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Brain tubulin and actin cDNA sequences: isolation of recombinant plasmids

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ABSTRACT

Rat brain mRNA enriched for tubulin and actin sequences was used to prepare double stranded cDNA. A library of recombinant clones was constructed by inserting the dsDNA into the Pstl site of pBR322 plasmid and transformation of *E.coli* x1776 host. Clones bearing sequences coding for tubulin and actin were identified and characterized.

INTRODUCTION

Microtubules are highly abundant in nerve processes and their concentration in axons increases with their development. Axon outgrowth and axoplasmic transport are also thought to depend on microtubule integrity (1). The concentration of tubulin, the subunit protein of microtubules, is much higher in the central nervous system than in other tissues. Maximal levels of cytoplasmic tubulin are found in newborn rats and by about day 10 after birth there is a progressive decrease in the level and rate of synthesis of tubulin until the rat reaches adulthood. This decline was found to result from a decrease of the corresponding translatable mRNA species (2). Recent studies showed that despite the overall decrease in tubulin synthesis during brain development, more tubulin mRNA species are expressed in the mature than in the prenatal brain.

These conclusions were reached from the observation that brain tubulin displays extensive microheterogeneity which is developmentally determined, increasing from five to six isotubulins prenatally to nine components during postnatal brain maturation (3). This phenomenon appears to be brain specific since it is not found in other organs such as liver and spleen. Other experiments show that mature brain mRNA when translated *in vitro* directs the synthesis of five tubulin forms. Prior fractionation of the mRNA by sucrose gradient centrifugation could partially resolve the mRNA species. Moreover, a marked age-dependent enhancement in the relative translation of a specific isotubulin has also been observed (4). Thus some of the age-dependent increase in tubulin

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microheterogeneity might be controlled at the mRNA level.

Another abundant cytoskeletal protein which plays an important role in governing cell shape and motility is actin. It is the major structural protein of the microfilaments found in the cytoplasms of all eukaryotic cells. In the cytoplasm of all mammalian cells two forms of actin, β- and γ-, are found while skeletal muscle is distinguished by the presence of α-actin (5). Like tubulin, a significant decrease in the rate of actin synthesis has been observed during brain development (2).

The mechanisms governing expression of tubulin and actin in the brain are currently unknown. On the basis of available information on this (4,6,7) and other systems (8,9), a likely possibility is that multiple genes exist for these proteins and that differential gene expression occurs during brain development. The availability of homogeneous DNA probes specific for the mRNA sequences coding for brain proteins is a prerequisite for the studies at the RNA and genomic level. In this report, we describe the cloning and identification of tubulin and actin sequences derived from rat brain mRNA.

MATERIALS AND METHODS

mRNA preparation, cell-free translation and product analysis

Brain RNA was prepared from 12 day old rats according to the method of Kirby (10). Poly(A)-containing RNA was isolated by oligo(dT)-cellulose chromatography.(11). Fractions enriched for particular mRNA species were obtained by sucrose gradient density centrifugation(5-25% sucrose in 0.1M NaCl, 10mM Tris pH 7.4 and 1mM EDTA)(4).Fractions of the gradient were analyzed by translation in the nuclease-treated rabbit reticulocyte lysate cell-free system (12). The cell-free products were analyzed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (13). Isoelectric focusing and two-dimensional gel electrophoresis were performed according to O'Farrel (14). Peptide mapping by partial proteolysis was performed according to Cleveland *et al.* (15).

Radiolabeled proteins and peptides were detected by autoradiography or fluorography (16) using Agfa Curix RP2 X-ray film.

Preparation of recombinant plasmids containing rat brain DNA sequences

The mRNA fraction enriched for tubulin and actin mRNA was used as template for the construction of a library of recombinant cDNA clones. Double-stranded cDNA was prepared by a procedure in which single-stranded cDNA is not purified prior to second strand synthesis (17). The second strand was synthesized with DNA polymerase I (gift from H. Schaller, University of Heidelberg)

and then treated with S1 nuclease. The double-stranded cDNA and the PstI-cut pBR322 plasmid were tailed by terminal transferase homopolymer addition of poly(dC) and poly(dG), respectively. Annealing of tailed plasmid with cDNA and transformation of competent *E.coli* strain x1776 were accomplished as described previously (18). All experiments were performed in accordance with the NIH guidelines for recombinant DNA work.

