

The Microtubule Binding Domain of Microtubule-associated Protein MAP1B Contains a Repeated Sequence Motif Unrelated to that of MAP2 and Tau

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Abstract. We report the complete sequence of the microtubule-associated protein MAP1B, deduced from a series of overlapping genomic and cDNA clones. The encoded protein has a predicted molecular mass of 255,534 D and contains two unusual sequences. The first is a highly basic region that includes multiple copies of a short motif of the form KKEE or KKE_V that are repeated, but not at exact intervals. The second is a set of 12 imperfect repeats, each of 15 amino acids and each spaced by two amino acids. Subcloned fragments spanning these two distinctive regions were expressed as labeled polypeptides by translation in a cell-free system *in vitro*. These polypeptides were tested for their ability to copurify with unlabeled brain microtubules through successive cycles of polymeriza-

tion and depolymerization. The peptide corresponding to the region containing the KKEE and KKE_V motifs cycled with brain microtubules, whereas the peptide corresponding to the set of 12 imperfect repeats did not. To define the microtubule binding domain *in vivo*, full-length and deletion constructs encoding MAP1B were assembled and introduced into cultured cells by transfection. The expression of transfected polypeptides was monitored by indirect immunofluorescence using anti-MAP1B-specific antisera. These experiments showed that the basic region containing the KKEE and KKE_V motifs is responsible for the interaction between MAP1B and microtubules *in vivo*. This region bears no sequence relationship to the microtubule binding domains of kinesin, MAP2, or tau.

MICROTUBULES prepared *in vitro* by successive cycles of assembly and disassembly consist largely of α - and β -tubulin, together with a number of other proteins that are collectively defined as microtubule-associated proteins (MAPs)¹ (for reviews, see 32, 35, 52). Because neuronal cells are rich in microtubules, the best characterized MAPs are those from brain. Conventionally, these MAPs have been classified according to their molecular mass: the high molecular mass MAPs, MAP1A, MAP1B, MAP1C, and MAP2 (in the range 200–350 kD), and the low molecular mass MAPs, or tau proteins, a heterogeneous group of proteins in the range 35–40 kD (51). MAP3 and MAP4 are less abundant than these; MAP3 is primarily expressed in astroglia and some neurons (21), while MAP4 is expressed in glia, vascular, and some other tissues (37). With the exception of MAP1C, which has been identified as cytoplasmic dynein (38, 49) the function of brain MAPs is essentially unknown, though many have been shown to promote microtubule assembly upon addition to purified tubulin *in vitro*.

Among the high molecular mass MAPs, MAP1B (7) (which is referred to variously as MAP1.2 [16], MAP1(X) [9], and MAP5 [40]) differs from the others in its high abundance, its prominence both in association with microtubules

and in soluble form, and the relative inefficiency with which it cycles with tubulin, particularly during the first cycle. Nonetheless, MAP1B is generally regarded as an authentic MAP because it does cocycle with purified tubulin and promote microtubule assembly *in vitro* (40) and because antisera specific for MAP1B (both polyclonal and monoclonal) identify characteristic microtubule networks in neuronal (and other) cells (6, 12). MAP1B is expressed in the axons and dendrites of neurons, as well as in glia and other cells. Because it is especially prominent in axons during their initial outgrowth (40, 43), it has been suggested that MAP1B plays a role in neurogenesis.

Several years ago, we isolated a set of cDNA clones encoding a portion of a MAP1 subspecies (28). Subsequently, an antiserum (3d2) raised against a fusion protein derived from one of these clones was shown to uniquely recognize MAP1B on Western blots of brain microtubules and of total protein from PC12 cells (1). The same antiserum also gives a staining pattern identical to that of other anti-MAP1B antisera on brain sections (A. Matus and N. J. Cowan, unpublished observations), thereby proving that our original set of cDNA clones were derived from MAP1B mRNAs. We have now obtained two genomic clones and further overlapping cDNA clones that together encode the entirety of MAP1B. Here we report the complete sequence of MAP1B, deduced from these clones. The encoded protein has a molecular mass of 255,

1. Abbreviation used in this paper: MAP, microtubule-associated protein.

534 D and contains two unusual sequences. One is a set of 12 imperfect repeats that do not occur in any other sequenced protein, each 15 amino acids long, and spaced by 2 amino acids. The other is a highly basic region with many copies of the sequence KKEE and KKE_V, repeated but not at fixed intervals. We show that it is this latter region which is responsible for the binding of MAPIB to microtubules both in vitro and in vivo.

Materials and Methods

cDNA and Genomic Clones

cDNA from 5-d-old mouse brain mRNA was prepared as described (26), except that half was ligated to Eco RI linkers (Boehringer Mannheim Biochemicals, Inc., Indianapolis, IN) and half to Xba I linkers, and each portion cloned into the vector λGem (Promega Biotec, Madison, WI). The resulting libraries were screened (4) as described in the text, using purified cDNA restriction fragments ³²P-labeled by nick translation (41). Hybridizing clones were subcloned into pUC, restriction-mapped, and, in some cases, sequenced. Sequencing of the complete set of overlapping clones on both strands was accomplished by subcloning bal31-generated fragments (27) into M13, and using the dideoxy chain terminator method (42). Four overlapping genomic clones containing the 5' end of the MAPIB gene were obtained by screening a mouse genomic library in the EMBL4 vector, kindly provided by P. D'Eustachio (New York Medical Center). The cosmid clone C (Fig. 1), including the 6-kb MAPIB exon, was isolated by screening a library (provided by S. Tonegawa, Massachusetts Institute of Technology) with clone 141A (Fig. 1).

Constructs for Translation, Fusion Proteins, and Transfection Experiments

Two constructs encompassing repeated sequences were made for in vitro transcription, translation, and cycling experiments as follows: (a) clone 72 was digested with Xho I, blunt-ended with the Klenow fragment of DNA polymerase I, cut with Bgl II, and the coding fragment (Fig. 1, 5' arrow) cloned into the transcription vector pGEM3 (Promega Biotec) cut with Hinc II and Bam HI; (b) clone 141A was digested with Pst I and Eco RI, and the purified fragment (Fig. 1, 3' arrow) cloned into pGEM3 cut with the same restriction enzymes. For both constructs, an in-frame translational start signal was provided by the pGEM vector. The insert from the first of these pGem clones, including the initiator AUG, was cloned into a pSV vector (34) for expression in cultured cells (Fig. 1, construct X). In addition, constructs were made from the 5' and 3' end of the set of MAPIB cDNAs to place the

translational start and stop signals: (a) the insert from clone 12X was subcloned into the Xba I site of pGEM1; and (b) the Sac I/Eco RI fragment was cloned into pGEM3 cut with Acc I, blunt ended with Klenow and then digested with Eco RI; this procedure supplied an AUG codon for translational initiation in frame with the MAPIB coding sequence.

