MOLECULAR AND CELLULAR BIOLOGY, Dec. 1981, p. 1125-1137
0270-7306/81/121125-13$02.00/0

Vol. 1, No. 12

# Sequence Heterogeneity, Multiplicity, and Genomic Organization of α- and β-Tubulin Genes in Sea Urchins

DESPINA ALEXANDRAKI* AND JOAN V. RUDERMAN

*Department of Anatomy and Department of Cell and Developmental Biology, Harvard Medical School, Boston, Massachusetts 02115*

Received 8 May 1981/Accepted 12 August 1981

We analyzed the multiplicity, heterogeneity, and organization of the genes encoding the α and β tubulins in the sea urchin *Lytechinus pictus* by using cloned complementary deoxyribonucleic acid (cDNA) and genomic tubulin sequences. cDNA clones were constructed by using immature spermatogenic testis polyadenylic acid-containing ribonucleic acid as a template. α- and β-tubulin clones were identified by hybrid selection and in vitro translation of the corresponding messenger ribonucleic acids, followed by immunoprecipitation and two-dimensional gel electrophoresis of the translation products. The α cDNA clone contains a sequence that encodes the 48 C-terminal amino acids of α tubulin and 104 base pairs of the 3′ nontranslated portion of the messenger ribonucleic acid. The β cDNA insertion contains the coding sequence for the 100 C-terminal amino acids of β tubulin and 83 base pairs of the 3′ noncoding sequence. Hybrid selections performed at different criteria demonstrated the presence of several heterogeneous, closely related tubulin messenger ribonucleic acids, suggesting the existence of heterogeneous α- and β-tubulin genes. Hybridization analyses indicated that there are at least 9 to 13 sequences for each of the two tubulin gene families per haploid genome. Hybridization of the cDNA probes to both total genomic DNA and cloned germline DNA fragments gave no evidence for close physical linkage of α-tubulin genes with β-tubulin genes at the DNA level. In contrast, these experiments indicated that some genes within the same family are clustered.

The α and β tubulins are the major structural components of the microtubules of all eucaryotic cells. As such, they are involved in a wide variety of developmental events and cellular functions; these include meiotic and mitotic spindle formation and chromosomal segregation, ciliary and flagellar motility, axonal transport, programmed modifications of cell shape during both normal morphogenesis and neoplastic transformations. Also, tubulins have been implicated in various membrane-modulated events (34, 40, 42). Over the past 10 years, workers have accumulated considerable, although indirect, evidence for the existence of multiple α- and β-tubulin subtypes, which vary in primary structure and are specific for each of the different microtubule-containing structures (4, 15, 17, 21, 39, 43, 44, 51). This idea has been supported by the demonstration of heterogeneous α- and β-tubulin messenger ribonucleic acids (mRNA's) in some cases (9, 29). These findings suggest that there are multiple heterogeneous α- and β-tubulin genes and that the expression of these genes is modulated selectively during embryonic development and tissue differentiation. In fact, the presence of multiple tubulin genes (or pseudogenes) has been demonstrated recently in chickens (12) and *Drosophila* (37).

In sea urchins, different subsets of α and β tubulins are required at several stages of development. Tubulins specific for mitotic spindles must be produced to sustain the rapid cleavages of early embryos. Ciliagenesis in hatching blastulae requires the synthesis and assembly of ciliary tubulins that are specific for the central pair and for the outer doublet A-tubule and B-subfiber. During spermatogenesis, still another group of tubulin subtypes, which are specific for the central pair and the outer doublet of the flagella, are synthesized (3, 4, 42, 44). These findings suggest that the tubulins are encoded by a multigene family whose members are under strict development regulation. Furthermore, all microtubule-containing structures examined to date contain equal amounts of α tubulin and β tubulin assembled as heterodimers. Production of the various microtubule-specific heterodimers requires coordinate expression of the appropriate α- and β-tubulin genes at some level. These considerations raise several questions. How many different tubulin genes are there, and how many copies of each exist in the genome? How