Selection of clones containing inserts complementary to brain Poly(A)$^+$ RNA sequences

Bacterial colonies resistant to tetracycline but sensitive to ampicillin were grown in 96-welled microtiter plates in FB medium. (FB medium contains per liter 25 g trypton, 7.5 g yeast extract, 1 g glucose, 6 g NaCl, 0.05M Tris-HCl, pH 7.6, 100 mg diaminopimelic acid and 50 mg thymidine). Replicas of the microtiter plates were transferred to FB agar and then to nitrocellulose filters on which their DNA was denatured (19,20). The filters were air-dried and then baked in a vacuum oven at 75°C for 2 h. The filters were hybridized with [^{32}P]-labeled cDNA probe made on a template of mRNA isolated from rat brain or liver. Radioactive cDNA probes were prepared by incubating 1μg of poly(A)$^+$ RNA in a final volume of 10μl in the presence of 50mM Tris-HCl (pH 8.0), 10mM $MgCl_2$, 100mM KCl, 100μM dATP, 100μM dTTP, 60 μg/ml oligo(dT) primer, 60mM DTT, 33 μg/ml actinomycin D, 33 μg/ml bovine serum albumin, 20mCi/ml of both [^{32}P]dGTP and [^{32}P]dCTP (at 300-400 Ci/mmol) and 200μg/ml of avian myeloblastosis virus (AMV) reverse transcriptase (kind gift of Dr. J. Beard). Following 1 h incubation at 42°C, 3 μl of 1M NaOH were added to each tube and incubation continued for 20 min at 70°C to hydrolyze the mRNA template. Methyl red was added as a pH indicator followed by 1M HCl to neutralize the NaOH. The reaction products were then passed over a column of Biogel P60 equilibrated in 10mM triethanolamine carbonate buffer (pH 8.5). The excluded material was pooled and used for colony hybridization. Hybridizations were carried out for 18 h at 65°C in 0.9M NaCl/0.09M Na citrate (6xCCS) containing Denhardt solution(21 50 μg/ml of denatured salmon sperm DNA, 0.5% $NaDodSO_4$ and [^{32}P]-labeled cDNA (0.5×10^6 counts/min) per filter. Following hybridization, the filters were washed twice at 65°C in 2 x SSC for 1 h followed by a 20 min wash with 0.1 x SSC, after which the filters were dried and exposed to autoradiography with preflashed Agfa Curix RP2 films for 2 days at -70°C with an intensifying screen.

Preparation of plasmid DNA

For preliminary characterization of clones suspected of containing brain DNA nucleotide sequences the rapid screening procedure of Birnboim and Doly was

employed (22). Plasmids were isolated from 3 ml of non-amplified liquid cultures by selective alkaline denaturation of the bacterial high molecular chromosomal DNA. The size of the EcoR1 digested plasmid as well as the size of the Pst1 liberated insert was analyzed by electrophoresis on 1% and 1.4% agarose gels respectively.

For large scale preparations of purified plasmid, *E.coli* x1776 cells containing recombinant plasmids were grown to a density of 0.5-0.6 OD_{550}/ml in FB medium containing tetracyclin (12.5μg/ml). Chloramphenicol (12.5 μg/ml)was added and incubations were continued for additional 15 h. Cleared lysates were prepared as described by Katz *et al.* (23). Supercoiled plasmid DNA was purified by equilibrium banding in CsCl/ethidium bromide gradients centrifuged in a Beckman type 65 rotor at 40,000 rpm for 48 h at 15°C. The lower band containing the plasmid was removed, and the ethidium bromide was extracted with isoamyl alcohol saturated with CsCl. The plasmid DNA solution was dialyzed successively against 4 x SSC, 2 x SSC and 10mM Tris (pH 8.0)/1mM EDTA.

