUB1 proteins, exhibiting 44% identity and 66% similarity with mouse BUB1p (Fig. 1B) across this region. To test further whether this 496-nucleotide fragment was derived from a rat BUB1 gene, we performed cross-species hybridization to the mouse multiple-tissue Northern blot. The rat probe identified only a single band, with size and tissue distribution identical to the higher molecular weight mRNA of mouse BUB1 (Fig. 3A). Therefore, we conclude that the rat 3i3 sequence indeed represents a segment of the rat BUB1 gene.

The mRNA accumulation in c-Myc-transformed cells implied by RDA recovery of rat BUB1 in the original screen was verified by Northern blot analysis of total mRNA from Rat1a and Rat1a-myc cell lines cultured with or without anchorage to the substratum. Quantitative analysis of rat BUB1 mRNA levels (Fig. 4) indicates a 7.9-fold elevation in the rat BUB1 hybridization signal in Rat1a-myc cells grown on soft agar. For comparison, the mRNA for lactate dehydrogenase A, a direct transcriptional target subject to upregulation by c-Myc (Tavtigian *et al.*, 1994; Shim *et al.*, 1997), was detected at levels 6-fold higher in the Rat1a-myc cell line in a similar experiment (Lewis *et al.*, 1997). Rat1a cells overexpressing the human oncogene ras (Rat1a-ras; Q. Li and C. V. Dang, unpublished results) also exhibit the soft agar growth transformation phenotype. Rat BUB1 mRNA exhibits a 5.3-fold elevation in Rat1a-ras cells, indicating that the increase in accumulation of rat BUB1 mRNA is not c-Myc specific and may simply correlate with proliferative state of the cells assayed. Whether expression of the BUB1 gene plays a role in the transformation process in this system as well is a question that remains to be directly addressed.

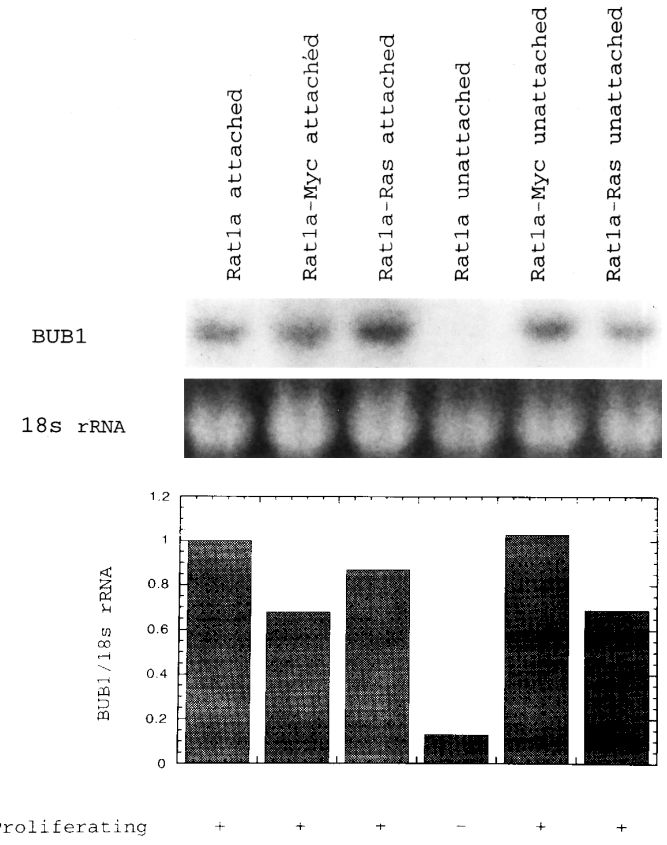


FIG. 4. Correlation of rat BUB1 mRNA accumulation with proliferation status of cultured fibroblasts. Rat1a, Rat1a-myc, and Rat1a-ras cells were cultured under conditions allowing attachment to the culture dish substratum (left three lanes) or in the presence of an agar layer preventing attachment (right three lanes). The fraction of proliferating cells in each culture was determined by BrdU incorporation and flow cytometry. The presence of 25–45% incorporating cells is indicated by a plus sign, whereas the presence of less than 5% incorporating cells is indicated by a minus sign. The autoradiographic signal indicating the levels of rat BUB1 mRNA (**top**) was quantitated by phosphorimager analysis. Quality and amount of total RNA loaded into each lane was independently determined by analysis of ethidium bromide staining of the 18S rRNA using NIH Image software. Ratios of these values are shown in the histogram, using ratios of Rat1a attached cells as baseline. Similar results were obtained from analysis of rat BUB1 mRNA in comparison to vimentin, RPL, and GAPDH mRNA hybridization signals (data not shown).