are these genes organized? Are there clusters of $\alpha$ variants and clusters of $\beta$ variants, or are the various $\alpha$ and $\beta$ genes scattered separately throughout the genome? Alternatively, are there pairs of microtubule-specific $\alpha$ and $\beta$ genes that are closely linked, possibly under the control of spatially common regulatory regions? How is the expression of the different $\alpha\beta$ gene pairs regulated at the different development stages? What mechanisms modulate coordinate expression? In this work we used cloned $\alpha$- and $\beta$-tubulin sequences to investigate the heterogeneity and multiplicity of tubulin genes in the sea urchin genome. We also describe some preliminary analyses of the organization of the two gene families at the deoxyribonucleic acid (DNA) level.

## MATERIALS AND METHODS

**Materials.** *Lytechinus pictus* was purchased from Pacific Biomarine. pBR322 DNA was provided by L. Villa-Komaroff, as was the bacterial strain used for transformation, *Escherichia coli* HB101 (*hsm hrs recA gal pro* Str$^r$) (7). The bacterial strains used as hosts for the λ clones were *E. coli* KH802 (*har ham*$^+$ *galK* SuII *lac*$\gamma$ *met*) (F. R. Blattner, protocol that accompanies the Charon λ phages) and *E. coli* CSH18 (Δ[*lac pro*]XIII *supE* [F′ *lacZ Y*$^+$ *proA*$^+$*B*$^+$]) (5), which were gifts from Gordon Moore and Tom Eickbush, respectively.

Avian myeloblastosis virus reverse transcriptase was obtained from J. W. Beard. Terminal deoxynucleotidyl transferase was generously provided by R. L. Ratliff. S1 nuclease was a generous gift from A. Efstratiadis. Restriction endonucleases were purchased from New England BioLabs, DNA polymerase I was from Boehringer Mannheim Corp., *Staphylococcus aureus* was from Enzo-Biochem nitrocellulose filters were from the Schleicher & Schuell Co., oligodeoxythymidylic acid$_{12-18}$ and oligodeoxythymidylic acid cellulose $T_3$ were from Collaborative Research, Inc., and dextran sulfate from Pharmacia. Some of the $\alpha$-$^{32}$P-labeled deoxynucleoside triphosphates (specific activity, 300 Ci/mmol) used were prepared and provided by M. DePamphilis. Some $\alpha$-$^{32}$P-labeled deoxynucleoside triphosphates (specific activity, >300 Ci/mmol) and [$^{35}$S]methionine (specific activity, >500 Ci/mmol) were purchased from New England Nuclear Corp.

**Preparation of RNA.** Sea urchin egg and embryo cytoplasmic RNAs were prepared as previously described (36). Testis RNA was extracted from a 15,000-×-*g* supernatant fraction of immature testes homogenized in 20 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.6)–50 mM magnesium acetate–0.2 M NaCl–0.25% diethylpyrocarbonate. An equal volume of 2× sodium dodecyl sulfate (SDS) buffer (1× SDS buffer contains 0.5% SDS, 0.1 M NaCl, 20 mM Tris-hydrochloride, pH 7.4, and 2 mM ethylenediaminetetraacetate [EDTA]) was added to the 15,000-×-*g* supernatant fraction. The sample was deproteinized by repeated extractions with phenol-chloroform-isoamyl alcohol (50:48:2) and ethanol precipitated. Polyadenylic acid-containing [poly(A)$^+$] RNA was obtained by oligodeoxythymidylic acid cellulose chromatography at 4°C (1), using 0.5 M ammonium acetate as the binding buffer and water as the elution buffer; 1 ml of packed testes usually yielded about 1 mg of cytoplasmic RNA, 1% of which fractionated as poly(A)$^+$ RNA.