The full coding sequence of MAPIB was assembled, first by joining clones 12X and 1R at an internal Pflm I site, then by digesting the resulting construct with Xho I and Eco RI, and finally by adding both the Xho I/Ssp I fragment from cosmid clone C and the Ssp I/Eco RI fragment from clone 166 in a three-way ligation. The clones and cloning sites are depicted in Fig. 1. The full cloning region was subcloned into a pSV vector using a unique Sal I site in the 5' flanking pUC polylinker, and a unique Dra I site in the 3' untranslated region. Deleted versions were obtained by (a) joining a Msc I site to a Pvu I site blunt ended with T4 DNA polymerase (Fig. 1, 1B-H); (b) deleting an ~1-kb Msc I fragment by self-ligating a gel purified fragment from a partial Msc I digest (Fig. 1, 1B-K); and (c) by joining the Xho I and Bg II sites, by blunt ending each with the Klenow fragment of DNA polymerase I (Fig. 1, 1B-X).

To raise an antiserum specific for the second set of repeats, a Pst I/Eco RI fragment (Fig. 1, 3' arrow) from clone 141A was subcloned into a pATH vector (29) that was used to express a fusion protein whose amino terminal part is *Escherichia coli* TrpE, and whose carboxyterminal part is derived from MAPIB. Fusion protein excised from SDS polyacrylamide gels was used to immunize rabbits. The resulting serum is called YXY; its specificity is demonstrated in Fig. 7.

Transcription, Translation, and Microtubule Cycling Experiments

pGEM subclones were linearized at the 3' end and transcribed in vitro using SP6 polymerase as described (33). The capped mRNAs were translated in rabbit reticulocyte lysate (Promega Biotec) supplemented with either [³⁵S]methionine or [³H]lysine. Translation products were resolved on SDS polyacrylamide gels, which were fluorographed, dried, and exposed to film. Microtubule cycling experiments were performed as described (30).

Hybrid Selection and Primer Extension

Clone 5X (Fig. 1) was used to select MAPIB-specific mRNA from total mouse brain RNA (100 μg/ml/2.1-cm nitrocellulose filter) by methods described previously (17). The selected RNA was used in a primer extension reaction containing an antisense oligonucleotide, 5'CCTGAGAGAAGT-GTTCCT3' (corresponding to nucleotides 17-34 of the sequence shown in Fig. 4), ³²P-end labeled with polynucleotide kinase. Reaction conditions and analysis of the extended product were as described (3).

Transfection Experiments and Immunofluorescence

Hela cells were grown in DME supplemented with 5% FCS (Hyclone

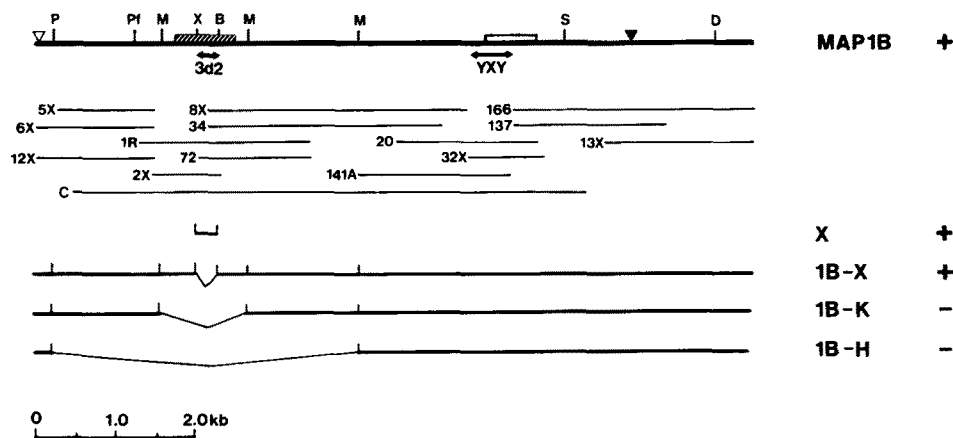


Figure 1. MAPIB clones and constructs. The top line is a composite map of our set of MAPIB clones. The hatched and open rectangles represent regions encoding the KKEE/KKE_V repeated motifs and the 15-amino acid repeats, respectively (see Fig. 4). The start codon (▽) and stop codon (▼) are indicated, as are some salient restriction sites: P, Pvu I; P₁, Pflm I; M, Msc I; X, Xho I; B, Bgl II; S, Ssp I; D, Dra I. The arrows indicate fragments subcloned for in vitro cycling experiments, and for the generation of antisera 3d2 (1) and YXY (see Materials and Methods). Thin lines show the extent of cDNA and genomic clones which span the coding region of MAPIB. Clones with names ending in X came from an Xba I-linked cDNA library, while those with names ending in R came from an Eco RI-linked cDNA library (see Materials and Methods). Other clones have been previously described (28). Deletion constructs created for transfection experiments are indicated by heavy lines, and the results of these experiments are shown at the right: +, microtubule binding; -, no microtubule binding.

+

Laboratories, Logan UT) in a 10% CO₂ atmosphere; 3T3 cells were grown under the same conditions, except 10% FCS was used. Cells growing on glass coverslips were transfected with DNA by the procedure of Chen and Okayama (10), and after 72 h fixed in one of three ways: (a) with paraformaldehyde (36); (b) with cold methanol for 6 min; or (c) with cold methanol following detergent extraction with 0.2% Triton X-100 in a microtubule stabilizing buffer (47). Immunofluorescence was performed using one of the two rabbit anti-MAP1B antisera, 3d2 (1) or YXY (this paper). The regions of MAP1B to which these sera bind are shown in Fig. 1. A mouse anti- α -tubulin monoclonal antibody (Amersham Corp., Arlington Heights, IL) was used for double labeling; FITC-conjugated goat anti-rabbit and rhodamine conjugated goat anti-mouse second antibodies were from Boehringer Mannheim Biochemicals. All antibodies were used at a dilution of 1:500.

Results and Discussion

Isolation of Clones Encoding MAP1B

With a view to elucidating the structural and functional domains of MAP1B and its relationship to other MAPs, we first sequenced our original set of overlapping MAP1B-encoding cDNA clones. This analysis showed that the set of clones contains a long open reading frame of 5.4 kb, extending from the 5' end of the overlapping set to a location 1.5 kb from the 3' end. Because this open reading frame encodes a polypeptide of at most \sim 198,000 D, we extended our collection of overlapping cloned cDNAs in the 5' direction by further "walking" experiments. To facilitate the search for overlapping clones, we constructed two mouse brain libraries using different linkers for cloning. These were first screened with a small fragment from the 5' end of our 5' most overlapped cDNAs (clone 72; reference 28), and twice more, each time with fragments obtained from the new set of clones. Representative examples from the complete set of overlapped cDNA clones are depicted in Fig. 1.