DISCUSSION

Analysis of the cellular response to spindle damage indicates that conserved functions can be identified in comparisons between organisms as divergent as mammals and budding yeast (reviewed in Rudner and Murray, 1996; Chen *et al.*, 1996; Li and Benezra, 1996; Taylor and McKeon, 1997). Recent rapid growth of the sequence databases, in particular the mammalian segment of dbEST and the large-scale sequence efforts directed at model organism genomes, has greatly facilitated the identification of conserved genes exhibiting sequence similarity suggestive of homologous function (see, e.g., Waterston and Sulston, 1995; Miklos and Rubin, 1996; Rubin, 1996; Bassett *et al.*, 1997; Botstein *et al.*, 1997). In the past year, vertebrate proteins with both structural and functional homology to two budding yeast genes have been described: MAD2 from human (Li and Benezra, 1996) and frog (Chen *et al.*, 1996) and BUB1 from mouse (Taylor and McKeon, 1997). In each case, results from intervention in experimental cell culture or cell extract systems strongly support conservation of function for these proteins. Here we report sequence data on candidate orthologous BUB1 genes from budding yeast, nematode, mouse, rat, and human genomes; map positions for human and mouse BUB1 genes; and expression data in human and mouse tissues.

How well does the sequence similarity of these related proteins in multiple organisms support the hypothesis that these represent true orthologues? At the

level of sequence analysis, yeast and mouse predicted proteins provide the most informative comparison, exhibiting 55.7% similarity over 1078 amino acids with strong conservation of a motif at the amino-terminus and a distinctive kinase domain at the carboxy-terminus. Moreover, when the mouse polypeptide was used to search the GenBank nonredundant protein database using the BLAST algorithm, yeast BUB1p was the best match with a *P* value 13 orders of magnitude lower than all other matches. GenBank NRDBp currently contains all *Saccharomyces cerevisiae* proteins identified by the full genomic sequence of this organism (Goffeau *et al.*, 1996). Therefore, yeast BUB1p clearly provides the most significant match to murine BUB1 within the entire budding yeast protein repertoire. While sequence comparisons of this kind cannot rigorously rule out the existence of another highly similar mouse protein yet to be described, the simplicity of the results from moderate-stringency Southern blot hybridization utilized in the mapping analysis argue against the presence of highly related gene family members in mammals. These facts taken together with the evidence for functional conservation between the mouse and the budding yeast proteins (see below) comprise a compelling argument in support of an orthologous relationship.

Evidence for a mitotic checkpoint function in cell culture has recently been presented for the mouse BUB1 gene product (Taylor and McKeon, 1997). Analysis of subcellular localization of BUB1 protein by immunofluorescence revealed an accumulation coincident with the positions of chromosomal kinetochores in prophase and prometaphase cells. This staining was no longer evident in metaphase or anaphase, consistent with a previous result reported for another conserved mammalian checkpoint protein, HsMAD2 (Li and Benezra, 1996; see also Chen and Murray, 1996). A determinant of kinetochore localization that mapped within the N-terminal third of the predicted mouse protein provided a dominant negative activity when overexpressed in HeLa cells. The mutant phenotypes observed included diminished premitotic accumulation of cells treated with microtubule poison, accelerated entry into mitosis in untreated cells, and protection from the apoptosis that normally ensued upon prolonged mitotic arrest of this cell type. In this context, we note that the highly homologous protein kinase from the *C. elegans* genome sequence lacks the N-terminal motif. Perhaps this reflects a difference in the organization of centromere-associated proteins in holocentric mitosis (see Albertson *et al.*, 1997). In addition, the 2-kb variant species of mRNA we have observed in mouse testis may indeed encode a similar protein kinase lacking the N-terminal domain.