**In vitro translation.** In vitro translation assays were performed by using a nuclease-treated reticulocyte lysate system (32). Usually, 0.5 μg of poly(A)$^+$ RNA (measured by absorbance at 260 nm) or 5 μg of total cytoplasmic RNA was assayed in a 10-μl reaction mixture containing [$^{35}$S]methionine (final specific activity, 0.5 mCi/ml).

**Immunoprecipitation assay.** Immunoprecipitation assays were performed by using *S. aureus* as the immunoadsorbent (23) according to the modified procedure of Thireos et al. (46). Specific tubulin antiserum was raised in rabbits against vinblastin sulfate crystals of sea urchin (*Strongylocentrotus purpuratus*) cytoplasmic tubulin and was generously provided by Keigi Fujiwara (16).

**Polyacrylamide gel electrophoresis of proteins.** In vivo translation products were analyzed in SDS–10% polyacrylamide slab gels (24). Two-dimensional gel electrophoresis was performed as described by O'Farrell (31). Radioactively labeled proteins were detected by fluorography (6, 25). Tubulin markers, which were generously provided by R. W. Linck, were prepared from axonemes of *L. pictus* flagella.

**Construction of the cDNA clones.** Complementary DNA (cDNA) complementary to testis cytoplasmic poly(A)$^+$ RNA was synthesized in 50-μl reaction mixtures containing 200 mM Tris (pH 8.3), 10 mM $MgCl_2$, 28 mM β-mercaptoethanol, 0.5 mg of RNA per ml, 0.5 mg of oligodeoxythymidylic acid$_{12-18}$ per ml, four deoxynucleoside triphosphates (each at a concentration of 1.2 mM), four $\alpha$-$^{32}$P-labeled deoxynucleoside triphosphates (25 μCi each; >300 Ci/mmol), and 800 U of reverse transcriptase per ml at 42°C for 3 h (14; A. Efstratiadis, personal communication). After the RNA template was removed by alkali digestion, the second DNA strand was synthesized under the same conditions that were used for making the first strand. S1 nuclease treatment yielded blunt-ended double-stranded DNA molecules with sizes ranging from 0.2 to 1.5 kilobases (kb), as determined in native and denaturing 1% agarose gels (30). Approximately 15 deoxycytidine molecules were added to the 3′ ends of double-stranded cDNA, and 15 deoxyguanidine residues were added to the 3′ ends of *Pst*I-linearized plasmid pBR322 by using terminal transferase at 10°C (35). The tailed double-stranded cDNA molecules were annealed with 20 times (wt/wt) the amount of tailed plasmid DNA, as described previously (48). *E. coli* HB101 cells were transformed with the hybrid molecules (50). Tetracycline-resistant transformants were picked and screened by using the method of Grunstein and Hogness (19). The labeled single-stranded cDNA probes used for the screening procedures were synthesized by the above-described procedure.

**Screening of the λ recombinant library.** A recombinant phage λ library of sea urchin sperm DNA partially digested with *Eco*RI was kindly provided by

G. Moore and E. H. Davidson. Screening of this library for tubulin sequences was performed as described by Benton and Davis (2) and Maniatis et al. (27).

**Hybrid selection cell-free translation.** Plasmid DNAs were purified by the method of Clewell (13). A total of 4 μg of DNA was linearized, denatured, and spotted onto nitrocellulose filters by the method of Kafatos et al. (20). Hybrid selection translations were performed by a modification of the procedure of Ricciardi et al. (33). The filters were baked, prehybridized for 1 h at 54°C in 50 μl of a solution containing 0.6 M NaCl, 10 mM PIPES [piperazine-*N*,*N*'-bis(ethanesulfonic acid)] (pH 6.4), 50% deionized formamide, and 100 μg of poly(A) per ml, and hybridized for 2 h at 54°C in 30 μl of prehybridization buffer in which the poly(A) was replaced by 3 μg of testis poly(A)$^+$ RNA. Subsequently, the filters were washed five times in 1 ml of 1× SSC–0.5% SDS (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C and twice in 1 ml of 10 mM Tris–2 mM EDTA (pH 7.8) at room temperature. The hybridized RNAs were eluted by boiling the filters in 100 μl of water. The eluted RNAs were ethanol precipitated after the addition of 3 μg of transfer RNA as a carrier and 0.1 volume of 2 M sodium acetate (pH 5.5). RNA pellets were suspended in 2 μl of water and used in 15-μl reticulocyte lysate translation reaction mixtures (32). Translation products were analyzed in SDS–10% polyacrylamide gels after they were treated with 1 μg of deoxyribonuclease I and ribonuclease A.