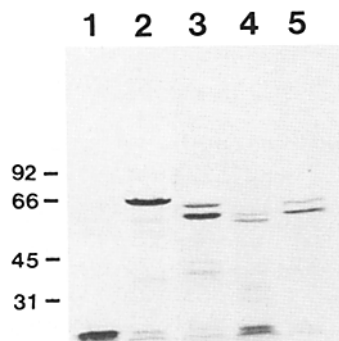


Figure 2. Determining the placement of the start and stop codons within the MAP1B transcript. Fragments from the 5' and 3' end of our MAP1B cDNA set were cloned into transcription vectors; capped RNA was synthesized and translated in a cell free system containing [³⁵S]methionine (see Materials and Methods). The resulting labeled translation products were resolved on an 8.5% SDS polyacrylamide gel. Lane 1, no RNA control; lane 2, RNA from clone 12X (nucleotides 1-1,574); lane 3, RNA from clone 137, linearized with Eco RI (Fig. 4, nucleotide 6,304-3' end of clone); lane 4, RNA from clone 137 linearized with Kpn I (Fig. 4, nucleotides 6,303-7,333); lane 5, RNA from clone 137 linearized with Nae I (Fig. 4, nucleotides 6,303-7,454). In lanes 3-5, the slowest migrating species results from initiation at an AUG triplet provided by the vector (see Materials and Methods), whereas the second prominent band presumably results from initiation at an internal AUG codon.

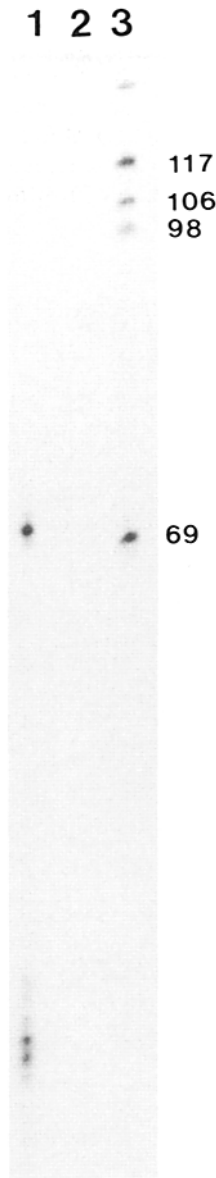


Figure 3. The MAP1B transcriptional initiation site lies 35 nucleotides upstream from sequences represented by overlapping cloned cDNAs. A synthetic antisense oligonucleotide corresponding to nucleotides 17-34 of the sequence shown in Fig. 4 was end-labeled with polynucleotide kinase and used in a primer extension reaction with RNA hybrid selected with clone 5X (Fig. 1) (see Materials and Methods). The reaction products were analyzed on a 6% polyacrylamide sequencing gel. Lane 1, selected RNA; lane 2, control (*E. coli* tRNA); lane 3, molecular size markers (shown, in nucleotides, right).

All attempts to extend this cloned set further in the 5' direction resulted in the isolation of numerous unrelated and nonoverlapping sequences that were presumed to be cloning artifacts generated by the unavoidable adventitious ligation of unrelated cDNA sequences during construction of the cDNA library, or by "hairpinning" of cDNA generated during the synthesis of the second strand. Sequence analysis of the overlapping 5' cDNA clones extended the long open reading frame to 7.5 kb. However, because this open reading frame extended to the extreme 5' end of our overlapped cDNAs, we cannot be certain whether the first AUG triplet indeed encodes the initiator methionine residue; it is possible that further upstream sequences encoding additional aminoterminal MAP1B residues might be unrepresented in our cDNA libraries. However, we did three further experiments which strongly suggest that translation of MAP1B does begin at the first AUG triplet found in our cDNA clones. Firstly, a fragment extending from the extreme 5' end of the overlapped cDNAs (Fig. 1) was cloned into a vector

