

of copies of Ty-*TRP1* cassettes per genome ranged from 1 to 10 or more, averaging 2.3.

The number of copies of Ty-*neo*, Ty-*TRP1*, and Ty-*lacO* cassettes incorporated per genome is not very different, although the number of nucleotides inserted into the Ty in these constructs is very different (Table 2). Presumably, the packaging limit for Ty RNA is considerably larger than 7 kb. Inserts as large as 2.3 to 2.7 kb have been successfully transposed (9, 10). The size limit to DNA that can "hitchhike" on Ty elements remains to be determined.

These findings have many potential applications. (i) They provide a convenient phenotypic assay for transposition that does not rely on Southern blotting, allowing many colonies to be screened for genetic transposition defects in both the transposon-carrying plasmid and in the host, particularly when combined with 5-fluoro-orotate selection, which selects against the *URA3* marker in the plasmid (11). (ii) This approach may be useful in screening for pharmacological agents that interfere with transposition. (iii) Plasmid pGTyH3-*neo* (and similar plasmids) have been used as transposon tags in cloning yeast genes (12). (iv) These pGTy plasmids should be useful in constructing yeast strains bearing multiple dispersed copies of useful genes. Vectors specifically designed for such applications are being developed; a pGTyH3 vector carrying an *ARG3* promoter-*Escherichia coli galK* cassette supported its transposition (10). Quantitation of galactokinase activity (performed in *gal1Δ* strains) suggests that these cassettes are expressed well; four different strains suffering single insertions of a TyH3-*ARG3-galK* cassette produced 12,400, 12,300, 23,800, and 20,400 units of galactokinase per milligram of protein; a control cassette (not imbedded in a Ty) on a centromeric plasmid produced 22,200 units per milligram of protein (10). (v) Amplification of useful genes with pGTy plasmids may be more desirable than with high copy number plasmids, particularly in large-scale fermentations where instability can cause problems. Preliminary studies indicate that strains containing 20 or more copies of marked chromosomal Ty elements are stable for dozens of generations by Southern blot pattern (8); mitotic loss of inserted sequences by  $\delta$ - $\delta$  recombination or gene conversion events does not invalidate this approach. Genes may need special engineering to allow maximal expression within the confines of a Ty element, although *neo* and *TRP1* are expressed after transposition. Their expression could presumably be increased further by fusion to a strong promoter prior to its insertion into the pGTy plasmid.

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14. The *TRP1* gene in p981 was derived from Yrp7 (15) as follows: Yrp7 was digested with Pst I, releasing *TRP1* coding sequences and flanking vector sequences. "Slow" BAL-31 (International Biotechnology) was used to produce deletions extending into the 3' end of *TRP1* (removing termination signals). Bam HI linkers were added; the DNA was cut by Eco RI and Bam HI, releasing the *TRP1* fragment. The *TRP1* fragment was ligated into plasmid pSP64 (Promega Biotech) previously digested with Eco RI and Bam HI. Selection for Trp<sup>+</sup> transformants in *E. coli* MH5 (*pyrF::Tn5*, *trpC9830*, *lacX74*) ensured that only deletions expressing functional *TRP1* would grow. This selection produced p981; its deletion extends to *TRP1* position 722 [coordinates from (15)] and, therefore, deletes the last codon of *TRP1*.
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16. We thank N. Grindley for providing pGH54, M. Hall for providing MH5, and D. Garfinkel for helpful discussions. Supported in part by NIH grants GM36481 (J.D.B.) and GM35010 (G.R.F.), in part by a Searle Scholars Program award (J.D.B.), and by an American Cancer Society professorship (G.R.F.).

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## Long-Term Facilitation in *Aplysia* Involves Increase in Transmitter Release

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In a variety of vertebrates and invertebrates, long-lasting enhancement of synaptic transmission contributes to the storage of memory lasting one or more days. However, it has not been demonstrated directly whether this increase in synaptic transmission is caused by an enhancement of transmitter release or an increase in the sensitivity of the postsynaptic receptors. These possibilities can be distinguished by a quantal analysis in which the size of the miniature excitatory postsynaptic potential released spontaneously from the presynaptic terminal is used as a reference. By means of microcultures, in which single sensory and motor neurons of *Aplysia* were plated together, miniature excitatory postsynaptic potentials attributable to the spontaneous release of single transmitter quanta from individual presynaptic neurons were recorded and used to analyze long-term facilitation induced by repeated applications of 5-hydroxytryptamine. The results indicate that the facilitation is caused by an increase in the number of transmitter quanta released by the presynaptic neuron.