**DNA electrophoresis, blotting, and hybridization procedures.** The cloned DNA fragments, which were used for restriction analyses or as labeled probes were separated in Tris-borate-EDTA-polyacrylamide gels (28) and were eluted from gels as described by Gilbert and Maxam (18); 0.8% agarose gels (thickness, 1.5 to 3 mm) were used to electrophorese DNAs which were to be transferred to nitrocellulose (38). After electrophoresis, the gels were treated as described by Wahl et al. (49). Then the DNAs were transferred by the procedure of Southern (41), using 1 M ammonium acetate in the transfer buffer (20). Hybridizations were carried out as described previously (19) by using 10% dextran sulfate (49). Dot hybridizations were performed with sheared or digested sperm DNA and linearized plasmids which were denatured, spotted onto nitrocellulose filters (20), and hybridized as described above. $^{32}$P-labeled probes were prepared by nick-translation (28). Autoradiography was performed by exposing the nitrocellulose filters to preflashed Kodak XR5 X-ray film with Du Pont Cronex Lightning Plus intensifying screens at −80°C.

**Preparation of high-molecular-weight genomic DNA.** High-molecular-weight genomic DNA was prepared by a modification of the method of Britten et al. (8). Sperm from individual animals (*L. pictus*) was collected by 0.55 M KCl stimulation, repeatedly washed with membrane-filtered (Millipore Corp.) seawater, and disrupted by adding 100 volumes of 0.2 M Tris–0.2 M EDTA (pH 7.8) and 2% (final concentration) SDS. The preparations were incubated at 60°C for 10 min, and this was followed by pronase digestion (1 mg/ml) at 37°C overnight. $NaClO_4$ was added to a final concentration of 1 M, and the DNA was extracted repeatedly with neutralized phenol-chloroform-isoamyl alcohol and dialyzed extensively against 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA at 4°C. A total of 30 to 50 mg of DNA was obtained from each 1 ml of packed sperm.

## RESULTS

**Tubulin mRNA levels in development.** To identify the mRNA population that was most appropriate for use in constructing cDNA clones containing α- and β-tubulin sequences, we assayed RNAs from several developmental stages and from immature spermatogenic testes. RNAs were extracted from post-mitochondrial supernatant fractions and translated in vitro in reticulocyte lysates in the presence of [$^{35}$S]methionine, and the translation products were examined by one-dimensional SDS gel electrophoresis in parallel with authentic tubulin markers. As Fig. 1A shows, the levels of translatable α- and β-tubulin mRNA's were low in the unfertilized egg and early cleavage stages and rose during the later stages, becoming prominent in ciliated hatching blastulae. The concentrations then declined slightly in the interval from hatching to gastrulation. These changes probably reflect the increased need for mitotic spindle tubulins during the later cleavages and the need for the synthesis of ciliary tubulins at hatching. Not surprisingly, immature spermatogenic testes, which synthesized relatively large amounts of flagellar tubulins, were an especially rich source of tubulin mRNA's (Fig. 1B). The identities of tubulins in the testis mRNA translation products were confirmed by electrophoretic comigration with marker tubulins on two-dimensional gels and by specific immunoprecipitation (see below). Accordingly, testis poly(A)$^+$ mRNA was used to construct tubulin cDNA clones.

