Differential Expression of Alternatively Spliced Forms of MAP4: A Repertoire of Structurally Different Microtubule-Binding Domains[†,#]

Steven J. Chapin,[‡] Chia-Man Lue,[§] M. T. Yu,[∥] and Jeannette Chloe Bulinski*[,§,⊥]

Departments of Anatomy & Cell Biology, Pathology, and Genetics & Development, College of Physicians & Surgeons, Columbia University, 630 West 168th Street, New York, New York 10032, and Department of Biology, University of California, Los Angeles, California 90024

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ABSTRACT: We previously reported that the microtubule (MT)-binding domain of microtubule-associated protein 4 (MAP4) contains three 18-amino acid imperfect repeats that are homologous to the repeats found in the MT-binding domains of the neuronal MAPs, MAP2 and tau [Chapin, S. J., & Bulinski, J. C. (1991) *J. Cell Sci. 98*, 27−36]. Here we report the isolation of clones encoding additional isoforms of MAP4, which differ in the number of repeated elements contained within their MT-binding domains. In addition to clones encoding three repeats, we isolated clones encoding a four-repeat isoform, whose MT-binding domain bears a striking similarity to the four-repeat isoform of tau. Other MAP4 clones that we isolated encode five repeats. The additional repeat in the five-repeat isoform of MAP4 is quite unusual in its amino acid sequence; this unusual repeat was also found by Aizawa et al. [Aizawa, H., et al. (1990) *J. Biol. Chem. 265*, 13849−13855] among the repeats encoded by bovine MAP4 clones possessing four repeats. In humans, MAP4 was recently shown to be encoded by a single-copy gene [West, R. R., et al. (1991) *J. Biol. Chem. 266*, 21886−21896]; we demonstrated that the human MAP4 gene is located on human chromosome 3p21. Expression of multiple MAP4 isoforms from this gene, which appears to result from alternative RNA splicing, was investigated by RNase protection analysis of mammalian cell lines and rat tissues. The five-repeat isoform was the only form detectable in most cell lines, and it was the most abundant isoform expressed in rat lung, liver, kidney, spleen, and testis. However, in rat brain, heart, and skeletal muscle, although the five-repeat isoform was expressed at all developmental stages examined, the tau-like four-repeat isoform was also expressed, and its expression increased during development. The three-repeat isoform was expressed in heart and, to a lesser extent, in brain, skeletal muscle, and lung. Our results demonstrate that several different MAP4 isoforms are expressed in the rat in different tissues and at various developmental stages. Furthermore, our data suggest that differential expression of MAP4 isoforms possessing distinct MT-binding domains may be involved in the changes in MT dynamics or function that are known to accompany differentiation.

Differentiation of the microtubule (MT) cytoskeleton during morphogenetic events is believed to depend on the function of assembly-promoting, microtubule-associated proteins (AP-MAPs). For example, MAP2 and tau expression has been shown to be integral to the formation of neurites and axons (Dinsmore & Solomon, 1991; Caceres & Kosik, 1990). While the detailed mechanisms by which these MAPs effect morphogenesis are not clear, MT stabilization is likely to play a central role (Chapin & Bulinski, 1992). The MT-binding domains of MAP2 and tau are homologous in a region near the C-terminus, which contains three imperfectly repeated sequences of 18 amino acids each, separated by interrepeat segments of 13−14 amino acids (Lee et al., 1988; Lewis et al., 1988). Juvenile and adult isoforms of MAP2 and tau are derived from a single gene by alternative RNA splicing (Garner & Matus, 1988; Kosik et al., 1989). In the case of tau, the adult-specific isoform contains an additional, or fourth, repeat within its MT-binding domain (Kosik et al., 1989; Goedert et al., 1989). Expression of this four-repeat tau isoform has been correlated with increased MT stability in vivo (Hanemaaijer & Ginzburg, 1991), and the presence of the four-repeat isoform appears to confer a greater degree of MT stabilization than the three-repeat isoform in vitro (Goedert & Jakes, 1990) and in vivo (Lee & Rook, 1992). While expression of MAP2 and tau is limited to neuronal tissue (Lewis et al., 1986; Binder et al., 1985), MAP4 is an AP-MAP with widespread cell and tissue distribution (Bulinski & Borisy, 1980a; Parysek et al., 1984; Huber & Matus 1990; Aizawa et al., 1990). Thus, MAP4 is likely to play an important role in morphogenetic events in a variety of cell types.

We have previously reported that the MT-binding domain of MAP4 contains repeats homologous to those of MAP2 and tau (Chapin & Bulinski, 1991a). The MAP4 cDNA whose sequence we reported encodes a three-repeat isoform of MAP4. Here we report the isolation of cDNAs encoding additional MAP4 variants, including a five-repeat isoform first documented by West et al. (1991) and a four-repeat isoform similar to the tau four-repeat isoform. Bovine cDNA

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* Author to whom correspondence should be addressed at the Department of Anatomy & Cell Biology, Columbia University. Telephone: (212) 305-5113. Fax: (212) 305-3970.

[‡] University of California at Los Angeles.

[§] Department of Anatomy & Cell Biology, Columbia University.

[∥] Department of Genetics & Development, Columbia University.

[⊥] Department of Pathology, Columbia University.

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clones isolated by Aizawa et al. (1990) encode yet another isoform of MAP4, a different four-repeat isoform. Thus, four different forms of the MT-binding domain of MAP4 have been identified, and we suggest here that these variants arise by alternative splicing of RNA transcribed from the single-copy MAP4 gene, which we have localized to human chromosome 3p21. We have examined the patterns of MAP4 protein expressed from both the endogenous MAP4 gene and from single human cDNAs transfected into nonprimate cells. We have also studied the cell and tissue distributions of mRNAs encoding MAP4 isoforms, by using RNase protection to assay rat tissues at various developmental stages. We hypothesize from our data that regulated expression of MAP4 isoforms that may be functionally distinct may play a role in the regulation of MT dynamics during differentiation of tissues.

MATERIALS AND METHODS

Screening of cDNA Libraries. A λ gt-11 human fetal brain cDNA library and an unamplified λ ZAPII HepG2 cDNA library were the generous gifts of Dr. Rachel Neve (Harvard University) and Dr. Eugene Marcantonio (Columbia University), respectively. Immunoscreening was performed as described previously (Chapin & Bulinski, 1991a). Screening by hybridization was performed using random-primed cDNA probes (Feinberg & Vogelstein, 1984) or oligonucleotides labeled by terminal transferase. Inserts from clones isolated from λ gt-11 were subcloned into the *Eco*RI site of pGEM7Zf(+). λ ZAPII clones were converted in vivo to the plasmid form (pBluescriptSK(−)) according to protocols provided by Stratagene (La Jolla, CA).

Peptide and DNA Sequencing. HeLa MAP4 was gel-purified from a heat-stable MAP fraction digested with V8 protease during reelectrophoresis, and peptides were subjected to N-terminal sequencing according to Matsudaira (1987), as described previously (Chapin & Bulinski, 1991a). Overlapping cDNA subclones generated by exonuclease III deletion (Henikoff, 1984) or restriction enzyme digestion were sequenced as described previously (Chapin & Bulinski, 1991a).

Construction of Fully Encoding Human MAP4 Clones and Expression in Nonprimate Cells. The following strategy was used to construct clones fully encoding MAP4 isoforms: Clones Hep1 (in pBluescriptSK(−)) and Hfb2 (in pGEMZf-(+)) were cleaved with *XBa*I (which cleaves once within the polylinker of each plasmid) and *Nco*I (which cleaves at a unique site in the MAP4 cDNA; see Figure 1). The small fragment from Hep1 was ligated into the large fragment of pGEM7Zf(+)-Hfb2 and subcloned. The result was to join the two clones in frame at their shared *Nco*I site; the new fully encoding MAP4 clone contains the initiator ATG of MAP4 (see Results) and features a three-repeat MT-binding domain. This clone, designated pGEMMAP4fI, is flanked on its 3′-end by the pGEM polylinker *Hin*dIII site. Cleavage with *Xba*I, followed by conversion to blunt ends and ligation in the presence of *Hin*dIII linkers, resulted in a MAP4 cDNA that was flanked on both sides by *Hin*dIII sites. The MAP4 insert was then excised with *Hin*dIII and ligated into the *Hin*dIII site of the expression plasmid pMMTV or pRSVi-*Hin*dIII-SP (Chin & Liem, 1989; these were kindly provided by Dr. Steve Chin, Columbia University), placing the MAP4 cDNA under the control of the mouse mammary tumor virus promoter or the Rous sarcoma virus promoter, respectively. A construct encoding the tau-like four-repeat isoform of MAP4 (form III) was prepared by replacing the repeat-containing *Bgl*II/*Bst*EII fragment of pGEMMAP4fI with that of clone Hfb1. Likewise, a full-length cDNA encoding the five-repeat isoform of MAP4 (form IV) was prepared by replacing the *Bgl*II/*Bst*EII fragment of pGEMMAP4fI with that of clone Hfb6. Stable Ltk− and CHO cell lines expressing human MAP4 were obtained by cotransfecting supercoiled pMMTV or pRSVi plasmids containing MAP4 cDNA constructs and pSV2i-NEO using the calcium phosphate procedure (Parker & Stark, 1979). Colonies resistant to G418 were isolated, using cloning rings, and cells were replated at clonal density. Resistant clones were then isolated and screened for reactivity with primate-specific MAP4 antiserum by immunofluorescence (Chapin & Bulinski, 1991a); for glucocorticoid-inducible pMMTV transfectants, 1 μM dexamethasone (Sigma Chemical Co., St. Louis, MO) was added 4−20 h before immunoscreening, in order to induce the synthesis of transfected MAP4 forms. Clones exhibiting MAP4 immunofluorescence were analyzed further by Western blotting.