LONG-LASTING SENSITIZATION OF the gill withdrawal reflex in *Aplysia* involves a corresponding enhancement of the synaptic transmission between siphon sensory and gill motor neurons (1, 2). This long-term facilitation of synaptic transmission could be achieved in two ways. (i) The properties of the postsynaptic cell could change so that it could respond more effectively to the same amount of transmitter. Such changes could involve an increase in the affinity or density of the receptors for the transmitter or an increase in the input resistance of the postsynaptic neuron. (ii) The presynaptic cell could be altered to release more transmitter. This could be accomplished by increasing the amount of transmitter released from a constant number of synaptic contact points or by forming, as

a result of growth, new release sites between the pre- and postsynaptic neurons. To distinguish between these two possibilities, we have studied the synapses between siphon sensory and gill motor neurons in *Aplysia*. These neurons are involved in mediating the gill withdrawal reflex, and long-lasting facilitation of their synaptic connections contributes to long-term behavioral sensitization, an elementary form of long-term memory (1, 2).

To determine whether the change in synaptic strength at this connection involves an increase in transmitter release, we have carried out a quantal analysis. Such an analysis

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is greatly strengthened if the unitary miniature synaptic potentials, which represent spontaneous quantal release from a single presynaptic neuron, can be detected. In vivo, postsynaptic cells typically are innervated by many presynaptic neurons, all of which release transmitter spontaneously, making it difficult to distinguish the spontaneous release from any one presynaptic neuron. However, the sensory and motor neurons of *Aplysia* are large and readily identifiable on the basis of their size, position, and axonal arborization. It is therefore possible to isolate them individually and to grow a single sensory neuron and a motor neuron together in dissociated cell culture where they readily form synapses (3). This has enabled us to record the spontaneous miniature excitatory postsynaptic potentials (mepsps) released by single sensory neurons, uncontaminated by release from other sensory or interneuronal cells. The synapses formed between cultured neurons undergo short-term facilitation lasting minutes after a single application of 5-hydroxytryptamine (5-HT) in the bathing medium (4), and long-term facilitation, lasting at least 1 day, after four or five consecutive applications of 5-HT (5). The cultured neurons therefore offer a useful preparation for studying long-term synaptic facilitation.

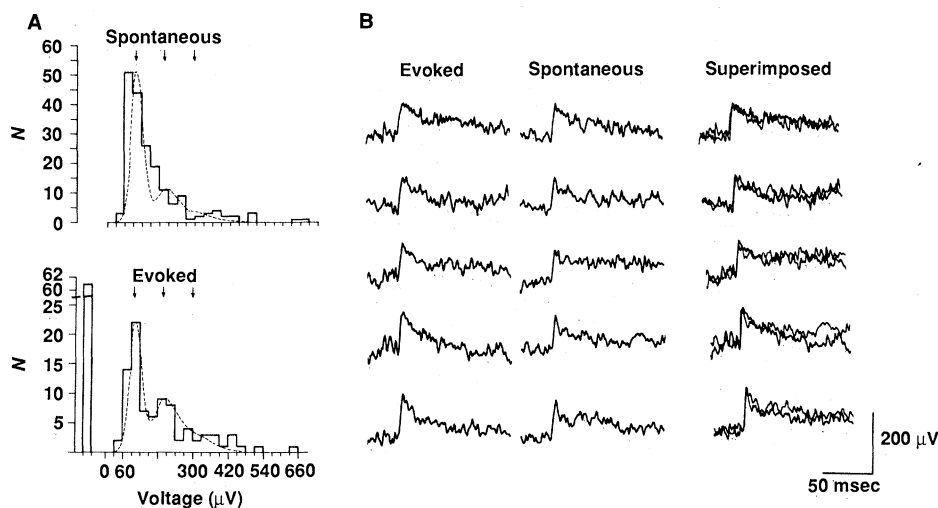
In cocultures of single sensory and motor cells, mepsps could readily be observed (6) and unambiguously attributed to release from a single neuron. Their frequency of occurrence could be increased by depolarizing the sensory neuron. Moreover, the mepsps are not due to the generation of spontaneous action potentials in the terminals of the sensory neuron. Cultured sensory neurons do not fire spontaneously or undergo spontaneous fluctuations of membrane potential. Furthermore, the mepsps persisted in the presence of concentrations of tetrodotoxin (TTX) high enough to block the generation of action potentials. Thus, the mepsps seem to represent, like the miniature end-plate potentials of the neuromuscular junction, the spontaneous release of transmitter quanta from the presynaptic terminals.