**Isolation and characterization of α- and β-tubulin cDNA clones.** Testis cDNA clones containing α- and β-tubulin sequences were constructed as described above. Tubulin cDNA clones were chosen by differential screening, using first labeled testis cDNA as a probe and then cDNA complementary to embryonic chicken brain poly(A)$^+$ RNA, which is also highly enriched in tubulin mRNA's (9, 11). Tubulin clones pα1 and pβ1 were identified positively by their abilities to hybridize selectively to template-active mRNA's which coded for α tubulin and β tubulin, respectively. The identifications of the putative α- and β-tubulin translation products were confirmed by immunoprecipitation followed by one-dimensional gel electrophoresis (Fig. 2) and by two-dimensional gel electrophoresis (Fig. 3). The cDNA insertions of pα1 (300 base pairs) and pβ1 (450 base pairs) do not represent full-length copies of α- and β-tubulin

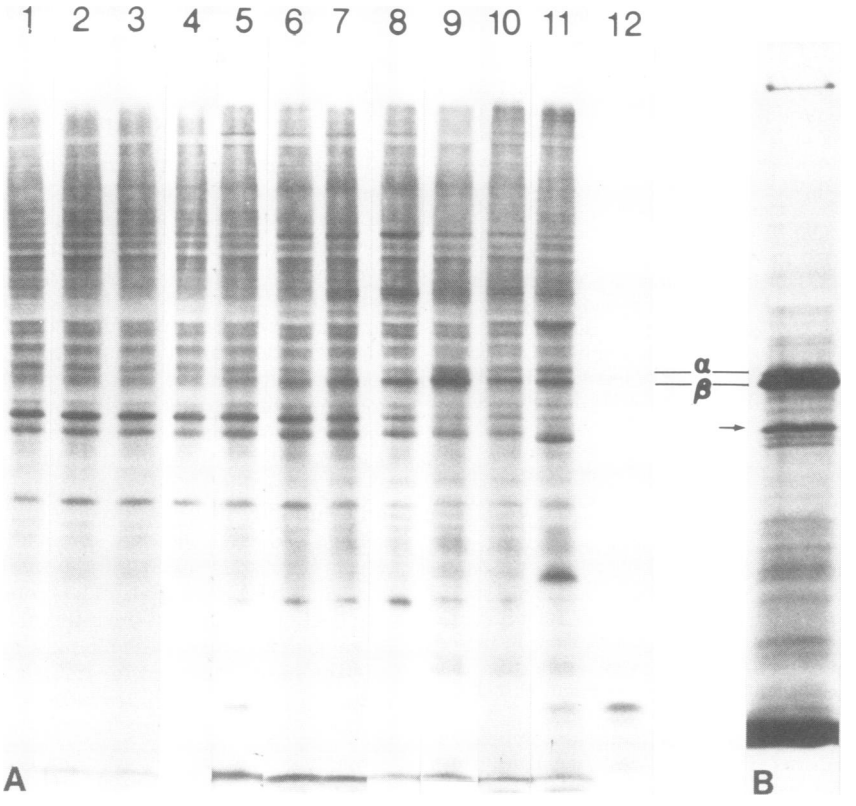


FIG. 1. *Cell-free translation of RNAs from early embryonic stages and testes of sea urchins. (A) SDS-polyacrylamide gel electrophoresis of [$^{35}$S]methionine-labeled in vitro translation products of total cytoplasmic RNA from eggs (lane 1), first cleavage (lane 2), fourth cleavage (lane 3), sixth cleavage (lane 4), 7 h post-fertilization (lane 5), 9 h post-fertilization (lane 6), 11 h post-fertilization (lane 7), 12.5 h post-fertilization (50% hatching blastula) (lane 8), hatching blastulae (lane 9), mesenchyme blastulae (lane 10), gastrulae (lane 11), and endogenous reticulocyte lysate incorporation (lane 12). (B) SDS-polyacrylamide gel electrophoresis of [$^{35}$S]methionine-labeled in vitro translation products encoded by testis cytoplasmic poly(A)$^+$ RNA. α and β indicate the translation products that comigrated with authentic α and β tubulins (molecular weight, 55,000). The observed lower intensity of the α-tubulin product compared with β tubulin in part reflected the lower methionine content of α tubulin. The arrow indicates an endogenous reticulocyte lysate polypeptide.*