In Situ Hybridization to Human Chromosomes. pGEM7Zf-(+) containing cDNA clone Hfb2 was labeled with digoxygenin dUTP (Boehringer Mannheim, Indianapolis, IN) by nick translation. Probe was denatured by heating at 75 °C for 7 min in hybridization buffer containing 50% formamide. Metaphase chromosomes from human lymphocytes or hybrid cell lines were hybridized to the probe at a concentration of 10 μg/mL at 37 °C for 2 days. Slides were then washed in 2× SSC containing 50% formamide at 42 °C, followed by washes in 2× SSC without formamide. Probe was detected with peroxidase-conjugated anti-digoxygenin antibody (Boehringer-Mannheim) using a DAB-silver enhancement kit (Amersham, Arlington Heights, IL).

Cell Culture. HeLa cells were cultured as previously described (Chapin & Bulinski, 1991b). All other cells were grown in DME supplemented with 10% FBS (Gibco, Grand Island, NY). L_6 cells were differentiated for the indicated number of days by substituting 2% horse serum (obtained from Hyclone, Inc., Logan, UT; heat-inactivated at 50 °C for 30 min) for FBS, as previously described (Gundersen et al., 1989).

Western Blots. All chemicals used in these procedures were obtained from Sigma Chemical Co. (St. Louis, MO). Cultured cells were solubilized with SDS sample buffer (Laemmli, 1970) containing chymostatin, leupeptin, antipain, and pepstatin at 0.1 μg/mL each and 0.2 mM phenylmethanesulfonyl fluoride (PMSF) and analyzed by Western blotting using a primate-specific MAP4 antiserum, as described previously (Chapin & Bulinski, 1991a). Induction of human MAP4 synthesis in Ltk−cells transfected with pMMTV-MAP4 constructs was effected by treatment with 1 μM dexamethasone for 16 h prior to solubilization.

Northern Blots. Total RNA from cells and tissues was isolated as described by Cathala et al. (1983) or Chomczynski and Sacchi (1987). Rat tissues were frozen in liquid nitrogen and then quickly pulverized before homogenization in lysis buffer using a Tekmar Tissuemizer (Fisher Scientific, Springfield, NJ). RNA was electrophoresed in formaldehyde−agarose gels, transferred onto nylon membranes (Genescreen, NEN, Norwalk, CT), and cross-linked to the membrane using a Stratalinker device (Stratagene, La Jolla, CA). cDNA

Table 1

primer	orientation	sequence	position (in human MAP4)
PCR1	sense	5′-TGACTTGAGTCGCCCAAAGAGCA	2566−2590
PCR2	antisense	5′-TGGATCTGGCTGTCCAAGGTCT	3422−3443
PCR3	antisense	5′-CTGCCACCGCCCTCAGTCTTCA	3257−3278
insert 1	antisense	5′-GCTGTAGCTCACTTTTTTGGAGACTATCTGGAC	2860−2892
insert 2	antisense	5′-AGCAGCTGCCTCTGTTTTTTTCTCTACTTTGGC	2973−3005
junction-spanning	antisense	5′-TTCTTGTTCTGAATCTGAACCCGGCCTCCTCCA	2846−2859/3066−3085

probes were prepared by the random-priming method (Feinberg & Vogelstein, 1984). Blots were hybridized to cDNA probes as described previously (Chapin & Bulinski, 1991a). Oligonucleotides (American Synthesis, Inc., Pleasanton, CA) were labeled with [α-^{32}P]dATP (6 Ci/mol) using terminal transferase. Hybridization was performed according to Collins and Hunsaker (1985). The appropriate washing conditions for each oligonucleotide probe were determined empirically using Southern blots containing the appropriate cDNAs or by running formaldehyde-denatured cDNAs in lanes adjacent to RNA samples. Autoradiography of blots hybridized to oligonucleotide probes was performed using two intensifying screens, usually for 5−7 days. For blots that were hybridized sequentially to multiple probes, membranes were stripped according to the manufacturer's protocols before rehybridization.

Oligonucleotides for PCR and Hybridization. The primers were purchased from American Synthesis, Inc. (Pleasanton, CA) and are shown in Table 1.

PCR Amplification and Cloning of MAP4 cDNA from L_6 Cells. We used PCR to amplify cDNA from various sources to assay expression of MAP4 isoforms. PCR primers 1 and 2 were selected because they spanned identical sequences in human and bovine MAP4, suggesting that they could be used to amplify MAP4 cDNA from various species. Reverse transcription of 1 μg of total RNA using random primers and amplification of the resulting cDNA with MAP4-specific primers were performed essentially according to Kawasaki and Wang (1989), except that Promega (Madison, WI) Taq polymerase and buffer were used in some of the amplification reactions. Sequential amplifications using primers PCR1 and PCR2, followed by PCR1 and insert 2, demonstrated that proliferating cells of the rat myogenic cell line, L_6, contained RNA encoding the five-repeat isoform of MAP4 (form IV). We cloned this repeat-encoding region from L_6 cells for use in RNase protection analysis of rat cells and tissues. An 875 bp L_6 PCR product, obtained with primers PCR1 and PCR2, was digested with *Pst*I in an attempt to clone a *Pst*I fragment encoding the repeat-bearing region of the MT-binding domain into the *Pst*I site of pBluescriptSK(−). This attempt failed because the *Pst*I site at position 2647 of the human sequence is not present in cDNA from rat L_6 cells. However, we serendipitously isolated a rat clone representing a *Pst*I fragment located 3′ to the repeats (corresponding to nucleotides 3241−3553 of the human sequence), which was most likely generated by false priming of the PCR2 (antisense) primer at position 3558. This clone, designated L_6*Pst*I, was sequenced, and the sequence information was used to select a new antisense primer (PCR3), whose sequence is conserved between rat and human MAP4. The new antisense primer was chosen to avoid the mispriming mentioned here and to preclude having to amplify the region between PCR2 and PCR3 (this region is quite GC-rich, and this fact may have contributed to the low yields we had noted in previous PCR reactions). Using PCR1 and PCR3 as primers, we were able to obtain and clone rat PCR products that spanned a region corresponding to positions 2568−3245 of the human MAP4 sequence. Briefly, this was accomplished by conversion of the ends of the PCR products to blunt ends with Klenow enzyme, ligation of the products to *Hin*dIII linkers, and digestion with *Pst*I (which cuts the rat PCR product internally at a position that corresponds to nucleotide 3142 of the human sequence) and *Hin*dIII, followed by ligation into a pGEM3Z plasmid that had been cut with *Pst*I and *Hin*dIII. The resulting plasmids were made linear with *Hin*dIII prior to transcription of nuclease protection probe, using T7 RNA polymerase.

RNase Protection Assays. (A) Preparation of Templates. Template for human RNA probes was prepared by cleaving plasmid pGEM7Zf(+)-Hfb6 with *Bam*HI, followed by self-ligation of the appropriate fragment, such that the SP6 promoter of the plasmid was juxtaposed to the 3′-side of the unique *Bam*HI site in the cDNA, which resides within the fifth repeat. After subcloning, template DNA was cut at the *Bgl*II site at position 2392 of the sequence to generate linear DNA. Next, transcription with SP6 RNA polymerase was used to produce an antisense transcript of 827 nucleotides, containing all of repeats one through four and most of repeat five. Template for rat probes was prepared by the cloning of PCR products of cDNA from L_6 cells (see above). To minimize possible PCR-induced mutations in the probe template, which would cause undersirable nicking of RNA duplexes, probes were prepared from multiple independent clones and tested for degradation due to mismatch in assays with rat tissue RNA. The human template described earlier begins at the *Bam*HI site instead of the *Pst*I site at position 3246 (see Figure 1A); since this probe overall is 28 nucleotides shorter than the corresponding rat probe, it results in protected fragments that are shorter by this increment for each isoform. For use as a size marker, and for increased usefulness of human sense RNAs as size markers in some RNase protections performed with rat samples (see below), we prepared an additional human template, originating from the *Pst*I site, whose 5′ end was identical in position within the rat template.