AGAGAGCGCGGAGAGAACTTCTCTCAGCGTTGACAGAGCGCGGAGAGGCGCA 40
 H A T 3
 CGTGGTGGTGAAGACCCAAACCGGAGCCATCGGCGAGCATCGCAACCCGCGCGCT 120
 V V V E A T E P E P B S B I G N P A A S 23
 CCACCTCGCCAGCCTTCCGACCGCTTCCGAGCAGCAAGTCTACTTCTGCTGGTGG 180
 T B P S L S H R F L D S K F Y L L V V V 43
 TCGCGAGACCGTAACCGAAGACCGTGGAGCGCCATCGGCAACATCGAGCTGGGGA 240
 G E T V T E E H L R R A I G N I E L G I 923
 TCGCATCTGGGACCAAACTGATGAGTCAACTTGGACGAGAACTCAAACTTTTCG 300
 R B W D T N L I E C M L D Q E L K L F V 83
 TATCTCGACACTCTGCAAGATCTCTCTGAACTTCCAGGACCAAAAGATTCTTCAACC 340
 S R H B A R F S P E V P G Q K I L M H R 103
 GAAGTGACTCTTGAAGAACTGTTCTGATCAACCTTTCAGATGAAGCCCTCAGCACTE 420
 S D V L E T V V L I N P B D E A V B T E 123
 AGTGGCTTGTGATCACTGACGCTGCCGCCATAAATGCTGGTCTCCCGGACAGT 480
 V R L M I T D A A R M K L L V L T G Q C 143
 GCTTTGAGAACTCGGAGCTCATCTGCAAGTCTGGCTTTCTCTTCCAGAACTTCA 540
 F E N T G E L I L Q G S G B F B F Q N F I 163
 TAGAGATTTTCAAGATCAAGAGATGTTGATCTTGAAGTCCCAACCCCACTGCAACA 600
 E I F T D Q E I G E L L S T H P A N K 103
 AAGCCAGCCTCACCTCTCTGTCGCCAGGAGGAGCTGGAAGAACTCCAACTTGACA 660
 A S L T L F C P E E G D M K N S N L D R 703
 GACACAATCTCAAGACTTCACTCAAGTCAAGTCAAGTCAAGTCTTCACTTCCAGAAA 720
 H N L Q D F I M I K L N B A S I L P E M 223
 TGGAGGAGCTTCTGAGTTCACCGATATCTCTGAGACTGTGAAGTCCCTCTCCCTC 780
 E G L B E F T V L S E B V E P B P F 243
 TTGACATCTGGAACCCCAACTCGGCGGCTTTGGAAGCTCTCAAGCTTCTGCTTT 840
 D I L E P P T B G G F L K L B K P C C Y 263
 ACATTTCCAGGGGCGCTGGGACTCGGCTGTTTCAAGTGAAGGATTCAGACTG 900
 I F P G G R G D S A L F A V N G F N H L 283
 TCACTCAAGGAGTCAAGGAAAGTCTGCTTCCGAACTCATCGGCACTTGGACC 960
 I N G G S E R K B C T F M K L I R H L D R 303
 GTGTGACTCCATCTGCTCACACATTTGGGATGACAATTTGCTGGATCAATAGCA 1020
 V D S I L L T H I G D D N L P G I N S M 323
 TGTGAGCGCAAGATGCAAGCTGGAAGAGGAGGCTCCAGGGTCCACAGCAACA 1080
 L Q R K I A E L E E R B Q S T S N S 343
 GTGACTGGATAAAACTCATCTCCCTGACTGGGGTGTGTTTCAAGTACTCE 1140
 D M K N L I S P D L G V V F L N V P E 363
 AAAAAGTGAAGAGCCAGCAACCAACTCAAGATGAAGAGAGATAGAGAAGCATGCT 1200
 N L K D P E P N I K M K R S I E E A C F 383
 TCACTCCAGTACTTAAACAACTGCTCAAGTGAAGCAGGCTCTTATGAAAGTGA 1260
 T L Q Y L N K L S M K P L F R B S V G 403
 GCAACCACTTGAAGCTGCTGCTGTTCCAGAAATGGAGTGGGAGGCTTGAAGAT 1320
 N T I E P V I L F Q K H G V G K L E H Y 423
 AGTGGTAAACCAAGTCAAGGAGCAAGGAAATGCAATTTTATGCAAGTGGACTE 1380
 V L N P V K B S K E M Q Y F M Q Q M T G 443
 GCAACCAAGAGCAAGCAACTAATCTTCCCAAGTGGTGAAGTGAAGTCACTCCGA 1440
 T N K D K A E L I C L P G Q E V D I P I 1640
 TTTCTACTTAACTCAGTCTCATCTTGTGTTGTTGTCATCGGCAACCCGCTGAGA 1500
 S Y L T S V B B L I V W H P A N P A E K 483
 AAATACTTGGGTTCTGTTCCGGAACAGTCAAGTCAAGTCAAGTCAAGTCAAGTCA 1560
 I I R V L F P G N S T Q V N I L E G L E 503
 AGAACTCAACATAGATTTCTAAAGCAGCACTGCCCACCCAAAAGATCTCACTE 1620
 K L H L D F L K Q P L A T Q K D L T G 523
 GCGAGTCCGACCCCGCAGTGAAGCAGTCAACTGAAACAGCGGCTGACAGCGAG 1680
 Q V P T P P V K Q V K L K Q R A D S R E 543
 AGATCTGAAGCAGCAAGCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCA 1740
 S L K P A T K P V A B K S V R K E S K E 563
 AGGAAACCCCTGAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCA 1800
 E T P E V T K A T B V E K T P K V E S G A 583
 AAGAGAAAGTGTAGTGAAGAAAGCAAGCTGTAAGACAGAAAGTAACTTCAAGTGA 1860
 E K V L V K K D K P V K T E S K P S V T 603
 CAGAAAAGAGTGTCCAGCAAGGAGCAGTCCCAAGTGAAGTGAAGTGAAGTGAAG 1920
 E K E V S S K E E Q S P V K A E V A E K 623
 AGCAAGCACTGAGGCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAGTCAAG 1980
 Q A T E S K P K V T K D K V V K K E I K 643
 AGCAAAACTCGAGAAAAGAGAGGAGGAGCCCAAGAAAGAGTGTAAAGAGGAG 2040
 T K L E E K K E E K P K K E E V V K K E D 663
 ACAAAACCCCTCAAGAAAGCAGAGGCAAGGAAAGGAGTGAAGAGGAGTCA 2100
 K T P L K K D E K P R K E E V K K E I K 683
 AGAAAGAACTCAAAAGAGAGAGAGGAGTGAAGAGGAGTGAAGAGGAGTCA 2160
 K E I K K E E R K K E E V K K E I K 703
 CCTGAGAGTCCCAAGAGGAGTGAAGAAAGAGGAGGAGGAGTGAAGAGGAG 2220
 L K D A K K E E V K K E E K K E E V K K E E 723
 AGAAGGACCCAAAGGAAATCAAGAGATCTCAAGGACATAAGAAATCAACTCTC 2280
 K E P K E I K K I S K D I K K S T P Q 743
 AGTCAACAAAGAGGAGTCTGCTTAAACAAAATAGCAAGGAGGAGGAGGAGTCCA 2340
 S D T K K P A L K P K A V A 763
 CTAAGAGGAGCTTCTGCTGAGAACTCAAGGACAGGAGGAGGAGGAGGAGGAG 2400
 K K E P L A A A G K L K D K G K V K V I K 783
 AAAAGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAG 2460
 K E G K T T E A A T A G T A G T A T A T A 803
 CTGCGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGG 2520
 A V V A A A G I A A S G P V K E L E A E 823