mRNA's (~1,700 and 1,800 nucleotides, respectively). As revealed by DNA sequencing (data not shown), pα1 contains a sequence that encodes the 48 C-terminal amino acids of α tubulin and 104 base pairs of the 3′ noncoding portion of the mRNA. The pβ1 insertion encodes the 100 C-terminal amino acids of β tubulin and also contains 83 base pairs of the 3′ nontranslated sequence. The correct coding frames of the pα1 and pβ1 sequences were identified by comparison with previously described chick α- and β-tubulin cDNA sequences (47). Within the regions compared, both α and β coding sequences are highly conserved; most differences are due to third base codon substitutions (Alexandraki and Ruderman, manuscript in preparation).

**Heterogeneous tubulin mRNA's.** Testis mRNA's hybridized with the α and β cDNA clones at 54°C encoded multiple polypeptides that could be separated on the basis of their isoelectric points (Fig. 3). However, mRNA's selected by pβ1 at increasingly higher temperatures (58 and 62°C) encoded fewer β-tubulin isoelectric forms. At the highest temperature used (62°C), essentially only one prominent β-tubulin translation product was detected. Densitometric measurements of the spot ratios at the three different temperatures confirmed this (data not shown). These results suggest that testis mRNA encodes heterogeneous but closely related β tubulins which differ in primary structure and are not the result of post-translational modifications that occur in the reticulocyte lysate. Similar results were indicated by the analysis of the pα1 clone (Fig. 3). However, streaking of α tubulins in the isoelectric focusing gel made

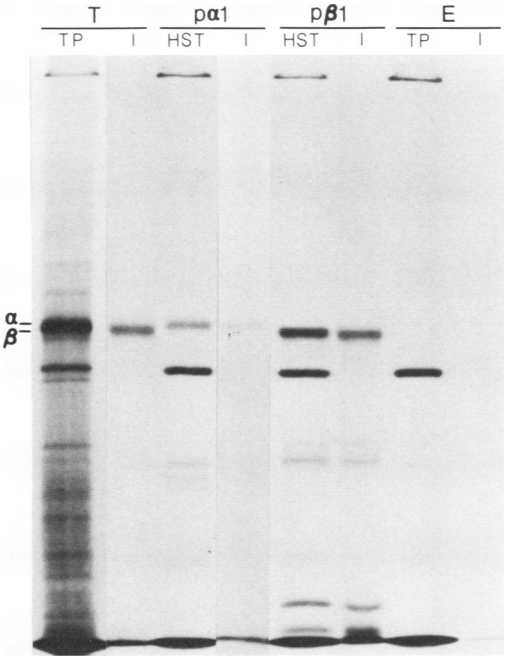


FIG. 2. *Identification of α- and β-tubulin cDNA clones by hybrid selection, cell-free translation, and immunoprecipitation. Testis mRNA's hybridized to pα1 and pβ1 DNAs were translated in vitro by using [$^{35}$S]methionine as a label, and the products were analyzed by SDS-polyacrylamide gel electrophoresis (lanes pα1-HST and pβ1-HST). Equal samples of these translation products were immunoprecipitated with specific αβ tubulin antiserum (lanes pα1-I and pβ1-I). Testis cytoplasmic poly(A)$^+$ RNA was translated in vitro (lane T-TP), immunoprecipitated (lane T-I), and electrophoresed in parallel. The products of reticulocyte lysate endogenous incorporation were analyzed similarly (lanes E-TP and E-I).*

it difficult to resolve distinct polypeptides encoded by mRNA's hybridized at the different temperatures.