(B) Preparation of Probes. RNA probes were prepared from human and rat templates according to Melton et al. (1984) using either T7, SP6, or T3 RNA polymerases in the presence of [α-^{32}P]CTP. Probes were purified from polyacrylamide−urea gels prior to hybridization with target RNA. In addition to the probe we designed, a truncated version of the antisense probe was obtained for both the human and rat MAP4 repeat regions. Apparently, transcription of this probe terminated at approximately position 2857 in both the human and rat sequences. Because this adventitiously terminated probe would be expected to yield a less complex pattern of protected species than the full-length probe, we chose to use it in the protection assays. Accordingly,

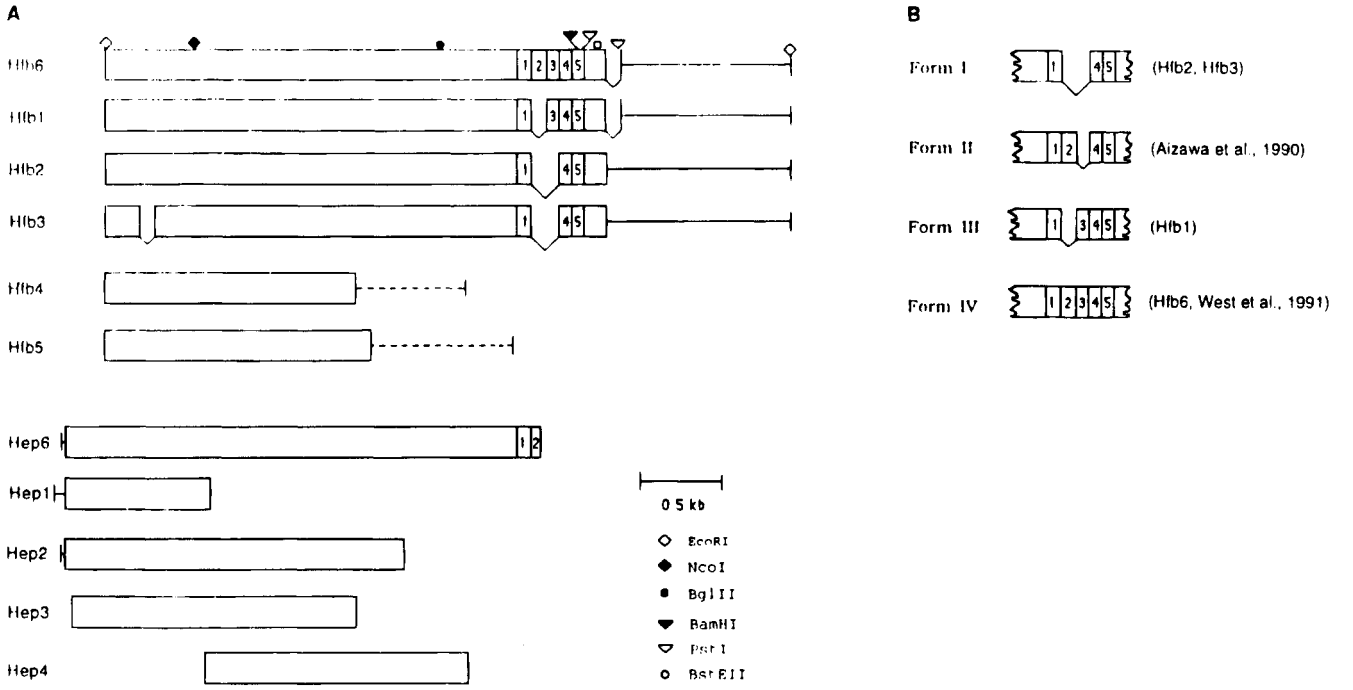

FIGURE 1: (A) Schematic map of MAP4 cDNA clones. The MAP4 coding region is indicated by open boxes. Each repeat and its preceding interrepeat segment in the region encoding the MT-binding domain are indicated by a numbered segment. The dashed lines in Hfb4 and Hfb5 represent sequences that diverge from sequences contained in other clones. The divergent sequence in Hfb4 probably represents an intron, while in Hfb5 it most likely indicates the presence of heterologous sequences ligated onto the MAP4 cDNA during preparation of the cDNA library (see Results). Positions of restriction sites used in the construction of clones for expression or nuclease protection are indicated. (For simplicity, additional *Pst*I sites are not indicated on the diagram.) Clone designations Hfb and Hep refer to the cDNA libraries (human fetal brain and HepG2, respectively) from which the clones were isolated. The 5′ ends of clones Hfb1−Hfb3 and Hfb6 are defined by *Eco*RI sites internal to MAP4 cDNA, which were cleaved during library construction. (B) Summary of MAP4 isoforms that have been identified by cDNA cloning. Four forms of the MT-binding domain on MAP4, which differ in the repeats they contain within their MT-binding domains, are defined by clones listed in part A, as well as by those isolated by Aizawa et al. (1990) and by West et al. (1991).

nuclease protections with the smaller probe, which was electrophoretically purified, are presented in the Results. RNase protection assays were performed exactly as described by Melton et al. (1984), using the amounts of total RNA from cells or tissues that are indicated in each figure legend. Double-stranded nuclease-protected RNAs were analyzed by electrophoresis on nondenaturing polyacrylamide gels followed by fluorography. Sense RNAs encoding forms I, III, and IV were transcribed from the appropriate human clones, subjected to RNase protection assays, and used as size markers for each gel. A schematic diagram of the nuclease protection assay is shown in Figure 6.

RESULTS

Isolation of Clones and Construction of Fully Encoding MAP4 cDNAs. A map of relevant MAP4 cDNA clones is shown in Figure 1. Previously reported clones (pMAP4.245 and pMAP4.220; Chapin & Bulinski, 1991a), which were isolated by immunoscreening a human fetal brain cDNA library, will be referred to here as clones Hfb2 and Hfb5, respectively. Clones Hfb1, Hfb3, and Hfb4 were also isolated from the same library by immunoscreening. As previously stated (Chapin & Bulinski, 1991a), the 5′ *Eco*RI sites of these clones are within the MAP4 coding region; these sites apparently were cleaved during construction of the human fetal brain cDNA library. Thus, a portion of the 5′ coding region of MAP4 is missing from clones Hfb1−Hfb5 (Figure 1). To obtain the 5′ coding region of MAP4, we screened an unamplified, random-primed λ ZAPII HepG2 cDNA library (kindly provided by Gene Marcantonio), using the entire clone Hfb2 as a hybridization probe. Four clones, Hep1−Hep4, were isolated. Three of these clones, Hep1−Hep3, as well as a subsequently isolated clone, Hep6 (see below), spanned the 5′ terminal *Eco*RI site of the Hfb clones, thus providing additional 5′ sequences. Next we used the short (380 bp) 5′ *Eco*RI fragment of Hep1 as a hybridization probe to rescreen the human fetal brain library. Six positive clones were analyzed; although these clones contained up to 3 kb of sequence 5′ to that of Hep1, only 80 bp could be confirmed as authentic MAP4 sequence. Other 5′ sequences were not shared by two or more clones (not shown) and may have resulted from heterologous sequences ligated to the 5′ ends of these clones.

We used the MAP4 cDNA clones we had isolated to try to verify the location of the AUG encoding the initiator methionine of MAP4, which had previously been reported by Aizawa et al. (1990) and West et al. (1991). A useful means of identifying the initiator methionine within a cDNA is to locate one or more in-frame stop codons 5′ of the putative initiator AUG codon. However, none of the MAP4 clones we isolated contained an in-frame stop codon near its 5′ end. We noted that the first AUG in-frame with the MAP4 coding sequence appears to be in a favorable context for initiation of translation (Kozak, 1987). Aizawa et al., (1990) originally stated that the first in-frame AUG within their bovine MAP4 cDNA, which corresponds to the first in-frame AUG contained in our clones, was the initiator codon for bovine MAP4, based on the presence of upstream in-frame stop codons in the bovine sequence. However, no upstream stop codons have been found in the reading frames