CAGATCACCTACTCTG66TGCACGTGCTCTCATATCACTGAAAACGG66CAACTGAA6 5100
 D H P T L G A S V L H I T E N G P T E V 1683
 T6GACTATAGTCCCTGTGATTCAG6GACTCTAGT7TTC6GATAGATCCCGCAAC66 5160
 D Y S P C D I Q O S S L S H K I P P T E 1703
 AAG6GACCTCTCACCCAGGATAAGTCTGTCTGAGCTCATCTCTGTCTCAG6T6 5220
 E P S Y T Q D N D L S E L I S V S Q V E 1723
 A66CTTCC66TCCACCTCTTCA6CTCACACCCCTCTCAGATAG6CCTCTCCTCTTCA66 5280
 A S P S T S S A H T P S I A S P L Q E 1743
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 D T L S D V V P P R E H S L Y A S L A S 1763
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 E K V Q S L E G E K L S P K S D I S P L 1783
 TCACCCAG6AG6TCTCACCCTTATATTCACCTGGCTTTCAGATTTCACTCTG6AG 5460
 T P R E S S P L Y A D S T S A A 1803
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 K E T A A A H G A S S P P I D A A T A 1823
 CAG6G6CCTAT6GCTTCC6CTCTCAAT6CTTTGATACAA6TCAAGCTCACTG6GCT 5580
 E P Y G F R S S M L F D T M Q H H L A L 1843
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 N R D L T T S S V E K T P G G 1863
 ACTTTAAGTCTGATCAAAAAGCT6GAGATG6C66GATCTCCAG6T6A6AAGAT 5700
 F N Y A Y Q K P E M A A G S P D E Y 1883
 AT6ACTAG6AATCTCA6G6AAG6GCTCC6GACCCAG6GCT6G6T6CTTACTACTG 5760
 D Y E S Q E K T I R T H O V V R Y Y Y E 1903
 A6AAG6CAG6AG6AACAATAAATCC6CATG6CAG6T6G6T6ACTCTCTAT6AG6CATTG 5820
 K T E R T I K S P C D S G Y S Y E I I E 1923
 A6AAG6CAG6AG6AACAATAAATCC6CATG6CAG6T6G6T6ACTCTCTAT6AG6CATTG 5880
 K T I T K T P E D G G Y T C E I T E K T I 1943
 CTC6G6CC6CAG6AG6G6T6G6T6TTCATATG6GAT6CAG6G6AAG6AC6CAG6G6C 5940
 R T P E E G G Y S Y E I S E K T I T R T P 1963
 CT6AAGT6AG6G6CTACACCTATGAAAG6CT6G6A6G6T6CAG6AG6G6T6G6T6G6A 6000
 E V B G Y T R B P D I S A Y C V D E T H E K 1983
 TTAG6CAAT6GCTAT6AT6GACT6G6T6G6C66CAG6CACTT6G6GACT6T6GACTACT 6060
 S M R Y D D T I A D S G Y L G D C S Y S 2003
 CCTAT6AAG6CT6G6AAG6AAT6AC6GCTTCTT6AG6T6G6AAG6CTATTCCTAC6G6A 6120
 Y E T T E K I T S F P E S E S Y S Y E I 2023
 CAAGTACAAAAACAAC6G6G6TCCAG6CACTCTG6CATCTGTTAC6G6CACTG6G6A 6180
 S T K T T R B P D I S A Y C V D E T H E K 2043
 AGATCAG6AAG6C66CAG6G6TCC6CATACTCTAT6G6G6T6CAG6C66G6T6G6T6 6240
 I T K T P G A S T Y S Y E T S D R C Y T 2063
 CTC6G6AAAAAG6TCC6CTCAG6G6C66CAG6G6T6G6T6T6G6T6G6T6G6T6G6T 6300
 T E K K S P S E A R Q D V D L C L V S S 2083
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 C E F K H P K T E L S P S F I N P N P L 2103
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 G6G6AG6C66C66C66CTT6CAG6G6AAG6AAG6C66G6G6AAG6T6G6AAG6C66C 6480
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 E C P S I T A D A M I D B E D E S E T I 2183
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 R S P S P R H P D V B H V D P D A L A V 2223
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 D Q N L G K A V K K D L K E K T K T K K 2243
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 P G T K T K S S S P V K K G D G K S K P 2263
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 L A A S P K P G A L K E B S D K V S R V 2283
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 A B P K K K E S V E K A T K T T T T P E 2303
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 CT6CATCAAATCAG6AAG6C66C66C66C66C66C66C66C66C66C66C66C66 7080
 A S K S A K T A T T G P G T T K T A K S 2343
 C6TCC6C66T6GCTC66G6C66CTC66T6G6T6T6G6C66T6G6T6T6G6C66C66 7140
 S T V P P G L P V Y L D L C Y I P N H S 2363
 GCAAC6AAG6AAT6G6T6G6T6G6T6G6T6G6T6G6T6G6T6G6T6G6T6G6T6G6 7200
 N S K N V D V E F F K R V R S S Y Y V V 2383
 T6AGT6G6AAG6AAG6C66CTC66C66G6G6AAG6AAG6AAG6AAG6AAG6AAG6AAG6 7260
 S G M D P A A E E P S R A V L D A L L E 2403
 AAG6AAG6CTCAGT6G6G6AAG6AAG6AAG6AAG6AAG6AAG6AAG6AAG6AAG6AAG6 7320
 G K A Q M G S M H Q V T L I P T H D S E 2423
 A6T6AT6AG6G6T6G6T6CAG6AAG6C66C66C66C66C66C66C66C66C66C66 7380
 V M R E W Y Q E T H E K Q Q D L N I M V 2443
 TCTT6AAG6C66CA6T6AG6T6G6T6G6T6G6T6G6T6G6T6G6T6G6T6G6T6G6 7440
 L A S B S T V V H Q D E S F P A C K I E 2463
 AACT6TAG6AAG6C66C66C66C66C66C66C66C66C66C66C66C66C66C66 7500
 L 2464
 GAATT6AAG6ACTCCTTTTCTAAAAC66C66C66C66C66C66C66C66C66C66 7560
 TACCTG6CAG6T6CTATACTATGTCATG6T6G6T6G6T6G6T6G6T6G6T6G6T6G6 7620

Figure 4. Complete sequence of MAPIB. The nucleotide sequence of our set of overlapping cDNA clones is shown together with the encoded amino acid sequence, in the one letter code. (The sequence of 1.4 kb of 3' untranslated region is not shown, but the entire sequence has been submitted to GenBank.) A repeated basic motif encoded toward the 5' end is underlined; a second set of repeats near the 3' end is indicated with dashed underlines. Possible phosphorylation sites for casein kinase II are indicated in two ways: predicted (●) and strongly predicted (▲) (see text).

designed for in vitro transcription. Capped mRNA synthesized from this construct was translated in a cell-free system containing [³⁵S]methionine, and the reaction products were analyzed by SDS-PAGE. The data from this experiment (Fig. 2) show an abundant translation product whose molecular size is consistent with initiation at the first AUG within the long open reading frame. Therefore, the 5' proximal AUG functions as an efficient translational initiator in vitro. The same AUG is also capable of efficient translational initiation in vivo (see below). Secondly, to examine the possible existence of MAPIB-encoding aminoterminal sequences upstream from the region covered by our overlapping cDNAs, we isolated a corresponding cloned genomic fragment by screening a mouse genomic library with a MAPIB cDNA probe. An antisense synthetic oligonucleotide corresponding to sequences close to the 5' end of overlapped MAPIB-encoding cDNAs was then used as a sequencing primer to determine upstream genomic sequences. This experiment showed that while the reading frame specified by the MAPIB amino acid sequence remains open, there are no potential upstream AUG codons; however, there is a canonical TATA sequence that could potentially form part of the promoter for the MAPIB gene (data not shown). Thirdly, we performed a primer extension experiment using an antisense oligonucleotide corresponding to nucleotides 17–34 of our MAPIB

cDNA sequence (see below) and RNA isolated (from total mouse brain RNA) by hybrid selection with clone 5X (Fig. 1). The result of this experiment is shown in Fig. 3: an extended product 69 nucleotides in length appears in the primed reaction containing selected RNA that is not present in a control reaction containing an equivalent mass of tRNA. Because the 5' end of the primer used in this experiment is located at nucleotide 34 in the sequence determined from overlapping cloned cDNAs, this experiment locates the MAPIB cap site only 35 nucleotides 5' to the beginning of the sequence shown in Fig. 4. Given the absence of upstream AUG codons in upstream genomic DNA and the very short 5' sequence not represented in our cloned cDNAs, the likelihood of translational initiation occurring at a site 5' to the first AUG present in the long open reading frame of our cloned cDNAs seems very remote.