Identification of clones containing tubulin and actin-specific DNA sequences

The purified plasmid DNA preparations were identified by a positive hybridization/translation assay (24). Between 5-20 μg of purified plasmid DNA was linearized with EcoR1 restriction enzyme and denatured by 1 min boiling at 100°C followed by quick freezing in liquid air. The denatured plasmid DNA preparations were immobilized on nitrocellulose filters presoaked in 6 x SSC by overnight air-drying and baking at 75°C for 2 h in a vacuum oven. The crushed plasmid-DNA filters were incubated at 50°C for 2 h in a 100 μl hybridization mixture containing 15-30 μg brain mRNA from 12 day old rats and 65% deionized formamide, 10mM Pipes (pH 6.4) and 0.4M NaCl. After incubation at 50°C for 2 h, the filters were washed as described. The hybridized mRNA was eluted and its encoded sequences were identified according to [^{35}S]methionine-labeled proteins synthesized in the reticulocyte cell-free system.

RESULTS

Colony hybridization analysis of recombinant plasmid containing brain-specific DNA sequences

Over two thousand tetracycline resistant, ampicillin sensitive bacterial clones were isolated. DNA from these bacterial clones was hybridized *in situ* with [^{32}P]-labeled cDNA complementary to total poly(A)$^+$ RNA from rat brain or liver. The rationale of such a selection procedure rests on the observation that brain mRNA isolated from 10-12 days rats is about 30-fold enriched for

tubulin mRNA sequences compared with that of liver mRNA, as judged by their *in vitro* translation products (data not shown). After stringent washing that yielded a maximal differential signal, we selected approximately 100 clones which could contain the desired inserted DNA sequences. Under these conditions no positive hybridization signal was observed with [^{32}P]-cDNA derived from muscle RNA enriched for α-actin sequences. Figure 1 shows the results of a hybridization experiment using brain- and liver-derived probes with duplicate nitrocellulose filters containing DNA from 96 recombinant clones. Clones 2C and 3H/4G were later identified as carrying recombinant plasmids bearing sequences hybridizing with tubulin and actin mRNAs respectively.

Identification of recombinant plasmids containing tubulin and actin specific DNA sequences

Following analysis of small preparations of plasmid DNA from the 100 candidate clones, those with longer and excisable Pstl inserts were used to prepare plasmid DNA by CsCl equilibrium density centrifugation. The various

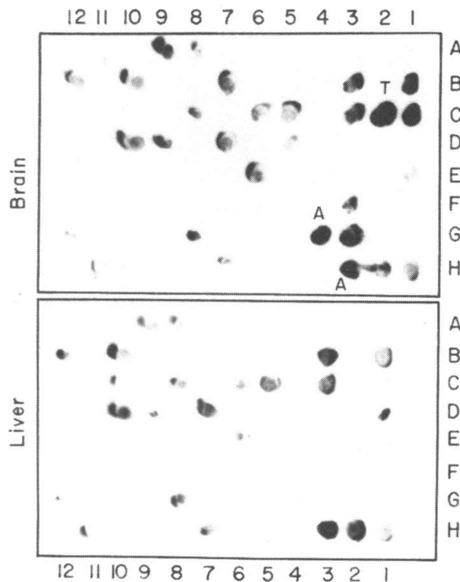

Fig. 1. Colony hybridization of recombinant clones. Recombinant clones derived from brain poly(A)-containing RNA were grown in duplicate in a 12x8 array in microtitre plates and transferred to nitrocellulose paper. Following lysis of the colonies, the filters were baked and hybridized with [^{32}P] labeled cDNA prepared from mRNA of brain and liver. Positive clones were detected by autoradiography of the filters. Clones subsequently identified as containing sequences coding for tubulin (T) and actin (A) are indicated.

DNA preparations were immobilized on nitrocellulose filters and used in turn to isolate specific mRNA chains. This method enabled us to isolate the mRNA sequences which hybridize to the recombinant plasmid DNA. The hybridized-selected RNAs were then eluted and translated in a cell-free system in order to identify their encoded polypeptide. The polypeptide products directed by the selected mRNA in the cell-free system were identified by their electrophoretic mobility in 8-12% $NaDodSO_4$-polyacrylamide slab gels. Figure 2 shows that the mRNA hybridizing to plasmid from clone No.25 stimulated the synthesis of two polypeptides with apparent molecular weights of 53,000 and 55,000, that comigrated with α- and β-subunits of rat brain tubulin. mRNA hybridizing to plasmid from clones No.1,3 and 72 stimulated the synthesis of a polypeptide with an apparent molecular weight of 43,000 which comigrated with the actin marker.