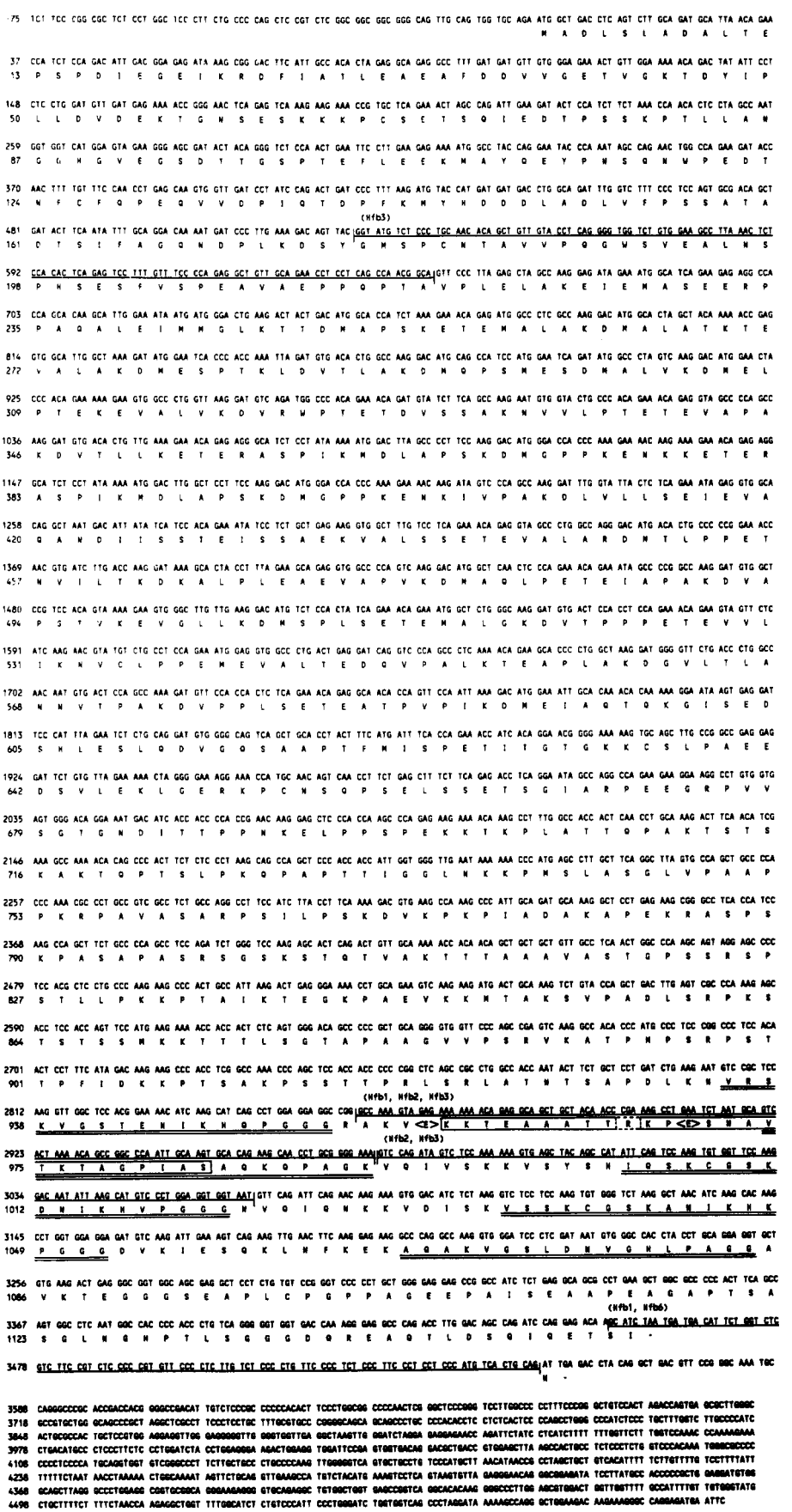

FIGURE 2: Nucleotide and amino acid sequence of MAP4. A sequence representing the region spanned by clones Hfb6 and Hep1 is shown. Translation is shown from the first in-frame ATG (see Results). The five 18-amino acid imperfect repeats are indicated by a double underline. Nucleotide deletions in cDNA clones are indicated by a single underline, with the name(s) of the clone(s) indicated at the beginning of the deletion. As shown, the deletion at the end of the coding region in Hfb1 and Hfb6 causes a frame shift that gives rise to an alternative C-terminus (N instead of SI). The region enclosed in boxes indicates an encoded sequence that matches a peptide sequence obtained from HeLa MAP4 at 25 out of 26 positions.

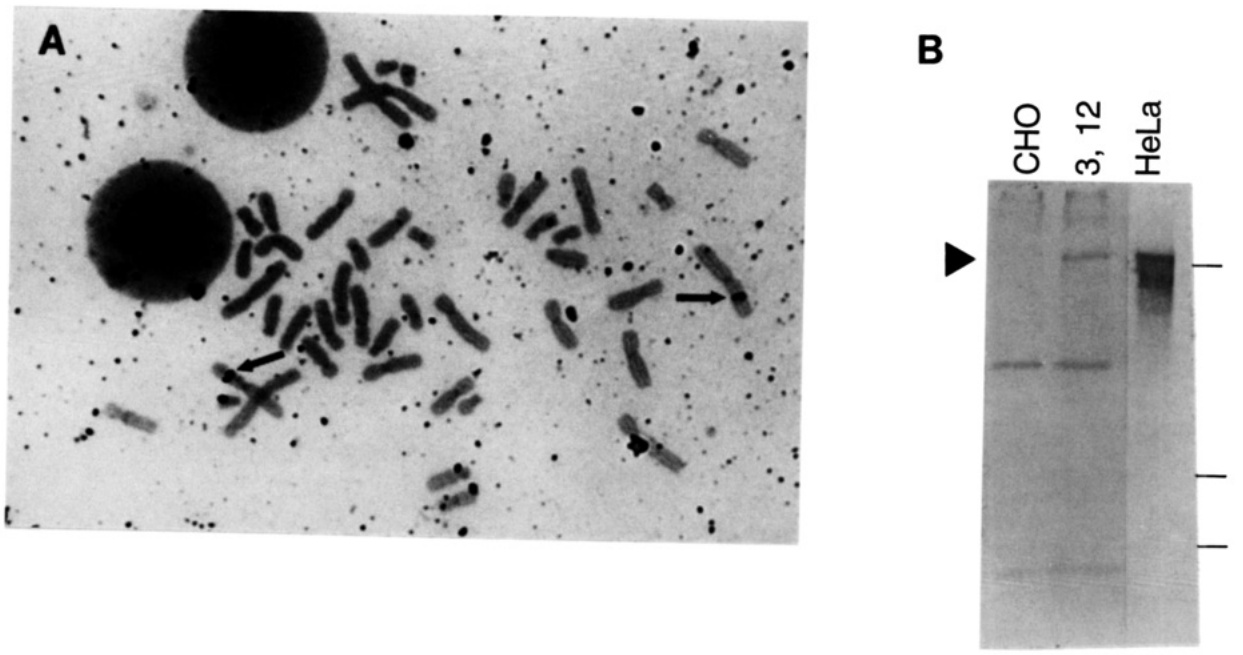

FIGURE 3: Chromosomal localization of the human MAP4 gene. (A) In situ hybridization to human chromosomes, as described in Materials and Methods, identifies the MAP4 locus as chromosome 3p21 (arrows). (B) Western blotting demonstrates that MAP4 is expressed in human/CHO hybrid cells containing human chromosome 3. Primate-specific MAP4 antiserum recognizes MAP4 (arrowhead) in a human/CHO hybrid cell line containing human chromosomes 3 and 12, as well as in HeLa cells, but does not react with CHO cells that lack human chromosomes. The lower M_r band present in all three lanes is due to nonspecific binding of antibody. Positions of molecular mass markers of 200, 116, and 94 kDa are shown as horizontal bars on the right.

of human or mouse MAP4 cDNAs, and the bovine MAP4 sequence of Aizawa et al. (1990) diverges from both the human and mouse MAP4 sequences at base position −19 (Figure 2; West et al., 1991). It is possible that the bovine clone whose sequence was reported contains an intron, since the sequence starting at position −19 closely resembles the vertebrate consensus sequence for intron/exon boundaries (Padgett et al., 1986). Thus, in-frame stop codons at the 5′ end of human or mouse MAP4 cDNAs cannot be used to demonstrate the authenticity of the putative initiator AUG.

A second means of verifying the location of the initiator AUG within a cDNA is to compare the electrophoretic mobility of the translation product of the putative full-length cDNA with the molecular mass predicted for the protein. This could be problematic for MAP4, however, since the sequence molecular weight expected if translation is initiated at the putative AUG predicts an M_r of approximately half that of biochemically isolated MAP4. All AP-MAPs studied thus far exhibit aberrant M_r's on SDS−PAGE, and the electrophoretic mobility properties of MAP4 appear to be even more aberrant than those of MAP2 and tau. Nonetheless, verification that the M_r of an MAP4 translation product matches that of bona fide MAP4 would provide convincing evidence that the position of the initiator AUG codon had been properly assigned.

In order to compare the translation product of our full-length MAP4 cDNAs with authentic MAP4, we first performed in vitro translation of a cDNA encoding a three-repeat isoform of MAP4. The product we obtained exhibited an M_r of about 200 000 on SDS−PAGE, approximately the same mobility as HeLa MAP4, although the presence of a number of smaller species, which we presumed to result from extensive proteolytic degradation, confounded our results (not shown). Additional evidence was provided by Western blot analyses of mouse and hamster cells that had been stably transfected with MAP4 cDNAs, which showed that the in vivo products of the transfected cDNAs are similar in apparent molecular weight to authentic HeLa MAP4 (Figure 4). In fact, although the products of MAP4 form I and II cDNAs migrate more rapidly than HeLa MAP4, the protein synthesized from MAP4 form IV cDNA is indistinguishable in its M_r from HeLa MAP4 (Figure 4B). Thus, our data suggest that clones containing the putative initiator AUG are translated efficiently in vivo and yield proteins whose M_r's are consistent with that of full-length MAP4.

Heterogeneity of MAP4 cDNA Clones. As we previously reported, clone Hfb2 contains three repeat sequences within its MT-binding domain, which are homologous with the repeats found in the brain MAPs, MAP2 and tau (Chapin & Bulinski, 1991a). Clone Hfb1, discussed previously (Chapin & Bulinski, 1991a), contains four, rather than three, repeats; its MT-binding domain repeats are similar to those of the four-repeat isoform of tau (Kosik et al., 1989; Goedert et al., 1989), as well as to a four-repeat isoform of MAP2c that was recently reported (Doll et al., 1993). Other MAP4 cDNA clones, which encode a different four-repeat isoform, were also reported by Aizawa et al. (1990). In order to identify additional forms of MAP4's MT-binding domain, we screened the HepG2 library we had used previously, using clone Hfb2 as a hybridization probe. One clone, Hep6, was isolated. This clone contained the first repeat encoded by each of our other clones, plus the beginning of the second repeat identified by Aizawa et al. (1990). We next re-screened the human fetal brain library; this time duplicate filters were hybridized in parallel with clone Hep6 and with an oligonucleotide corresponding to the unusual second repeat of clone Hep6 (insert 1). The clone isolated in both hybridizations, called clone Hfb6, contained all five repeats previously identified in MAP4 clones. Human and mouse five-repeat isoforms of MAP4 have previously been reported

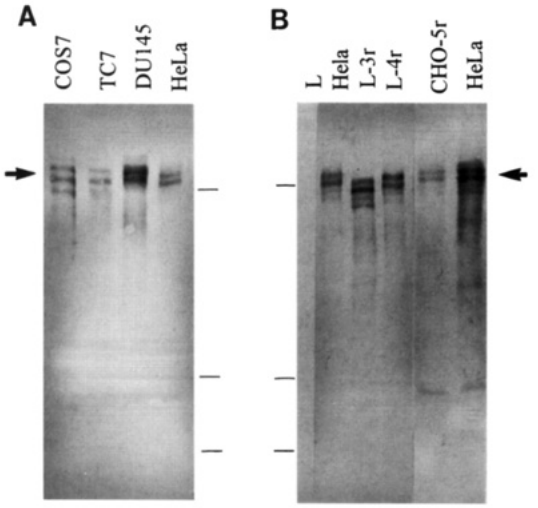

FIGURE 4: MAP4 heterogeneity in cultured primate cell lines and stably transfected rodent cells. (A) Western blot analysis of COS7, TC-7, DU145, and HeLa cells detects multiple MAP4 bands of M_r ~ 210 000 (indicated by arrow). (B) Ltk$^-$ cells (L) do not contain immunoreactive primate MAP4, while L cells stably transfected with MAP4 form I (L-3r) and form III (L-4r) and CHO cells transfected with form IV (CHO-5r) exhibit MAP4 species of electrophoretic heterogeneity similar to that of the MAP4 species visualized in HeLa extract samples (HeLa). Note that the electrophoretic migration of human MAP4 expressed in transfected cell lines varies slightly, depending on which isoform has been transfected, and the bands for forms I and II are slightly lower than the bands observed for form IV and HeLa samples (migration of MAP4 in HeLa is indicated by an arrow). Total cell extracts were electrophoresed on 5% SDS−PAGE and analyzed by Western blotting with primate-specific MAP4 antiserum. Positions of molecular mass markers of 200, 116, and 94 kDa are shown as horizontal bars on the right. A band of lower M_r (~100 000) present in most lanes of the blot is due to nonspecific binding of anti-MAP4 antisera.