The mean amplitudes of the spontaneous mepsps ranged from 92 to 156  $\mu\text{V}$  (7). To examine whether the quantal event underlying the mepsp is also the elementary unit of evoked release, we determined whether the synaptic potential evoked by action potentials in a sensory neuron is made up of integral multiples of these spontaneous mepsps. Five experiments were therefore carried out under conditions of low release (5 to 7 mM  $\text{Ca}^{2+}$ , 120 to 165 mM  $\text{Mg}^{2+}$ ) in

which the mepsps released spontaneously from a single cell were compared to the synaptic potentials evoked by single action potentials in the same cells (8). Under conditions of low release, each action potential in the presynaptic neuron led to the synchronous release of only a few quanta. In all five cases, the mean amplitude of the unitary evoked excitatory postsynaptic potential (EPSP), the EPSP containing only one quantum of transmitter, did not differ significantly from that of the spontaneous mepsp (Fig. 1A). The spontaneous mepsps were similar in shape and size to the evoked EPSPs and could be superimposed on them almost exactly (Fig. 1B). There were no significant differences between the mean rise and half-fall times of the two sets of synaptic potentials. Evoked EPSPs that were significantly larger than the unit size were integral multiples of the unit, since they were, for example, two or three times the amplitude of the unitary EPSP (Fig. 1A, bottom). Following the procedure of Boyd and Martin (9), we used the Gaussian distribution of the amplitude of the spontaneous mepsps to generate theoretical distributions of the amplitude distribution of the evoked EPSPs. We obtained good correspondence between these theoretical and the observed distributions by assuming that the evoked EPSPs consisted of integral multiples of underlying unitary events identical to the mepsp (Fig. 1A, bottom). We therefore conclude that the sensory neuron-motor neuron synapse resembles the neuromuscular junction in that the same quantal release event seems to underlie both the spontaneous mepsps and evoked EPSPs under conditions of low release and that evoked EPSPs seem to consist of a summation of integral multiples of this quantal unit.

The ability to examine spontaneous quantal release enabled us to study the mechanisms of the long-term facilitation by comparing the amplitude of the spontaneous mepsps and the evoked synaptic connection between sensory neurons and motor neurons, before and 1 day after five applications of 1  $\mu\text{M}$  5-HT (each application lasted 5 minutes and was separated by 15 minutes of washing) (10).

Confirming earlier work, we found that the repeated applications of 5-HT resulted in a facilitation of the evoked EPSP (Fig. 2A). In no case was the amplitude of the spontaneous mepsps significantly altered (Fig. 2, A and B). Overall the evoked EPSP was facilitated by 91% (SEM 25.7%,  $n = 12$ ), whereas the amplitude of the mepsps showed a statistically insignificant change of 1.5% (SEM 1.84%,  $n = 12$ ). This direct measurement of spontaneous release before and after facilitation indicates



**Fig. 1.** A comparison, at the same synapse, of spontaneous mepsps and EPSPs evoked in a medium containing 7 mM  $\text{Ca}^{2+}$  and 120 mM  $\text{Mg}^{2+}$ . (A) Comparison of the amplitude histograms for the mepsps and evoked EPSPs shows that the mean amplitude of the first peak is the same. Both distributions have smaller peaks occurring at 200 and 300  $\mu\text{V}$ , which appear to represent the release of two and three quanta of transmitter simultaneously. The smooth line is a curve that was computed on the assumption that each peak could be fitted by a Gaussian distribution and was obtained by adding three Gaussian distributions having means of 100, 200, and 300  $\mu\text{V}$  and standard deviations of 20, 40, and 60  $\mu\text{V}$ , respectively. The relative weight of each distribution was determined empirically to give the best visual fit, and we attribute no significance to the relative proportions of each peak. The arrows indicate the mean amplitude of each of the three peaks. The first column in (A) (bottom) represents the number of failures of synaptic transmission. (B) Comparison of individual mepsps and evoked EPSPs, recorded from the same cells in (A). They are very similar in shape and superimpose almost exactly. The evoked EPSPs had a mean rise time of 5.3 msec (SD = 1.1 msec,  $n = 15$ ) and half-fall time of 9.7 msec (SD = 3.22 msec,  $n = 15$ ) and did not differ significantly from the spontaneous mepsps, which had a rise time of 4.3 msec (SD = 1.73 msec,  $n = 19$ ) and a half-fall time of 9.1 msec (SD = 3.19 msec,  $n = 19$ ).

that long-term synaptic facilitation does not involve a change in the amplitude of the spontaneously occurring mepsp.