The products directed by the selected mRNA preparations were also characterized by two-dimensional gel electrophoresis (isoelectric focusing/$NaDodSO_4$ gels). Figure 3 shows that the putative tubulin polypeptides comigrated with the α- and β-subunits of purified tubulin marker (Figure 3C). The putative

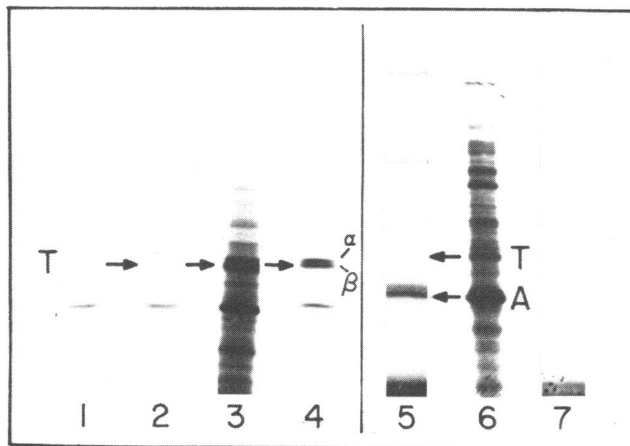

Fig. 2. Fluorogram of $NaDodSO_4$ polyacrylamide gel of *in vitro* synthesized tubulin and actin. Poly(A)-containing RNA and hybridized-selected mRNA were translated in the reticulocyte lysate cell-free system and translation products analyzed by electrophoresis on 8-12% acrylamide slab gel. The fluorogram shows the [^{35}S]methionine labeled translation products synthesized in the presence of: no added mRNA (lane 1), mRNA nonspecifically bound to uncloned parent plasmid pBR322 DNA (lanes 2 & 7), poly(A)-containing RNA (lanes 3 & 6), mRNA selected by plasmid DNA from clone No. 25 (lane 4) and mRNA selected by plasmid DNA from clone 72 (lane 5). The positions of tubulin (T) and actin (A) are indicated. Under the conditions of the separation the tubulin is resolved into two bands (α and β).