(West et al., 1991). Thus, it appears that the MT-binding domain of MAP4 exists in at least four different configurations of primary sequence. Three of these, possessing three, four, and five repeats, are represented in cDNA clones that we have isolated and discussed in this report, while the fourth configuration identified thus far, which contains four repeats, is represented in cDNA clones isolated by Aizawa et al. (1990). A schematic diagram of the four forms of the MT-binding domain of MAP4 is shown in Figure 1B.

Nucleotide and amino acid sequences of MAP4 are shown in Figure 2. This sequence represents the entire MAP4 cDNA sequence spanned by the clones in Figure 1. Within the MAP4 sequence, each 18-amino acid repeat unit is indicated with a double underline. The box denotes an amino acid segment identical to a sequence we obtained from proteolytic peptides of purified HeLa MAP4. Sites of V8 protease cleavage during the preparation of peptides for sequencing are denoted in parentheses. Two other HeLa MAP4 peptide sequences, which span amino acids 700−712 and 842−856, were reported previously (Chapin & Bulinski, 1991a). Although the protein encoded by the composite sequence shown in Figure 2 is not included in any single RNA identified by our clones (Hfb6 has a 3′ deletion), it is represented by at least one full-length clone isolated by West et al. (1991), which has five repeats and no 3′ deletion.

Heterogeneity in the cDNA clones isolated is also indicated in Figure 2. Deletions of nucleotides that occur in some clones are marked by a thin underline, and the names of the corresponding clones are denoted in parentheses at the beginning of each deleted region. The region indicated by a dashed line at the 3′ end of clone Hfb4 probably represents an intron because it bears a sequence characteristic of exon/intron boundaries (Padgett et al., 1986) at the site at which it deviates from the other clones. The region of Hfb5 represented by a dashed line is probably the result of ligation of heterologous sequences during library construction, as mentioned previously (Chapin & Bulinski, 1991a). The 3′ deletion in clones Hfb1 and Hfb6 places a premature stop codon in these clones, such that the C-terminal amino acids are changed from serine-isoleucine (SI) to an asparagine (N) residue. The fact that two independent clones exhibit identical deletions and the fact that the sequence at the deletion boundary agrees with the consensus sequence for exon boundaries in vertebrates (Padgett et al., 1986) both suggest that this deletion is more likely to have resulted from an alternative splicing event than from an artifact of the cloning process. Furthermore, the nucleotide sequence of a clone reported by Aizawa et al. (1990), containing the presumptive stop codon for bovine MAP4, markedly differs from our human MAP4 sequence at exactly the same site, again suggesting that this site could be a splice junction. The bovine C-terminal sequence is anomalous since, in contrast, human, rat (our unpublished data), and mouse (West et al., 1991) MAP4 sequences are highly conserved within both their C-terminal sequences (each contains SI or N at its C-terminus, in contrast to the bovine sequence, SKWLGLA) and their 3′ untranslated regions.

In contrast, a deletion we noted near the 5′ end of clone Hfb3 (spanning nucleotides 529−652; see Figure 2) *is* likely to represent a cloning artifact, since we have not found this deletion in any of the other MAP4 clones from our lab or others. In addition, RNase protection assays using a probe spanning this deletion detected only the undeleted form in RNA from HeLa and CaCo2 cells (data not shown).

Chromosomal Location of the Human MAP4 Gene. Southern blot data suggested that human MAP4 is encoded by a single gene (West et al., 1991; also our unpublished data). This hypothesis is corroborated by the facts that (1) the nucleotide sequence of each of more than 10 MAP4 clones that we isolated was invariant within all corresponding regions, and (2) extensive analysis of the entire MAP4 coding region by restriction fragment length polymorphism and single strand conformational polymorphism (Chiu, Bader, Minna, and Bulinski, unpublished observations) revealed few minor polymorphisms and point mutations. Thus, the differences within the MT-binding domains of MAP4 isoforms would have to arise from alternative splicing of MAP4 transcripts derived from a single-copy gene. To identify the chromosomal locus of the MAP4 gene, we performed in situ hybridization of a cDNA probe (clone Hfb1) to metaphase human chromosomes. As expected for a single-copy gene, we observed a single, discrete hybridization signal; this was localized to chromosome 3p21 (Figure 3A). In addition, human/CHO hybrid cells containing only human chromosomes 3 and 12 (Figure 3B) or chromosomes 3 and 6 (not shown) showed expression of human MAP4, as detected in Western blots or immunofluorescence with primate-specific MAP4 antibody (Figure 3B), as well as in RNase protection assays (not shown). These data are in agreement with earlier attempts to map the MAP4 locus using MAP4 immunore-

activity in a discordance analysis of human−mouse hybrid cell lines (Bulinski et al., 1980), in which it was concluded that the MAP4 gene(s) resided on chromosome 3, 8, or 10.

Expression of Single MAP4 cDNAs in Nonprimate Cells. The assembly-promoting MAP, tau, displays electrophoretic heterogeneity at the protein level; this is apparently contributed by alternative splicing of transcripts encoding both the MT-binding domain and other parts of the molecule, as well as by posttranslational modification [reviewed in Lee (1990)]. We hypothesized that the heterogeneity of the MAP4 protein pattern in electropherograms of cell extracts might result from the coexpression of isoforms of MAP4 that possess different MT-binding domains. A typical electrophoretic pattern of MAP4 expressed in human cell lines is shown in the immunoblot in Figure 4A. DU145 and HeLa cells each exhibit a major doublet of $M_r \sim 210\,000$, as well as a minor band of ~220 000 (Figure 4A, DU145 lane). Other primate cell lines, COS7 and TC-7, derived from African green monkey kidney, exhibit a slightly different MAP4 pattern, namely, a more widely spaced triplet centered around $M_r \sim 200\,000$, with an additional minor species of ~220 000. In order to test whether the synthesis of MAP4 proteins containing different forms of the MT-binding domain could give rise to the electrophoretic heterogeneity seen for MAP4, we examined MAP4 immunoblots of cells transfected with MAP4 cDNA clones. We transfected mouse Ltk$^-$ cells with DNAs encoding human MAP4 form I (three-repeat) or form III (four-repeat) under the control of the mouse mammary tumor virus promoter and prepared stable cell lines. We also prepared stable hamster (CHO) cell lines transfected with cDNA encoding human MAP4 form IV (five-repeat) under the control of the constitutive Rous sarcoma virus promoter. We analyzed the expression of transfected human MAP4 by probing Western blots of transfected cells with primate-specific MAP4 antibody.

As shown in Figure 4B, each of the three MAP4 isoforms expressed in transfected rodent cells gave rise to a doublet of MAP4 bands. The electrophoretic migration of each MAP4 doublet was similar to that of the HeLa MAP4 doublet shown in flanking lanes; untransfected rodent cells did not express primate MAP4, as expected. On these immunoblots, a low percentage of polyacrylamide (5%) was utilized in order to accentuate very slight differences in M_r within the ~200 kDa region. As shown in Figure 4B, the form I MAP4 species in the transfected cells manifested a slightly lower M_r than did form III, which in turn was lower (by approximately the same increment, as expected from their ~4 kDa difference in predicted molecular masses) than the M_r's of form IV and HeLa MAP4, which were indistinguishable from each other. Thus, in human cell lines, although the degree of electrophoretic heterogeneity observed does not provide information about which MAP4 isoform(s) is(are) expressed, slight differences in electrophoretic migration do provide this information. Additional evidence indicating which alternatively spliced MAP4 isoforms are expressed is derived from protein sequence data that we obtained from HeLa MAP4 (Figure 2; boxed sequence). The presence of this sequence in HeLa MAP4 protein demonstrates that either form II or form IV is expressed in this cell line, since forms I and III lack this sequence. However, for a more direct and convenient demonstration of which MAP4 isoforms are expressed in tissue culture cells and in tissues, we performed assays of MAP4-encoding transcripts, as described in the following.