**Cloned α- and β-tubulin sequences in pα1 and pβ1 do not cross-hybridize.** The use of cloned pα1 and pβ1 probes to follow differential tubulin gene expression in development, to select genomic clones containing α- and β-tubulin sequences, and to analyze the organization of the α- and β-tubulin genes in genomic DNA required that these two probes do not cross-hybridize. We detected no homology in the hybrid selection experiments (Fig. 2 and 3), nor was any homology suggested by the restriction enzyme maps (Fig. 4A). Nevertheless, it was essential to test for cross-hybridization at low criteria. This was done by using the dot hybridization method described by Kafatos et al. (20). As Fig. 4B shows, we detected no homology between pα1 and pβ1 even at a low hybridization temperature. This result agreed with the lack of cross-hybridization of chick α- and β-tubulin cDNA clones reported by Cleveland et al. (12).

**Multiple α- and β-tubulin genes exist in the sea urchin genome.** The results described above demonstrated the existence of several heterogeneous α- and β-tubulin mRNA's. Different mRNA's can arise from transcription of different genes or by differential splicing of the primary transcription product of a single gene. In the experiments described below, we estimated the numbers of α- and β-tubulin sequences in sea urchin germline DNA by using a straightforward dot hybridization assay (20) and the gel transfer method of Lis et al. (26). In these experiments, known amounts of sea urchin germline (sperm) DNA and pα1 or pβ1 DNAs (corresponding to 1, 2, 5, 10, and 20 copies per haploid genome) were bound directly to a nitrocellulose filter in adjacent dots of equal size or were electrophoresed in adjacent lanes in gels and transferred to nitrocellulose. To take into account the possible variations in the binding of DNA fragments of different sizes to nitrocellulose, we used genomic DNA prepared in the following three different ways: unsheared, randomly sheared (average length, 20 kb), and digested with *Pvu*II (1- to 20-kb fragments). The filters were hybridized with $^{32}$P-labeled pα1 or pβ1 insertions. We compared the amounts of probe hybridized by autoradiography and by liquid scintillation counting. The experimental details and results of these experiments are shown in Table 1. An example of the procedure for estimating gene copy number by the dot hybridization method is shown in Fig. 5. A densitometric analysis of the α autoradiograms (Fig. 5B and C) indicated that there were 9 to 11 copies of the α-tubulin gene and 12 or 13 copies of the β-tubulin gene per haploid genome. Quantification by liquid scintillation counting (Fig. 5D) gave very similar results. The estimates obtained by the gel transfer method were slightly higher (Table 1), possibly due to higher nonspecific backgrounds of hybridization, but they were in reasonably good agreement with the values from the dot hybridization experiments. We believe that for complex multigene families, the dot hybridization assay of gene copy numbers is more accurate than the gel transfer method, since a specific amount of DNA is applied directly and is bound to an adequate area of nitrocellulose. This both simplifies quantitative comparisons and avoids possible problems arising from unequal transfer efficiencies of DNA fragments of different sizes.

**Arrangement of tubulin genes in sea urchin DNA.** By analyzing both total and cloned segments of germline DNA with our cDNA probes, we obtained a general picture of the