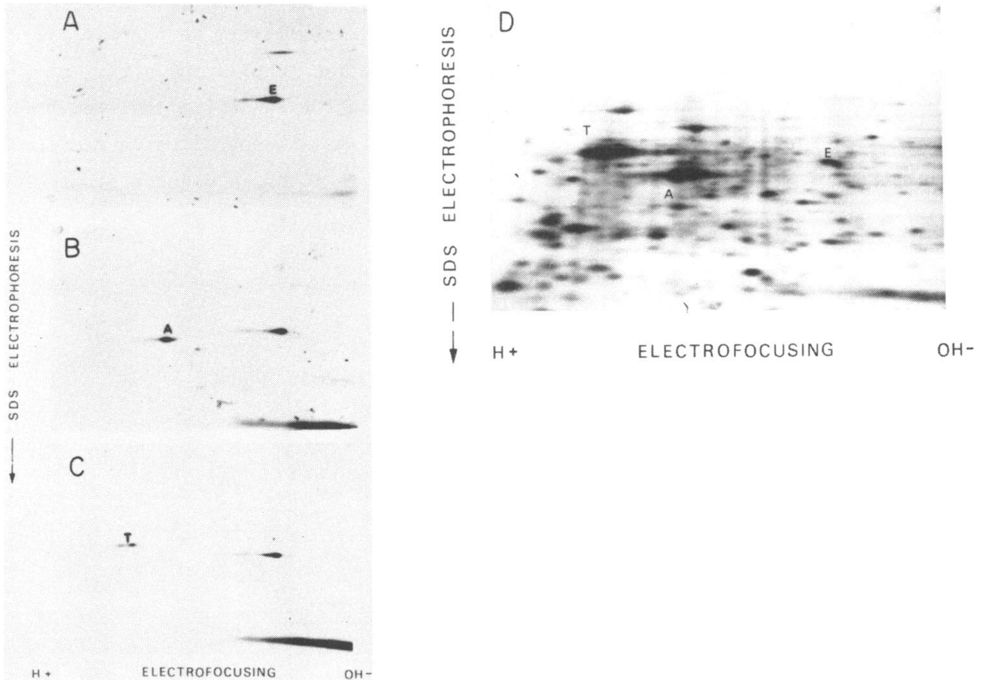

Fig. 3. Two-dimensional gel electrophoresis of cell-free translation products directed by hybridized-selected mRNA. Brain poly(A)-containing RNA was incubated with nitrocellulose filters containing immobilized DNA from recombinant plasmid. Specifically bound mRNA species were translated in the reticulocyte lysate cell-free system. Reaction mixtures were subjected to two-dimensional gel electrophoresis according to O'Farrel. The [^{35}S]methionine labeled translation products were visualized by fluorography of the dried gels. The fluorograms show translation products resulting from immobilization of: uncloned parent plasmid pBR322 DNA (A), plasmid DNA of clone No. 72 (B) and plasmid DNA of clone No. 25 (C). E = Endogenous protein of the cell-free system. A = Actin. T = Tubulin. (D) Products directed by poly(A)-containing RNA from brain.

actin comigrated on two-dimensional electrophoresis with non-muscle actin (Figure 3B).

The various [^{35}S]methionine-labeled proteins were further characterized by analysis of peptides derived from *Staphylococcus aureus* protease cleavage. For this purpose, the [^{35}S]-labeled products directed by the hybridized-selected mRNA species were first resolved by 5-15% $NaDodSO_4$-polyacrylamide gel electrophoresis. Under these conditions the α- and β-tubulin subunits were not separated and migrated as a single band. The single radioactive bands comigrating with tubulin and actin markers were excised and then subjected to limited *S. aureus*

protease digestion and further analyzed by 15-20% polyacrylamide/NaDodSO$_4$ gel electrophoresis (Figures 4A,B). There was a close similarity in the overall distribution of the cleaved peptides derived from the tubulin and actin products with the *in vivo* [^{35}S]methionine labeled tubulin and actin isolated from brain slices or rat glioma C6BU-1 cell cultures. All the peptides derived from digestion of the *in vitro* synthesized proteins were found in digests of *in vivo* labeled tubulin and actin. The digest of the *in vivo* labeled material contained

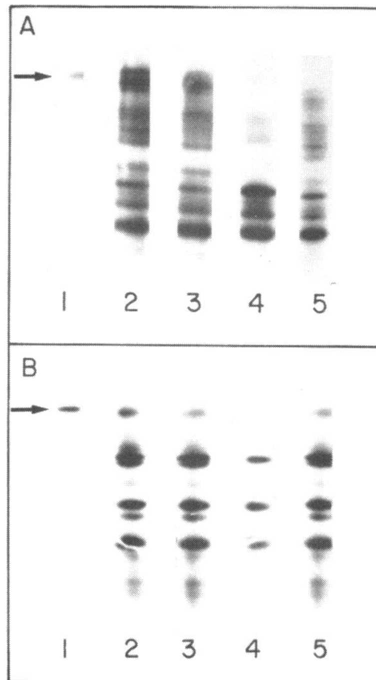

Fig. 4. Partial protease digestion patterns of *in vivo* and *in vitro* synthesized tubulin and actin. Tubulin and actin bands were cut from gels as described in Materials and Methods and partial digestion with *S.aureus* protease performed according to Cleveland *et al.* Radioactive proteins were visualized by fluorography. A. Lane 1: Tubulin from [^{35}S]methionine labeled brain slices with no added protease. Lanes 2-3: Digestion pattern of tubulin derived from [^{35}]methionine labeled brain slices (lane 2) and rat glioma C6BU-1 cell culture (lane 3). Lane 4: Digestion pattern of putative tubulin directed by mRNA selectively hybridized to plasmid DNA of clone No. 25. Lane 5: Digestion pattern of tubulin directed by poly(A)-containing brain mRNA. B. Lane 1: Actin from brain slices with no added protease. Lanes 2-3: Digestion pattern of actin derived from brain slices (lane 2) and rat glioma C6BU-1 (lane 3). Lane 4: Digestion pattern of putative actin directed by mRNA selectively hybridized to plasmid DNA of clone No. 25. Lane 5: Digestion pattern of actin directed by poly(A)-containing brain mRNA.

some additional bands probably resulting from a lesser degree of proteolysis.