To confirm the position of the translational termination codon, which our sequence analysis placed 1.5 kb from the 3' end of our cDNA clones, a restriction fragment spanning the region containing the 3' end of the long open reading frame was cloned into a vector designed for the transcription of RNA sequences. An initiator methionine codon was supplied in frame with the cloned insert via the AUG contained in the polylinker of the pGEM vector. This construct was linearized at restriction sites that we predicted would be either

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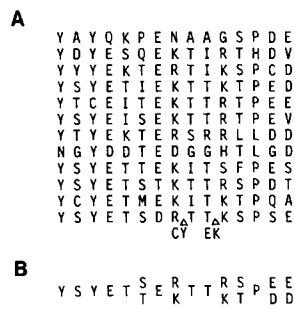


Figure 5. 12 imperfect repeats in the sequence of MAPIB. The repeats indicated by dashed underlines in Fig. 4 are aligned (A), and a consensus sequence is shown (B).

before or after the putative stop codon. These linearized DNAs were then used as templates for *in vitro* mRNA synthesis, and the mRNA was translated in *in vitro* in a rabbit reticulocyte lysate containing [³⁵S]methionine. As shown in Fig. 2, only the template cut 5' to the predicted stop codon (at Kpn I) yields a truncated product, thus confirming the existence of the stop codon at the position indicated in our MAPIB cDNA clones.

Because of the large size of MAPIB and the total absence of any protein sequence data, we checked the putative open reading frame in two ways, in addition to verifying the start and stop codons as described above. First, we verified that the reading frame in each of our original λgt11 expression clones matched the predicted long open reading frame. Second, we made seven *E. coli*-trpE fusion proteins with various fragments derived from the set of cDNAs in the reading frames predicted by our sequence data. In each case, the size of the fusion protein was in good agreement with that calculated from our sequence data (data not shown). Two of these fusion proteins were used to raise antisera: 3d2 (reported previously [1]) and YXY (see Fig. 1). These two fusion proteins were chosen because of their unusual amino acid sequences (see below). The sera generated using these fusion proteins are especially useful because they uniquely recognize two widely spaced and well defined regions of MAPIB. In particular, they are in all probability not against phosphorylated epitopes; antibodies to phosphorylated epitopes can produce confusing results, in part because the phosphorylation of MAPIB is dependent on developmental stage (43), and also because some of these antibodies cross react with phosphorylated epitopes on the neurofilament and other proteins (2, 31).

Deduced Sequence of MAPIB

The sequence of our overlapping MAPIB cDNAs together with the deduced amino acid sequence of MAPIB is shown in Fig. 4. The encoded protein is 2,463 amino acids long, with a molecular mass of 255,534 D. While this is smaller than its apparent size on SDS polyacrylamide gels, MAPIB (like tau, MAP2, and NF-M [18, 24, 25]) is known to run anomalously large in such gels (12). The predicted size of MAPIB is, however, ~25% larger than the calculated size of MAP2, which is consistent with their relative mobilities.

Two regions of repetitive amino acid sequence, each ~200 residues long, are evident in Fig. 4. One is a highly basic region containing many sequences of the form KKEX, where X is almost always an acidic or hydrophobic residue. These repeated motifs do not fall into any strict pattern, although they are clustered in the central part of a region containing

many basic amino acids. Indeed, the region from amino acids 589–787 has a calculated net charge of +30, and both the methods of Chou-Fasman (11) and Garnier et al. (15) predict that it folds into short, accessible hydrophilic α-helices, although these predictions are notoriously unreliable (50). The second repetitive region of MAPIB spans amino acids 1865–2070, and consists of 12 imperfect 15-amino acid-long repeats (Fig. 5); each repeat is separated by two nonconserved amino acids. This region is also quite hydrophilic, and is predicted to fall on a series of turns in the MAPIB molecule.

Recently, it has been discovered that MAP2 and tau have a highly similar carboxyterminal domain that contains three (or four [19, 20]) 18-amino acid-long imperfect repeats (24, 30). This domain has been shown to constitute the microtubule binding site of MAP2 and tau both *in vitro* (24, 30) and *in vivo* (S. A. Lewis, I. E. Ivanov, G. H. Lee, and N. J. Cowan, manuscript submitted for publication). However, the repeats in MAP2 and tau have no similarity to either the basic repeated motif of the 15-amino acid-long repeats in MAPIB, nor are there any regions of significant sequence homology between MAPIB and either tau or MAP2. Indeed, when the deduced amino acid sequence of MAPIB is compared with the PIR protein data base, no significant homol-

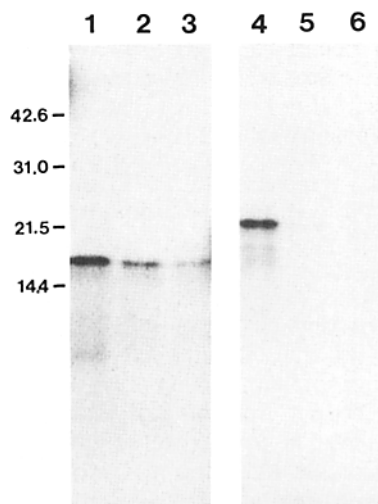


Figure 6. Translated protein corresponding to the first repeated motif in MAPIB cycles with microtubules *in vitro*. Capped RNAs transcribed from two regions (Fig. 1, arrows) were translated in a cell free system containing [³H]lysine or [³⁵S]methionine (see Materials and Methods). Translation products were cycled with microtubules, and at each stage, aliquots containing the same amount of protein (40 μgm) as judged by absorption at A₂₈₀/A₂₆₀ were analyzed on a 14% SDS-polyacrylamide gel. Lane 1, ³H-labeled protein derived from 5' subclone; lanes 2 and 3, 40 μg of microtubule protein after 1 and 2 additional cycles of assembly and disassembly in the presence of added ³H-labeled translation product. Lane 4, ³⁵S-labeled protein derived from the 3' subclone; lanes 5 and 6, 40 μg of microtubule protein after 1 and 2 additional cycles of assembly and disassembly in the presence of added ³⁵S-labeled translation product. The times of exposure have been adjusted to compensate for the difference in specific activity between [³H]lysine and [³⁵S]methionine in the cell-free system, the relative abundance of lysine and methionine residues in the respective peptides, and the relative fluorographic detection efficiency of the two isotopes.

ogies to other proteins are uncovered. However, the basic domain of MAP1B encompassing the KKEE and KKE_v repeated motifs shows some (probably fortuitous) sequence similarity to basic regions in other proteins, primarily to histones and other nuclear proteins but also to the highly charged tails of the neurofilament triplet proteins. This may account for the cross-reactivity of several anti-MAP1B antibodies with nuclear (12) or neurofilament (31) antigens.