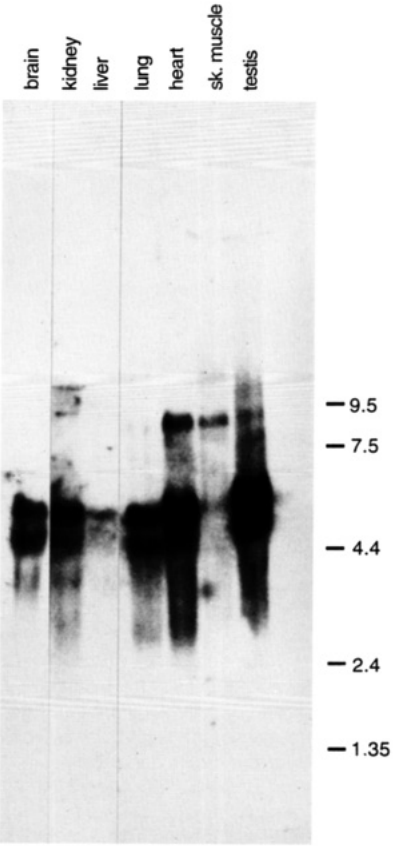

FIGURE 5: Northern blot analysis of rat tissues. Northern blot containing 25 μg of total RNA from rat brain, kidney, liver, heart, skeletal muscle, and testis and 10 μg of rat lung RNA was hybridized with a rat cDNA probe. Exposure for one-half the time interval of the other lanes was necessary for the brain RNA lane in order to show detail, since MAP4 RNA transcripts are very abundant in this tissue. Heterogeneous transcripts of ~5.5−6.5 kb are detected in all tissues except skeletal muscle and liver, in which the relative abundance of the ~5.5 kb transcript is reduced. Heart and skeletal muscle also contain a larger transcript (~8−9 kb); in skeletal muscle the ~9 kb species is the most abundant MAP4 message detected.

Cell and Tissue Distribution of Alternatively Spliced MAP4 Isoforms. (A) Northern Blots. Multiple MAP4 transcripts were previously reported on Northern blots of RNA from HeLa cells (Chapin & Bulinski, 1991a) and from mouse tissues (West et al., 1991). In order to study the relationship among the heterogeneous MAP4 cDNA clones we obtained, the transcripts we detected on Northern blots, and the heterogeneous protein species we observed in electropherograms, we hybridized Northern blots of several cell lines and rat tissues with MAP4 cDNA probes. Major RNA species of ~5.5−6.5 kb were detected in all cell lines (not shown) and in all rat tissues examined, except skeletal muscle (Figure 5). A minor amount of an ~8−9 kb MAP4 message species was also detected in heart, testis, and skeletal muscle; in the latter tissue, the large transcript was the most abundant message observed [Figure 5; also see West et al. (1991) for analogous results from several mouse tissues]. We also hybridized Northern blots with oligonucleotide probes complementary to the alternatively spliced, repeat-encoding exons; an oligonucleotide probe spanning the junction between repeats 1 and 4 did not specifically hybridize to any DU145 or L_6 cell transcripts (not shown), suggesting the transcripts

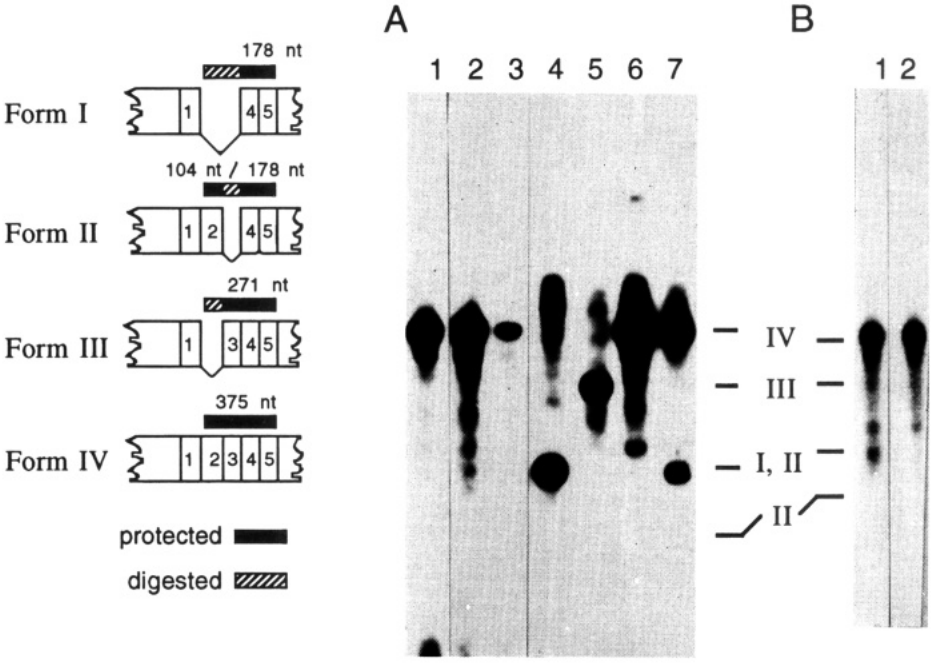

FIGURE 6: RNase protection assay of MAP4 isoforms in cell lines. Total RNA (30 μg, unless otherwise noted) was analyzed by an RNase protection assay, as described in Materials and Methods. The assay is illustrated schematically at left. Positions of fragments corresponding to forms I−IV are indicated. RNA samples assayed are as follows: (A) Lane 1, DU145; lane 2, HeLa; lane 3, HeLa, 10 μg; lane 4, sense RNA from Hfb2 (form I), 100 pg; lane 5, sense RNA from Hfb1 (form III), 100 pg; lane 6, sense RNA from Hfb6 (form IV), 100 pg; lane 7, sense RNA from Hfb2 and Hfb6, each 100 pg. (B) Lane 1, L_6, 10 μg; lane 2, NRK, 10 μg. Human and rat probes were used for parts A and B, respectively. The high molecular mass species (visible above the IV marking in lanes 4 and 5) are due to the presence of some large contaminating probe (see Materials and Methods for details). For all other experiments, electrophoretically purified small probe was used, in order to obtain the clearest results possible.

encoding the three-repeat isoform (form I) were not present at detectable levels in these two cell lines. In contrast, oligonucleotide probes corresponding to repeat 2 or 3, which are contained in forms II and IV or forms III and IV, respectively, hybridized with all of the same MAP4 transcripts as full-length cDNA probes, although the weaker signal obtained with oligonucleotide probes hampered the interpretation of these experiments (not shown). Although Northern blot data demonstrated the presence of heterogeneous MAP4 transcripts of two disparate size classes (~5.5−6.5 and ~8−9 kb), they provided only limited information on the expression of MAP4 isoforms. Therefore, in order to definitively resolve this issue, we performed RNase protection assays.

(B) RNase Protection Assays. RNase protection assays, utilizing a five-repeat (form IV) antisense RNA probe that spanned the alternatively spliced region, would allow one to determine unequivocally which isoforms of MAP4-encoding transcripts occur in a given cell or tissue type (see diagram in Figure 6). For RNase protection assays of human cell lines, an antisense RNA probe was prepared by in vitro transcription of the five-repeat-encoding clone (Hfb6), as described in Materials and Methods. Since a requirement of the RNase protection technique is that hybridization be complete, without even single base pair mismatches between probe and transcript, it was necessary to prepare a rat probe in order to analyze RNA isolated from rat cell lines and tissues. Accordingly, we prepared cDNA from the rat L_6 myogenic cell line and amplified the region encoding the MT-binding repeats by PCR. We then cloned a PCR product partially encoding the five-repeat isoform (form IV) of rat MAP4 and prepared an antisense probe by in vitro transcription.

Results expected from RNase protection assays are summarized schematically in Figure 6. Sense RNAs generated from clones Hfb6, Hfb1, and Hfb2 were used as positive controls for the five-, four-, and three-repeat isoforms of MAP4, respectively. Electrophoretic migrations of each of these fragments were used as size markers, as appropriate. By using the RNase protection technique with the rat probe, RNA transcripts encoding MAP4 isoforms are expected to give protected fragments as follows: form I, 178 nucleotides; form II, (two fragments) 104 and 178 nucleotides; form III, 271 nucleotides; and form IV, 375 nucleotides. Due to differences in the restriction sites used in cloning the 3′ ends of the rat and human templates, the rat probe should yield a protected fragment that is 28 nucleotides longer for each isoform than the fragments obtained with the human probe, with the exception of the 104-nucleotide form II fragment, which should be identical in length in both species (see Materials and Methods for details).

Results of RNase protection assays of cell lines suggested that only form IV RNA is expressed in proliferating cells. For example, as shown in Figure 6, form IV RNA was the major form detected in HeLa (cervical carcinoma), DU145 (human metastatic prostate tumor), L_6 (rat myoblast), and NRK (normal rat kidney fibroblast) cells. In other cell types, virtually identical results were obtained: in PC-12 (rat pheochromocytoma), CaCo2 (human intestinal epithelium), and H9C2 (rat cardiac myocyte) lines, form IV expression predominated (data not shown).