Extensive tubulin microheterogeneity is prominent in rat brain and to a much lesser degree in other organs. This microheterogeneity can be observed by separating tubulin on isoelectric focusing gels where the α-subunit is resolved into four components (isotubulins 1-4) while the β-subunit consists of five forms (isotubulins 5-9). Rat brain mRNA when translated *in vitro* directs the synthesis of five tubulin forms, namely isotubulins 1, 3, 4 (or 5), 6 and 7 (ref. 4). A similar pattern was observed upon analysis of the products obtained by the selected RNA. Figure 5 shows the autoradiogram of the [^{35}S]-labeled polypeptides stimulated by RNA selected by the DNA from tubulin clone No. 25. The labeled polypeptides were analyzed by isoelectric focusing as previously described (3). The most prominent labeled bands comigrated with isotubulins 1 and 6 by comparison with nonlabeled purified brain tubulin. These results confirm that the selected material contains both α- and β- tubulin mRNAs. A similar analysis of the putative actin clones, revealed that the *in vitro* products comigrated mainly with γ-actin and to a lesser extent with β-actin.

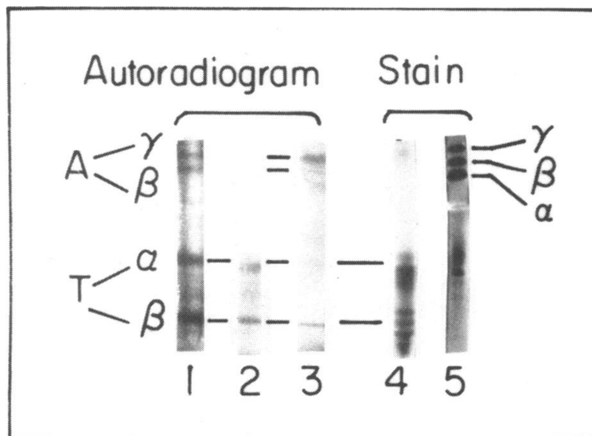

Fig. 5. Isoelectric focusing analysis of *in vitro* synthesized tubulin and actin. mRNA specifically hybridizing to plasmid DNA of clone No. 25 and clone No. 72 was translated in the reticulocyte lysate cell-free system. [^{35}S]methionine labeled products were separated by isoelectric focusing. Lane 1: Translation products of poly(A)-containing RNA. Lane 2: Translation products of RNA hybridizing to plasmid of clone No. 25. Lane 3: Translation products of RNA hybridizing to plasmid of clone No. 72. Lane 4: Vinblastin-purified rat brain tubulin. Lane 5: Mixture of purified α-actin from rat skeletal muscle and β- and γ-actin from rat brain. Lanes 1-3 are autoradiograms of labeled proteins. Lanes 4-5 show proteins stained by Coomassie Brilliant Blue. The positions of the α-, β- and γ-actin subunits (A) and the α- and β-subunits of tubulin (T) are indicated.

Hybridization of plasmid DNA to fractionated mRNA

We have characterized the recombinant plasmids by hybridizing plasmid DNA (clones No. 25 and No. 72) to fractionated mRNA from various sources. mRNA prepared from rat skeletal muscle, heart, stomach, brain and mononucleated (predifferentiation) L_8 cells (25), was electrophoresed on agarose gels in the presence of 7.5mM methyl mercuric hydroxide (26). The RNA was transferred to diazobenzyloxymethyl-paper and hybridized to [^{32}P]-labeled nick-translated plasmid DNA (27,28) (Figure 6). Using the tubulin probe we observed a single hybridization band corresponding to RNA of about 1800 bases. As expected, strongest hybridization was observed with brain mRNA (lanes 10,11). A lesser degree of hybridization was found with mRNA from prefusion myoblasts (lane 12) and almost insignificant hybridization with mRNA from stomach and heart (lanes 8,9).