The amplitude of a synaptic potential can be expressed as a product of the mean amplitude of the mepsp and the number of mepsp that contribute to the EPSP (the quantal content) (11). Since the amplitude of the mepsp remains constant as the average amplitude of the EPSP increases, this result implies that the quantal content of the EPSP increases with long-term facilitation and that the sensory neuron releases more transmitter. To test this possibility further and to estimate the quantal content of the EPSP, we next examined the quantal distribution of the evoked release of transmitter before and after exposure to 5-HT. In seven of the above experiments, we also examined the amplitude distributions of EPSPs evoked under conditions of low release (5 to 7 mM  $\text{Ca}^{2+}$  and 120 to 165 mM  $\text{Mg}^{2+}$ ) (8). The synaptic potential evoked by the first stimulus to the sensory neuron was facilitated by an average of 109% (SEM 41.6%,  $n = 7$ ), and the mean amplitude of the whole distribution of evoked EPSPs was increased by 49% (SEM 14.3%,  $n = 7$ , Fig. 2C) (12). Nonetheless, there was no significant change in the amplitude of the spontaneous mepsp. We obtained an estimate of the quantal content of the mean evoked EPSP by dividing the mean amplitude of the EPSP by the mean amplitude of the spontaneous mepsp. In every case, long-term facili-

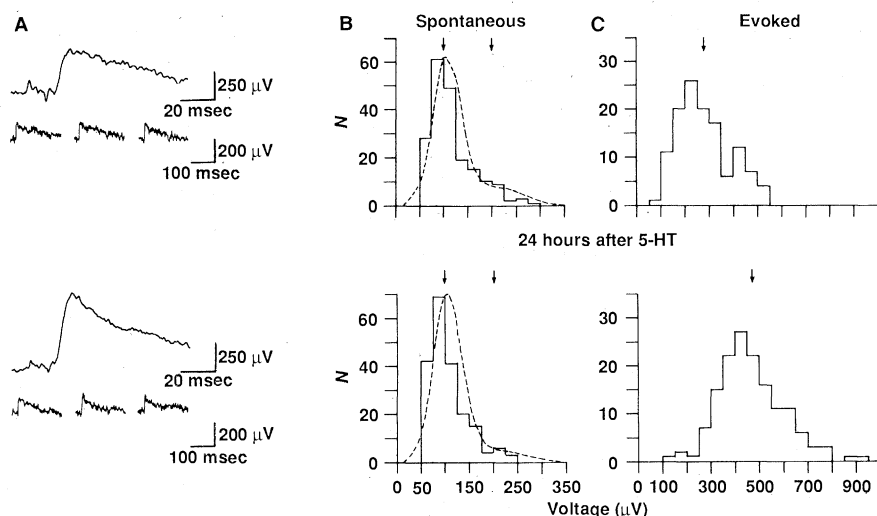
tation was accompanied by a comparable increase in quantal content (mean 50%, SEM 14.0%,  $n = 7$ ). Independent support for this finding came from an analysis of failures. In five other experiments under low-release conditions, the amplitude of the unitary evoked EPSP did not change significantly with long-term facilitation. However, there was a reduction in the number of failures in synaptic transmission (mean reduction 85%, SEM 8.9%,  $n = 5$ ). This further indicates that the quantal content of the EPSP was increased.

The evoked EPSP appears to be made up of elementary units identical to the spontaneous mepsp. Since the depolarization produced by the release of a single quantum of transmitter does not change significantly with long-term synaptic facilitation, the increase in the size of the evoked EPSP produced by the facilitation cannot be accounted for by a change in the amplitude of the underlying unitary mepsp. This suggests that neither the sensitivity of the postsynaptic receptors to the transmitter nor the density of the postsynaptic receptors changes with long-term facilitation. Since the quantal content of the evoked EPSP consistently increased with facilitation, we conclude that long-term synaptic facilitation involves an increase in the amount of transmitter released by the sensory neuron. These results are similar to those obtained for facilitation of the crustacean neuromuscular junction (lasting minutes to a few hours). Here also

the amplitude of spontaneous mepsp does not change with facilitation, which is associated instead with an increase in quantal content (13).