We also examined MAP4 forms expressed in eight rat tissues, using the RNase protection assay (Figure 7). In most tissues, form IV constituted the major, or only, form detected. However, heart, skeletal muscle, and brain contained roughly equivalent amounts of RNA encoding forms III and IV. In

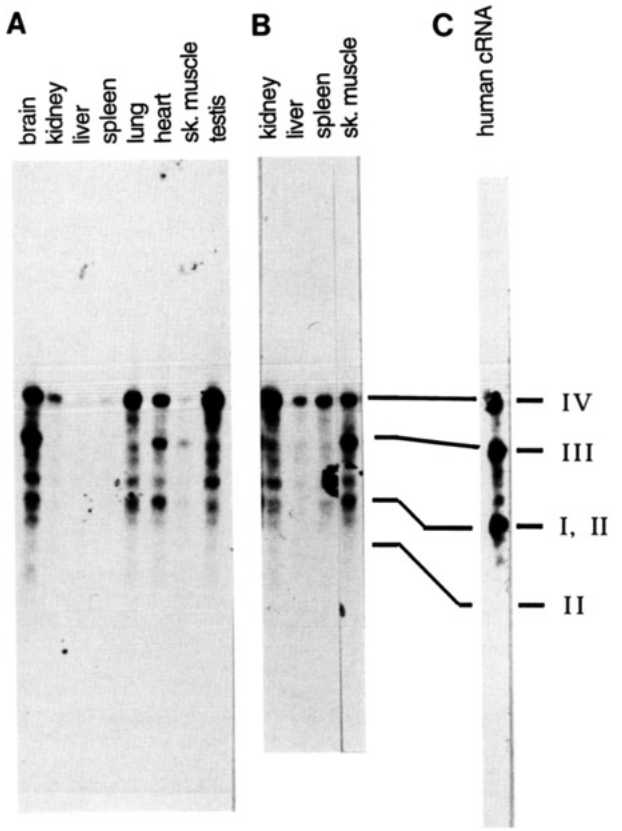

FIGURE 7: RNase protection assay of MAP4 isoforms in rat tissues. Total RNA from adult rats (30 μg) was analyzed by nuclease protection using the rat probe, as depicted in Figure 6. Positions of the fragments indicative of different isoforms are shown at right: (A) 5 h exposure; (B) 18 h exposure of kidney, liver, spleen, and skeletal muscle lanes. (C) A control RNase protection containing human forms I, III, and IV cRNA is shown (note that the human fragments are 28 nucleotides shorter due to a difference in the restriction site used in cloning human and rat templates; see Materials and Methods). Form IV is predominant in kidney, liver, spleen, lung, and testis, while form III can be seen in brain, heart, and skeletal muscle, and form I is present in heart and, as a more minor component, in brain, skeletal muscle, and lung.

addition, a band corresponding to the three-repeat isoform (form I) was detected in heart and, to a lesser extent, in brain, lung, skeletal msucle, and possibly testis. In no case did we detect a 104-nucleotide protected fragment that could have been attributed to the expression of form II RNA. In addition, when we used a larger, nontruncated RNA probe (see Materials and Methods), our results were qualitatively similar. In protection assays in which the large probe was used to assay brain, lung, and skeletal muscle RNA, we did not detect a 587-nucleotide fragment that would represent an unnicked hybrid composed of probe hybridized with form II RNA. In contrast, this longer probe did protect a 566-nucleotide fragment corresponding to an unnicked hybrid composed of form III RNA in brain and skeletal muscle, as well as a 680-nucleotide fragment corresponding to form IV RNA in all three tissues (data not shown).

We next addressed the question of whether alternative splicing of MAP4 transcripts is regulated temporally during development, as well as in a tissue-specific manner. Accordingly, we assayed RNA isolated from seven rat tissues at various developmental stages (Figure 8). In brain, heart, and skeletal muscle, form IV was the major species present at E15, while form III was first detectable 6 days after birth and either increased or remained approximately constant in level through adulthood. Assays of brain and skeletal muscle at postnatal day 1 and of heart at postnatal day 3 also showed that form III RNA was present at these early stages (data not shown). Form I RNA was a prominent species in heart as early as postnatal day 3; miniscule amounts of form I RNA could be detected in embryonic brain and skeletal muscle and also in lung. It is noteworthy that form I expression appears to be quite limited in embryonic rat brain, even though fully half of those cDNA clones encoding the MAP4 MT-binding domain that we isolated from a human fetal brain library encoded form I (Figure 1). In all other tissues, namely, kidney, liver, and spleen, form IV was the only MAP4 form detected at any developmental stage.

In our nuclease protection studies, we did not detect MAP4 form II, the other four-repeat isoform of MAP4 previously cloned by Aizawa et al. (1990), in any cell lines or in tissues at any developmental stage. There are several possible explanations for this: First, form II might be widely expressed among cells and tissue types, but this transcript might be present at very low levels relative to the transcripts encoding other MAP4 isoforms. Alternatively, form II might be expressed only at very early developmental stages, for example, at stages earlier than those we examined. Finally, it is possible that a relatively high level of expression of this isoform occurs only in certain species (e.g., in the bovine species) or in cell types that are not abundant in any rat tissue we examined. Aizawa et al. (1990) originally cloned form II from a library prepared from Madin−Darby bovine kidney (MDBK) cells, suggesting that this isoform might be abundant in all or in a subset of kidney polarized epithelia, at least in the bovine species. However, we did not detect MAP4 form II in RNA from whole rat kidney nor in the CaCo2 cell line, a human polarized epithelial cell line of intestinal origin. Further studies of developmental stages, tissues, and mammalian species not yet examined will be necessary to elucidate the expression pattern of MAP4 form II.

DISCUSSION

We previously showed that MAP4 contains an MT-binding domain possessing three repeated sequences that are homologous to sequences found within the nervous tissue MAPs, MAP2 and tau. In this paper, we report the isolation of cDNA clones encoding two additional forms of the MT-binding domain of MAP4. One isoform (form III, Figure 1B) contains four repeats; the additional repeat encoded by this clone closely resembles the alternatively spliced repeat present in adult-specific forms of tau and in a recently identified form of MAP2c (Doll et al., 1993). The other isoform we cloned contains five repeats (form IV); this form includes the tau-like repeat just described, as well as a repeat that is poorly conserved in sequence and contains a flanking interrepeat sequence that is peculiar in length and amino acid sequence. The unusual repeat was originally reported as the second repeat found in a four-repeat isoform (form II) of MAP4 encoded by bovine cDNA clones [Aizawa et al., 1990; MAP4 clones encoding five repeats were previously cloned by West et al. (1991)]. Thus, four different isoforms of the MAP4 MT-binding domain have been identified to date, and we have isolated cDNA clones encoding three of the four isoforms. All MAP4 isoforms appear to be encoded by a single gene located, in humans, on chromosome 3, and they

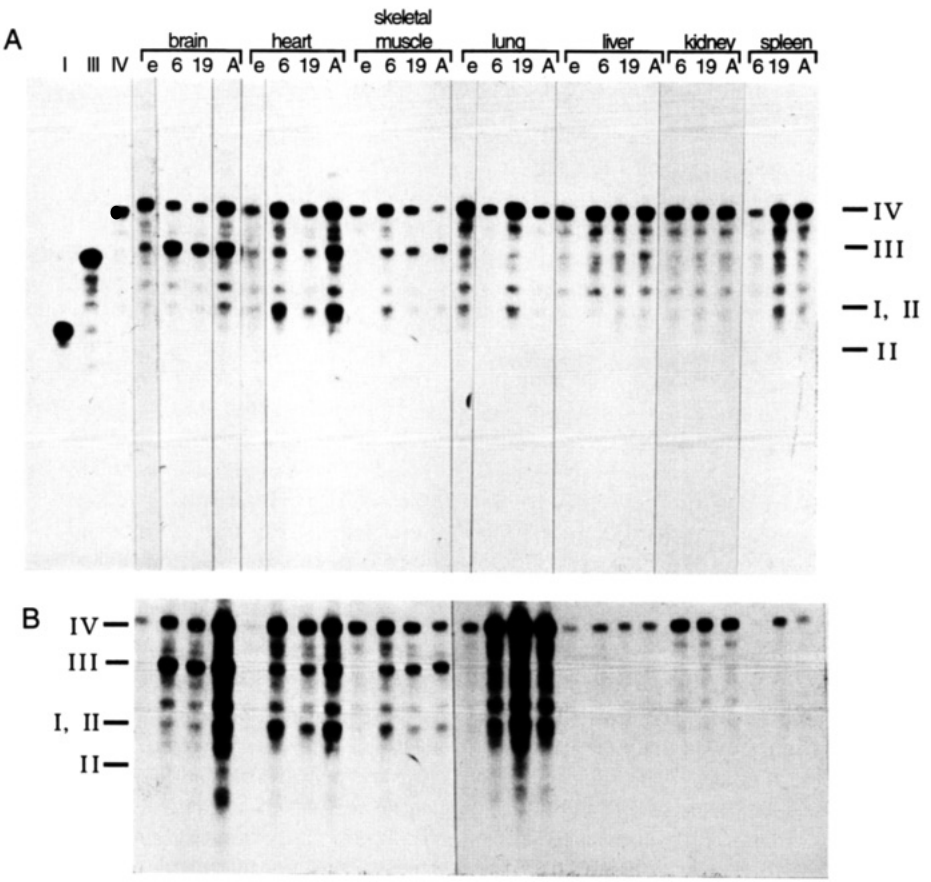

FIGURE 8: Analysis of MAP4 isoform expression during development of rat tissues. RNA was isolated from rat tissues at the indicated stages of development: embryonic day 15 (e); postnatal days 6 and 19; and adult (A). Thirty micrograms of each sample was analyzed by nuclease protection, as described in Figure 6. Lanes marked I, III, and IV contain control protections with human forms I, III, and IV RNA, respectively; note that each human protected fragment is 28 bp shorter than the corresponding rat protected fragment. (A) Different exposures (4, 11, or 36 h) are shown for different sample lanes, in order to normalize form IV band intensity and to permit comparison with the intensities of bands due to other expressed forms. Exposures are as follows: embryonic day 15 (e) for brain, heart, and lung and all lanes for liver and spleen, 36 h; brain 6 and 19, heart 6, 19, and A, and all lanes for skeletal muscle and kidney, 11 h; brain A and lung 6, 19, and A, 4 h. (B) Single exposure (11 h) of all rat tissue samples from the same experiment, in order to allow a comparison of relative MAP4 RNA abundance from sample to sample. Positions of fragments corresponding to each rat isoform are indicated on the right for A and on the left for B. Note that, after birth, form III expression is increased in brain, heart, and skeletal muscle, and form I expression is increased in heart.

appear to be derived by alternative splicing of two exons, each of which encodes a single repeat and an interrepeat sequence.