The actin probe hybridized to RNA band of about 2200 bases, characteristic of β- and γ-actin (18). Strongest hybridization was observed with mRNA

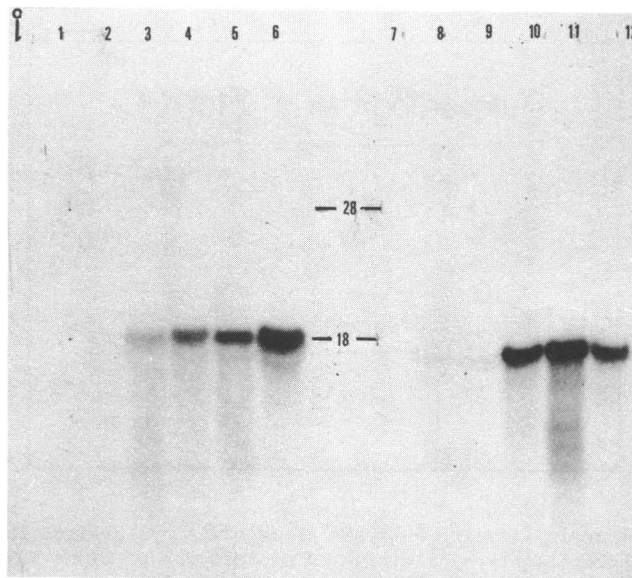

Fig. 6. Hybridization of plasmid DNA to size-fractionated RNA from rat muscle (lanes 1,7), heart (lanes 2,8), stomach (lanes 3,9), brain (lanes 4,5,10,11) and prefused L_8 cells (lanes 6,12). Twenty microgram of each RNA preparation was electrophoresed in 1% agarose gels and the RNA was transferred to diazobenzyloxymethyl-paper. Nick-translated plasmid DNA probes from actin clone No. 72 (lanes 1-6) and tubulin clone No. 25 (lanes 7-12) were used. The arrows indicate the position of 18S rRNA and 28S rRNA.

from prefusion myoblasts and brain with a lesser estent to stomach muscle and heart. No hybridization to mRNA from skeletal muscle with either the tubulin or actin probe was observed (lanes 1,7).

Sizing of DNA inserted into bacterial plasmids

Purified plasmid DNA preparations from tubulin No. 25 and actin No. 72 clones were cut with restriction endonuclease Pstl and run on 1.4% neutral agarose gels. The inserts were estimated to be 550-650 nucleotides long, by comparison with Hae III digested ΦX174 RF DNA marker.

DISCUSSION

The availability of homogeneous hybridization probes for tubulin and actin would be of value in analysis of brain cell differentiation. We have described the cloning of recombinant bacterial plasmids containing inserted sequences derived from DNA complementary to rat brain mRNA. We have identified clones which carry sequences which hybridize specifically with tubulin mRNA and non-muscle actin mRNA.

Tubulin, the subunit protein of microtubules, is a heterodimer composed of two subunits, α- and β-, with apparent molecular weights of about 53,000 and 55,000. Although very similar, they differ on the basis of immunological specificity (29), peptide mapping (15), isoelectric focusing (3) and their ability to undergo tyrosylation at the carboxy terminal end (4,30). The hybridization of clone No. 25 DNA with mRNA for tubulin α- and β-subunits indicates the extensive sequence homology of the two polypeptides. The coding region for tubulin, based on its molecular weight, is about 1200 nucleotides long while our estimated length of tubulin mRNA is about 1800 nucleotides. It is therefore likely that tubulin clone No. 25 which contains an insert of about 600 base pairs will hybridize with a part of the coding region of the tubulin mRNA.

The actin clones hybridize with brain mRNA coding for the non-muscle actin species β and γ. Katcoff *et al.* (18) have observed that α-actin clones containing coding sequences show a much greater extent of cross-hybridization with non-muscle actin than clones containing 3'-non-coding sequences. Our observation that DNA of clone No. 72 does not hybridize with mRNA from skeletal muscle confirms that plasmid of this clone contains sequences coding for β- and γ-actin. The DNA sequences of these isolated clones is now being analyzed. The availability of pure DNA probes will enable us to study the molecular control of nerve cell differentiation at the RNA level as well as allow identification of genomic clones carrying the various tubulin and actin genes.

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