Short-term facilitation of the *Aplysia* sensorimotor synapse also involves an increase in transmitter release (14). The short- and long-term modifications of this synapse therefore share a common locus and may, in consequence, share aspects of similar underlying mechanisms. However, the short- and long-term enhancements differ in one important respect: the long-term enhancement of synaptic transmission requires protein synthesis, whereas the short-term does not (5).

Our experiments do not, however, address other issues. In particular, our data cannot determine whether growth of more synaptic contact points might accompany the facilitation. Unless the new contact points evoked mepsp different in amplitude or time course (15) from the old, the method of analysis that we have used here would not detect them, and they would be manifest solely as an increase in the quantal content of the EPSP. Indeed, since in the intact animal prolonged training for long-term behavioral sensitization does seem to evoke morphological change in sensory neurons, including an increase in the number of varicose expansions (16), it would seem quite possible that long-term facilitation of the synapses of the cultured neurons also involves growth of new synaptic contact points.



**Fig. 2.** Long-term facilitation involves an increase in quantal content. (A) Repeated applications of 5-HT caused a long-lasting increase in the EPSP (evoked in a saline solution containing 165 mM  $\text{Mg}^{2+}$  and 5 mM  $\text{Ca}^{2+}$ ) of 51%, sample mepsp are shown underneath each EPSP. (B) The mean amplitude of the mepsp did not change significantly with facilitation, being 106  $\mu\text{V}$  before 5-HT treatment (SEM 2.2,  $n = 179$ ) and 100  $\mu\text{V}$  24 hours later (SEM 2.3,  $n = 190$ ). In both histograms, the smooth line was obtained by adding two Gaussian distributions with means of 100 and 200  $\mu\text{V}$  and standard deviations of 30 and 60  $\mu\text{V}$ , respectively. The arrows indicate the mean amplitude of the two peaks. (C) The distribution of the evoked EPSPs before and after long-term facilitation recorded from the same neurons. Before facilitation the mean of the distribution (indicated by arrows) was 279  $\mu\text{V}$  (SD 108  $\mu\text{V}$ ) and 24 hours later the mean had increased to 467  $\mu\text{V}$  (SD 135  $\mu\text{V}$ ). The quantal content of the EPSP had therefore increased by 78%.

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6. Standard electrophysiological techniques were used for recording from the cultured neurons. All results were stored on a seven-channel FM tape recorder (Racal Store 7DS); the records from the motor neuron were filtered at 300 Hz, 3 dB. Microelectrodes were filled with an electrolyte that consisted of 1M potassium acetate with 50 mM KCl adjusted to pH 7 with HCl. These electrodes had a starting resistance of 20 to 35 megohms, and those used to record from the motor cell were beveled to a resistance of 12 to 25 megohms to improve their electrical characteristics. The cells were recorded in a medium that consisted of equal volumes of artificial seawater mixed with L15 culture medium (with added salts to adjust it to marine saline conditions). *Aplysia* hemolymph was then added to this medium to give a final concentration of 2.5%.
7. In most experiments large spontaneous mepsp could occasionally be observed. These large mepsp

seemed to be integral multiples of the unitary mepsp, and we interpret them as the simultaneous release of more than one quantum of transmitter. In some experiments there were enough of the larger mepsp to form extra peaks in the amplitude histogram. In such cases the subsidiary peaks came out as multiples of the first main peak of the histogram, and the mean of the first peak was obtained by calculating the mean of all mepsp that had amplitudes within an upper limit that was to be chosen to include at least 2 standard deviations of the mean (that is, at least 98% of the Gaussian distribution forming the first peak).

8. Low-release salines were obtained by mixing isotonic  $MgCl_2$  in the appropriate proportions with the recording medium. The EPSPs were evoked at 10-second intervals, and 100 to 200 stimuli were delivered to the sensory neuron.
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facilitation was measured by comparing the amplitudes of the first EPSP evoked by intracellular stimulation of the sensory neuron before and 24 hours after 5-HT treatment. At least 5 minutes were allowed to elapse between the penetration of the sensory neuron and the measurement of the EPSP to allow any possible effects of impalement (such as injury discharge) to dissipate. Spontaneous mepsp were observed for a period of 10 to 20 minutes. All potentials were measured at the resting potential on the first day. If the resting potential recorded on the second day differed from that on the first by more than 5 mV, dc current was injected to move it to the same level.