We find that the distribution of accumulated BUB1 mRNA in mouse and human tissues shows conservation, exhibiting a markedly abundant representation of mRNA in testis. Human thymus also displayed a very high level of accumulated BUB1 mRNA (mouse thymus was not assayed), while transcripts were detected at much lower levels in other tissues at this film exposure. In concurrent experiments, we observed that the abundance of BUB1 mRNA is correlated with proliferation status of rat fibroblast cell lines. Indeed, the relative levels of human BUB1 expression roughly coincided with expression levels of a marker for cell proliferation, consistent with a role in a conserved mitotic checkpoint function required by cycling cells. The notable exceptions occurred in testis (which exhibited a striking BUB1 transcript abundance) and colon (which exhibited a relative paucity). We propose two hypotheses. In one scenario, these observations may reveal the presence of BUB1 mRNA regulation within mitotic cell cycles *in vivo*. For example, a segment of the cell cycle in which BUB1 mRNA is more abundant may be elongated in expressing cells of testis or shortened in cells from the colon. Alternatively, relative abundance of BUB1 mRNA may reflect a auxiliary role in some cell types. For example, the abundance of BUB1 mRNA in testis raises the possibility of an as yet uncharacterized meiosis-specific role. Interestingly, similar arguments must apply to the expression of human MAD2, as the mRNA distribution is coincident with that of BUB1.

A major advantage of establishing the map position of phenotypes and gene sequences early in their characterization is the ability to make the connection between them when they are generated in separate studies. Biochemical characterization of the yeast BUB1 gene product demonstrates that this protein acts as a serine–threonine protein kinase *in vitro* (Roberts *et al.*, 1994), and the biological functions affected in mutants are the spindle assembly checkpoint and chromosome stability (Hoyt *et al.*, 1991; Wang and Burke, 1996; Pangilinan and Spencer, 1996). Early characterization of human MAD2 and mouse BUB1 proteins has engendered discussion of candidate roles for these proteins in tumor development (Li and Benezra, 1996; Chen *et al.*, 1996) and protection from apoptosis (Taylor and McKeon, 1997). Clearly these may be related. The correlation of rat BUB1 mRNA accumulation and cell transformation may be consistent with this trend in candidate functions and warrants further consideration. In view of the hypothesis for a role in tumorigenesis, it is of interest that we find virtual identity between the protein predicted by BUB1 cDNA from normal mouse testis and the independently described predicted protein from a teratocarcinoma cell line (Taylor and McKeon, 1997).

Knowing the positions of BUB1 genes in both mouse and human genomes allows evaluation of phenotypes studied in both highly active genetics communities. For example, there are two candidate phenotypes of outstanding interest in human 2q11–q21 annotated as VIS1 (viral integration site 1; MIM 164755) and LCO (liver cancer oncogene; MIM 165320). Each of these genes has been described as having a potential role in neoplasia (Askew *et al.*, 1991; Ochiya *et al.*, 1986; Tokino *et al.*, 1988). Neither of these loci appears to correspond to human BUB1 upon further investigation. The human candidate oncogene VIS1 is clearly distinct

from human BUB1 because the map positions of the corresponding murine loci are separated by 44 cM. To evaluate human LCO, we obtained available 5′ gene sequence information (unpublished data kindly provided by T. Ochiya), which did not reveal significant similarity to murine BUB1 cDNA. Because the extant predicted sequence for murine BUB1 mRNA may not be full length, we have ascertained the position of LCO in the Stanford G3 radiation hybrid panel map for direct comparison with the location of human BUB1. LCO is tightly linked to the marker SHCG-14558 (0 $cR_{10,000}$ distance, lod score 1000), approximately 15 cM away from human BUB1, excluding LCO as a strong candidate phenotype for BUB1 defects. Other mapped phenotypes in the vicinities of the mouse and human BUB1 genes remain as candidates to be evaluated.

In summary, we have presented characterization of cDNA sequences related to yeast spindle assembly checkpoint gene BUB1, and we argue that these are highly likely to represent orthologues in human, mouse, rat, and nematode. The highly significant and extensive sequence similarity, the conservation of mRNA representation in multiple-tissue Northern blot analysis, and the absence of related genomic loci detectable by blot hybridization make a strong case for predicting orthologous function of the mammalian proteins. The murine and human genes have been placed in the public maps, which will facilitate evaluation of their roles in mammalian biology by interested laboratories. Detection of differential representation of the rat BUB1 transcript in cell lines that undergo a single-step transformation process provides a format in which to pursue the relationship between spindle assembly checkpoint function and cell proliferation control. Taken together, these results provide fundamental tools for molecular and genomic analysis of mammalian BUB1 genes and for the design of future experimental approaches addressing the *in vivo* role of the highly conserved BUB1 protein in mammalian systems.

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