We used RNase protection assays to examine the distribution of RNAs encoding each of the MAP4 isoforms in tissues and cell lines. In a variety of proliferating cultured cell lines of human and rat origins, in rat liver, spleen, kidney, and lung, and in human/CHO hybrids containing human chromosome 3 (not shown), we detected only RNA encoding the five-repeat isoform of MAP4 (form IV). RNase protection results are consistent with Northern blot data, which suggest that each of the multiple MAP4 transcripts seen in most cell lines and tissues encodes five repeats within its MT-binding domain.

We found that brain, heart, and skeletal muscle, unlike other tissues or proliferating cells, contain MAP4 form III, the tau-like four-repeat isoform, in addition to form IV. Moreover, expression of form III increases during the development of these tissues. In brain and skeletal muscle, form III is increased relative to form IV shortly after birth. In heart, form III RNA is expressed at a moderate level through postnatal day 19, but is present at a higher level in the adult. Form I RNA was most plentiful in heart, where its expression appeared to increase after birth; it was less abundantly expressed in brain (from which it was cloned) and lung.

Expression of the tau-like four-repeat form of MAP4 (form III) in adult brain and muscle tissues correlates with the occurrence of stable MTs during and following differentiation in those tissues [reviewed in Bulinski and Gundersen (1991)]. The other AP-MAPs may provide interesting paradigms here, because expression of the adult (four-repeat) tau isoform has been correlated with advanced development and increased MT stability in neurons (Lee, 1990), while expression of a four-repeat isoform of MAP2c has been correlated with glial cell differentiation (Doll et al., 1993). While we do not yet know the efficacy with which the various isoforms of MAP4 stabilize MTs, our preliminary transfection studies demonstrate that all MAP4 isoforms stabilize MTs in vivo (Lue, Chapin, and Bulinski, manuscript in preparation), as might be expected from MAP4's properties in vitro (Bulinski & Borisy, 1990b) and from its striking homology with MAP2 and tau. By analogy with tau, it is likely that the four-repeat isoform (form III) of MAP4 is a more potent MT stabilizer than form I. The second of the five repeats present in form IV, which also serves as the second of the four repeats contained in form II, is unusual because of its sequence divergence and the length of its interrepeat sequence (Chapin

& Bulinski, 1992). Given its unusual characteristics, it is possible that this repeat actually *detracts* from MAP4's capacity to stabilize MTs. According to this scenario, form III would actually be a more potent stabilizer of MTs than either form II or IV; this would be consistent with the predominance of form IV in proliferating cell lines and in many tissues in which MTs are known to be highly dynamic. Moreover, given the tissue distribution and developmental expression pattern of form III, it is possible that this isoform contributes to increased MT stability during differentiation of myoblasts in a manner analogous to the developmental switch of tau isoforms in neurons (Goedert et al., 1989). However, differential functions of MAP4 isoforms cannot be established until the efficacy of each MAP4 isoform to stabilize MTs has been compared carefully, both in vitro and in vivo.

A high level of expression of MAP4 has previously been noted in developing neurons of the rat, and changes in MAP4 protein heterogeneity have been examined during the course of rat brain development (Riederer & Matus, 1985; in these studies MAP4 was called MAP3). Riederer and Matus showed that a MAP4 species with slightly lower M_r appeared at birth and that this species, as well as a constitutively expressed MAP4 species of higher M_r, persisted through adulthood. In addition, it has been showed that both of these protein species are expressed in rat glia as well as in neurons (Huber et al., 1985). While the appearance of this additional MAP4 electrophoretic species is temporally correlated with the induction of form III RNA expression, at present this is only a correlation.

It is interesting to note that a 10-fold decrease in MAP4 expression was previously measured during prenatal rat brain development, and this decrease was ascribed to a decrease in the amount of MAP4 in neurons (Riederer & Matus, 1985). Our RNase protection studies indicate that expression of the four-repeat MAP4 (form III) transcript precedes expression of the four-repeat tau transcript; the latter begins to be expressed in rat brain at approximately postnatal day 8 (Kosik et al., 1989). The adult isoform of MAP2 (MAP2A), both transcript and protein, is also increased in expression during late embryonic development, concomitant with the decrease in MAP4 abundance in neurons (Riederer & Matus, 1985). Thus, it is likely that MAP4 is not coexpressed with either MAP2 or tau in neurons. MAP4 might play a role in the initial events of MT stabilization in neurons, during early stages of differentiation, before the adult neuronal MAPs are expressed. In contrast, in glial cells, it is possible that MAP4 could function concomitant with MAP2c, while in skeletal and cardiac muscle, in which MAP2 and tau are never expressed at any stage of development, MAP4 might constitute the primary MT-stabilizing factor duing the entire process of differentiation.

The RNase protection data we have presented here suggest that MAP4 abundance may be subject to posttranscriptional regulation. For example, MAP4 protein levels have been reported to decrease during rat brain development (Riederer & Matus, 1985), yet we did not observe a coincident decrease in MAP4 RNA levels. Further, MAP4 RNA is relatively rare in liver and relatively abundant in kidney (Figures 5−8), even though MAP4 protein abundance shows the reverse pattern (abundant in liver and present in trace amounts in kidney; Huber & Matus, 1990; Faire and Bulinski, unpublished observations).

In this study, we attempted to correlate MAP4 expression in embryonic muscle development with differentiation of the L_6 myogenic cell line. Although we detected abundant expression of MAP4 form III in muscle tissue during in vivo development (postnatal day 6, Figure 8), we detected only a small quantity of form III in cultured L_6 myoblasts before differentiation (Figure 6B, lane 1), and we noted no substantial increase during 14 days of differentiation (data not shown). Perhaps the low expression of form III in L_6 cells is a peculiarity of the L_6 cell line, or perhaps it requires a higher degree of differentiation than we achieved in these cultures. We previously showed that dramatic MT stabilization takes place during early stages of myogenesis (Gundersen et al., 1989); MAP4 form III might not contribute to this initial MT stabilization. On the other hand, in differentiating L_6 cells, we were able to detect the 8 kb MAP4 message characteristic of mature muscle, suggesting that unusual MAP4 transcripts—and perhaps MAP4 protein species—are expressed during differentiation of L_6 myoblasts. Taken together, our Northern blot and RNase protection data suggest that there is no correlation between the different size classes of MAP4 transcripts (~5.5−6.5 and ~8−9 kb) and the different MT-binding domains (forms I, III, and IV), that is, messenger RNA encoding each isoform appears to exist within each size class of RNA. We are currently investigating the possibility that a MAP4 isoform translated from the large mRNA expressed during muscle development might be functionally distinct, perhaps making a special contribution to the morphogenetic events of myogenesis.

Knowledge of the exact pattern of expression of each MAP4 isoform would permit us to formulate testable hypotheses of isoform-specific functions. However, to date, we have not detected expression of form II in any samples we assayed, and the only developmental change in form I expression we noted was in heart tissue. Further details of expression patterns of each isoform might be derived from further analysis. Our RNase protection studies have been limited to the assay of pure cell types and whole tissues at discrete stages; in situ analysis of MAP4 isoform expression might reveal expression patterns that are temporally or spatially more dramatic or might reveal populations of cells within a tissue that express an unusual MAP4 isoform.

In addition to the two alternatively spliced exons we identified in the region encoding MAP4's MT-binding domain, we also identified an alternatively spliced exon at the 3′ end of the coding sequence. Removal of this exon results in an alternative C-terminus, although this is a sequence difference of only two amino acid residues (SI vs N). Preliminary data suggest that the longer isoform (whose C-terminus is SI) is the predominant form expressed in HeLa and CaCo2 cells, while we detected expression of both isoforms in a number of rat tissues assayed by RNase protection (data not shown). At this time, we do not know whether the alternative C-termini encoded in MAP4 transcripts are functionally significant.

The electrophoretic doublet of MAP4 species produced from a single transfected cDNA (see Figure 4B) is striking and is consistent with the fact that most cell lines exhibit a doublet of MAP4 electrophoretic species, while they contain only a single MAP4 RNA transcript (encoding form IV). The generation of more than one distinct protein species from a single cDNA is also reminiscent of results obtained in transfection experiments performed with cDNAs encoding

tau (Kanai et al., 1988). Multiple species produced from either a transfected cDNA or an endogenous transcript may be due to alternative translational initiation sites within the cDNA or to posttranslational modification(s) such as phosphorylation. Heterogeneity in MAP4 proteins expressed from single cDNAs in bacteria supports the former idea (Bulinski, unpublished observations). MAP4 phosphorylation accompanying mitosis has been demonstrated to cause an observable decrease in mobility on SDS−PAGE (Vandre et al., 1991). Phosphorylated MAP4 in mitotic cells also migrates as a doublet, in which each band possesses a higher M_r than the M_r = 210 000 doublet one observes in asynchronous or interphase cells (Chapin and Bulinski, unpublished observations). Thus, the heterogeneous MAP4 species is most likely derived from alternative translational start sites and posttranslational phosphorylation, as well as alternatively spliced MAP4 transcripts. Currently, distinct roles that might be hypothesized for heterogeneous MAP4 forms or MAP4 differentially phosphorylated during the cell cycle [e.g., Vandre et al. (1991)] have yet to be established. However, changes in both the expression and modification of MAP4 forms have the potential to modulate MT dynamics or functions in response to cellular signals that occur concomitant with motility, the cell cycle, or morphogenetic events.

ADDED IN PROOF

Sequences shown in Figure 2 were submitted to Genbank on January 10, 1995.

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