The phosphorylation of MAP1B increases during neurite outgrowth, both in PC12 cells induced to differentiate with nerve growth factor (1, 5, 14) and in neuroblastoma cells that generate neurites when serum is removed from the growth medium (13). MAP1B is also found in phosphorylated form in developing axons in vivo (43). These facts, together with the elevated levels of MAP1B protein found in developing axons (or neurites) in vivo and in vitro (14, 16, 43), and the increased affinity of phosphorylated MAP1B for microtubules (13), suggest that phosphorylated MAP1B plays a role in the cytoskeletal changes that accompany neurite extension. A casein kinase II type activity is implicated in MAP1B and β -tubulin phosphorylation both in brain and in cultured differentiating neuroblastoma cells (13, 46). Casein kinase II phosphorylates serine and threonine residues in sequences of the form $\frac{X}{Y} \times \frac{R}{S}$ where $X \neq K, R$; however, only those sequences that fall on β turns in a protein appear to be phosphorylated (39). The primary recognition sequence for casein kinase II occurs 81 times in the MAP1B sequence; in 30 cases these sequences are predicted to form β turns by the Chou-Fasman and Garnier algorithms, and, in seven of these cases, turns are strongly predicted. These 30 potential phosphorylation sites are indicated in Fig. 4; nine are clustered in the region of MAP1B containing the 12 imperfect repeats.

The Microtubule Binding Domain of MAP1B

There exists within the MAP1B amino acid sequence two regions containing repeated motifs (discussed above). The fact that the microtubule binding regions of both MAP2 and tau contain repeated sequences (24, 30), and that microtubules are themselves composed of repeating subunits suggested that one of these regions might constitute the microtubule binding domain. To test the ability of these domains to bind to microtubules, restriction fragments encoding regions spanning each set of repeats (Fig. 1, *arrows*) were subcloned into RNA transcription vectors; a translational start signal in the correct reading frame was in each case provided by the vectors. The resulting constructs were used as templates for the transcription of capped mRNA, and the transcription products used to direct protein synthesis in vitro in a rabbit reticulocyte lysate containing either [³H]lysine (in the case of the first repeat region, which contains no methionine or cysteine residues) or [³⁵S]methionine (in the case of the second repeat region). The translation products were added to two-cycle-purified, depolymerized bovine brain microtubules, which were then taken through two further cycles of polymerization and depolymerization. After each cycle, aliquots containing the same amount (40 μ gm) of protein were removed and analyzed by SDS-PAGE, to see whether either labeled polypeptide could coassemble with the unlabeled carrier microtubules. The result of this experiment is shown in Fig. 6. A labeled polypeptide derived from the first repeat region was observed to cycle with brain microtubules, though

there is a significant loss (of $\sim 60\%$) of labeled polypeptide with each successive cycle; in contrast, a labeled polypeptide derived from the second repeat region completely failed to cycle. The relative inefficiency with which the labeled polypeptides from the first repeat region cycles with microtubules in vitro could result from competition with endogenous unlabeled MAP1B present in the microtubule preparations used in these experiments, or from the inherently poor cycling efficiency of MAP1B with microtubules in vitro (6), or a combination of both these factors. Nonetheless, these data suggest that the region containing the basic repeated sequence motifs is responsible for the cycling of MAP1B with microtubules during their purification.

To determine whether this domain is also responsible for MAP1B-microtubule interactions in vivo, a construct designed to express the entirety of MAP1B was made and transfected into cultured cells. To facilitate the assembly of a full-length MAP1B coding region, we isolated a mouse genomic clone from a cosmid library containing a 6-kb exon (Fig. 1, *clone C*). After joining this exon to cDNAs derived from the 5' and 3' ends of the MAP1B transcript, the full coding sequence was inserted into a vector containing promoter and terminator sequences derived from SV40. The final construct was transfected into HeLa and 3T3 cells and the disposition of MAP1B in transfected cells was assayed by double label immunofluorescence, using either of the anti-MAP1B antisera 3d2 and YXY, described above, and a mouse anti- α -tubulin monoclonal antibody. The observed patterns of MAP1B staining were dependent on the fixation conditions: no MAP1B was observed bound to detergent extracted cytoskeletal preparations, but in cells fixed directly in methanol (in which case proteins are fixed and extracted simultaneously) MAP1B-decorated microtubules were observed (Fig. 7, *a* and *b*). Notably, in cells fixed directly with paraformaldehyde, MAP1B filled the cytoplasm, obscuring the microtubule-bound MAP1B (data not shown). This implies that much or most MAP1B in transfected cells is in soluble form. Thus, in contrast to MAP2 (S. A. Lewis, I. E. Ivanov, G.-H. Lee, and N. J. Cowan, manuscript submitted for publication), MAP1B binds to microtubules weakly, and the equilibrium favors the unbound state since none remains bound to microtubules when the unbound portion is removed by detergent extraction. This is consistent with the behavior of MAP1B in brain (32), and the poor cycling efficiency of MAP1B with microtubules in vitro (7).

This assay for the binding of MAP1B to microtubules in vivo allowed us to define the microtubule binding domain by making constructs expressing deleted forms of MAP1B, and transfecting them into cultured cells. The results of these experiments are summarized in Fig. 1; representative immunofluorescence data are shown in Fig. 7. Deletion of the 5' half of the MAP1B coding region (construct 1B-H) or of those sequences encoding amino acids 524-848 (construct 1B-K) results in the expression of truncated forms of MAP1B that have completely lost the capacity to bind to microtubules (e.g., Fig. 7, *c* and *d*). Thus, the region of MAP1B containing the repeated KKEE and KKE_v motifs is alone responsible for microtubule binding both in vitro and in vivo.