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## The Primary Structure and Heterogeneity of Tau Protein from Mouse Brain

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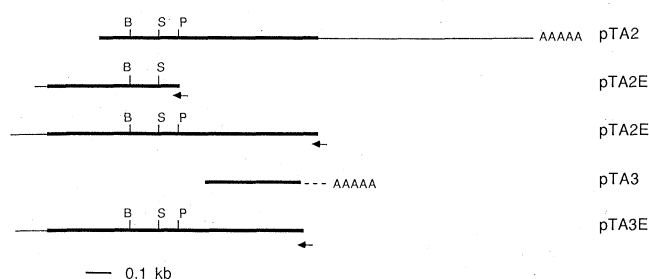
Tau protein is a family of microtubule binding proteins, heterogeneous in molecular weight, that are induced during neurite outgrowth and are found prominently in neurofibrillary tangles in Alzheimer's disease. The predicted amino acid sequences of two forms of tau protein from mouse brain were determined from complementary DNA clones. These forms are identical in their amino-terminal sequences but differ in their carboxyl-terminal domains. Both proteins contain repeated sequences that may be tubulin binding sites. The sequence suggests that tau is an elongated molecule with no extensive  $\alpha$ -helical or  $\beta$ -sheet domains. These complementary DNAs should enable the study of various functional domains of tau and the study of tau expression in normal and pathological states.

**M**ICROTUBULES ARE ASSEMBLED from tubulin, which is a dimer of two polypeptides that are members of distinct multigene families (1). A high degree of conservation exists within these families and the various polypeptides form copolymers in vivo and in vitro (2). Despite this similarity of tubulin polypeptides, microtubules exhibit much diversity in structure and function, suggesting that other proteins must be present that determine the properties of different microtubules. Among the factors thought to regulate microtubule structure and function are the microtubule-associated proteins (MAPs) that copurify with microtubules (3). Two major classes of MAPs have been identified from vertebrate brain: high mo-

lecular weight MAPs and tau protein. Tau protein promotes microtubule assembly in vitro and limits the growing and shrinking phases of dynamic microtubules (4). Tau co-

localizes with microtubules in cells (5) and is induced along with MAP1 during neurite outgrowth from rat pheochromocytoma cells (6). Microinjected tau protein increases tubulin polymerization and decreases the rate of microtubule depolymerization, suggesting that tau protein can regulate microtubule assembly in vivo (7).

A striking feature of tau protein is its extensive heterogeneity. In adult porcine brain, it is comprised of at least four related phosphoproteins, 55,000 to 62,000 daltons in size (8). Tau proteins were initially thought to be the result of artifactual proteolysis of a common precursor protein; however, translation of messenger RNA (mRNA) in vitro shows that this is not the case (9). Other proteins reacting with tau antibodies have been detected in brain, neuroblastoma cells, spinal ganglia, and coated vesicles (6, 9, 10). In addition, tau protein



**Fig. 1.** Schematic representation of cDNA clones used to determine tau protein sequence. Heavy line indicates open reading frame regions, thinner line and dotted line indicate noncoding regions of pTA2 and pTA3, respectively. The pTA2 and pTA2E are pBR322 clones; pTA3 is a  $\lambda$ gt11 clone, and pTA3E and pTA2E' are

pUC9 clones. Restriction sites are Bam HI (B), Sma I (S), and Pst I (P). AAAAA indicates a poly(A) stretch of 18 to 19 bases. Arrows indicate the location of specific oligonucleotide primers used to initiate cDNA synthesis for library construction. The isolation of pTA2 was as described (9). The pTA2E was isolated from a pBR322 primer extension library constructed with cDNA primed by a 22-base oligonucleotide (5'-GACATTCTTTAGGTCTGGCATG-3') (20). The pTA3 was isolated as described (21); antibody employed was affinity-purified anti-tau (10). The pTA3E was isolated from a pUC9 primer extension library (22) with size-selected cDNA primed by a 21-base oligonucleotide (5'-TTGACTGCCCTGGGAGCCTGA-3'). Two additional libraries were constructed in the manner described for the pTA3E library: one primed with a 21-base oligonucleotide from the 3' untranslated region of pTA2 (5'-GGCAGAGGTCCCCAAGAGGC-3'), from which pTA2E' clones were isolated, and the other primed with the 22-base oligonucleotide used for the pBR322 library above. In cDNA synthesis, primers were preincubated with mRNA prior to reverse transcriptase reaction. The cDNAs were dC-tailed for insertion into dG-tailed plasmid vectors.

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