Two further constructs, X and 1B-X (Fig. 1), show that the microtubule binding domain is divisible. The protein expressed from X includes only amino acids 646-732, whereas that expressed from 1B-X contains all of MAP1B with the ex-

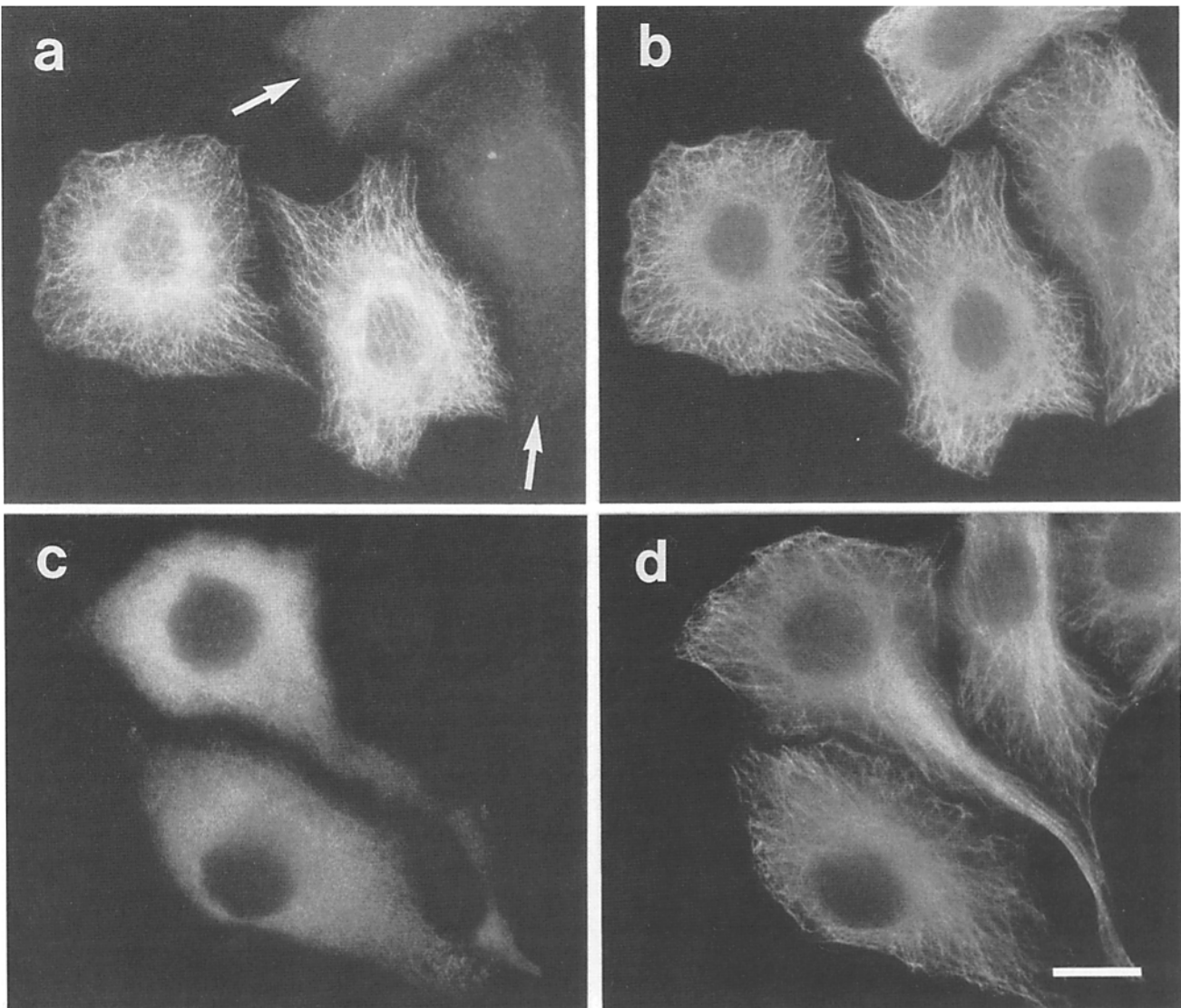


Figure 7. Characterization of the MAPIB microtubule binding domain *in vivo*. Constructs MAPIB and 1B-K (Fig. 1) were transfected into HeLa cells. After 72 h, cells were fixed with methanol, and analyzed by indirect double label immunofluorescence using the YXY antibody (*a* and *c*) directed against a segment of MAPIB (see Fig. 1), and an anti- α -tubulin monoclonal antibody (*b* and *d*). *a* and *b*, Cells transfected with MAPIB; *c* and *d*, cells transfected with the 1B-K construct (Fig. 1); untransfected cells in *a* are indicated by arrows. Bar, 10 μ m.

ception of this region. Surprisingly, the protein expressed from each of these complementary constructs binds to microtubules with approximately the same efficiency (within the limits of our assay) as intact MAPIB. This implies that at least two subregions from the KKEE and KKE_v-containing region bind independently to microtubules. It therefore seems likely that MAPIB binds to at least two tubulin subunits in the polymer, and that this bridging of subunits is involved in nucleating microtubule polymerization and in stabilizing microtubules.

Conclusions

We have cloned and sequenced cDNAs spanning the entire coding region of the MAPIB gene, and shown that a basic region of the MAPIB protein, containing many repeats of the form KKEE or KKE_v, is the MAPIB-microtubule binding domain. This was accomplished experimentally *in vitro* by

cycling translated fragments with microtubules, and *in vivo*, by transfecting constructs expressing full-length and deleted forms of MAPIB into cultured cells. The MAPIB binding domain is completely unrelated to the highly similar microtubule-binding domains of MAP2 and tau, although like those domains, it has a net positive charge and contains repeated elements. Thus, in common with MAP2 and tau (44, 45) MAPIB probably interacts ionically with the negatively charged carboxyterminal portion of α - and/or β -tubulin, though it has not been shown whether these molecules compete for the identical site on microtubules. The binding of MAPIB to microtubules is weak compared with MAP2, and unlike MAP2 (S. A. Lewis, I. E. Ivanov, G.-H. Lee, and N. J. Cowan, manuscript submitted for publication) expression of high levels of MAPIB in cultured cells does not result in any obvious cytoskeletal changes (Fig. 7, *a* and *b*). However, MAPIB has two light chains that also associate with

MAP1A (22, 23, 43); these and/or other coexpressed proteins may alter the properties of MAP1B.

The data presented here for MAP1B, together with that reported previously for MAP2 and tau (19, 20, 24, 30) and for kinesin (53), define three structurally unrelated kinds of microtubule binding domains. These differences must in some way reflect the different functions served by the interaction of each MAP with microtubules. For example, kinesin, which acts as a motor for anterograde transport along microtubules (8, 48), would be expected to have completely different binding properties from MAP2, which crosslinks and stabilizes microtubules in dendrites (S. A. Lewis, I. E. Ivanov, G.-H. Lee, and N. J. Cowan, manuscript submitted for publication). Therefore, it is not surprising that their binding domains are completely dissimilar. It seems probable that each of these three binding domains is representative of a separate family of functionally related microtubule-associated proteins.

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