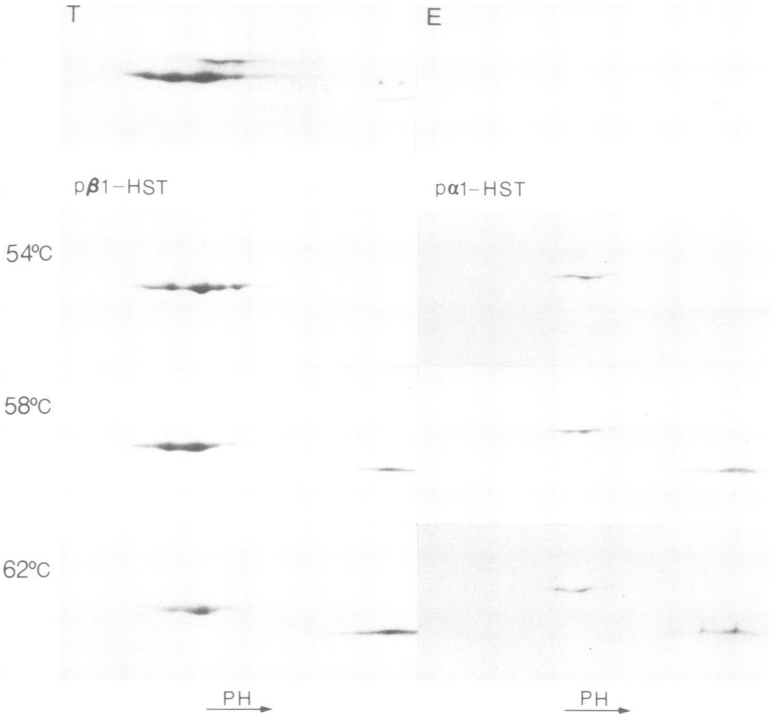


FIG. 3. *Two-dimensional electrophoretic analysis of translation products encoded by mRNA's hybrid selected by pα1 and pβ1 at different temperatures. Testis mRNA's were hybridized to pα1 and pβ1 DNAs at three different temperatures (54, 58, and 62°C in 50% formamide–0.6 M NaCl), translated in vitro in the presence of [$^{35}$S]methionine (pα1-HST and pβ1-HST) and analyzed by two-dimensional gel electrophoresis. T and E indicate the in vitro translation products of testis cytoplasmic poly(A)$^+$ RNA and reticulocyte lysate endogenous incorporation, respectively. A pH range of 4 to 8 was used for isoelectric focusing. Only the parts of the gels that resolved the tubulins are shown.*

organization of the tubulin genes in the sea urchin genome. To analyze total genomic DNA, we digested equal portions in separate incubations with several hexamer restriction enzymes which do not cut inside the cloned α and β cDNA sequences. All digestions were carried out to completion, as judged by the extent of digestion of the λ or pBR322 DNA standards included in parallel reactions. Restricted DNAs were electrophoresed in agarose gels along with *Sal*I-linearized pα1 or pβ1 DNAs loaded in amounts that were equivalent to 1, 2, 5, and 10 copies per haploid genome. The DNA fragments were transferred to nitrocellulose and hybridized with a pα1 or pβ1 insertion probe (Fig. 6A through D). Both probes hybridized to several DNA fragments, a finding consistent with the existence of multiple genes. The complexity of the hybridization patterns indicates that all of the genes are not present as tandemly repeated identical units. The various hybridized fragments could have contained either identical sequences or closely related variants. Bands that showed hybridization intensities higher than one copy equivalent may have resulted from the presence of clustered genes (identical or different) within a single fragment or from the existence of several fragments having the same electrophoretic mobility, each containing one or more genes. Fragments with hybridization intensities lower than one copy equivalent very probably resulted from cross-hybridizations to tubulin sequences of considerable mismatch. This interpretation is consistent with the hypothesis that variant mRNA's are transcribed from different genes. Most of the hybridized fragments represented different genes rather than portions of an individual gene (or genes) interrupted by introns containing the relevant restriction sites within the regions spanned by our probes; both the 5′ half and the 3′ half of the pβ1 insertion hybridized to the same sets of fragments generated by *Pvu*II, *Bgl*II, and *Ava*I digestions (Fig. 6E). We do not know whether the same is true for the α genes, since it was difficult to obtain an unambiguous genomic hybridization pattern with the 3′ half of the pα1 probe. (The 3′ half of the pα1 probe consists mainly of the 3′ nontranslated sequence.)

Comparisons of the genomic hybridization

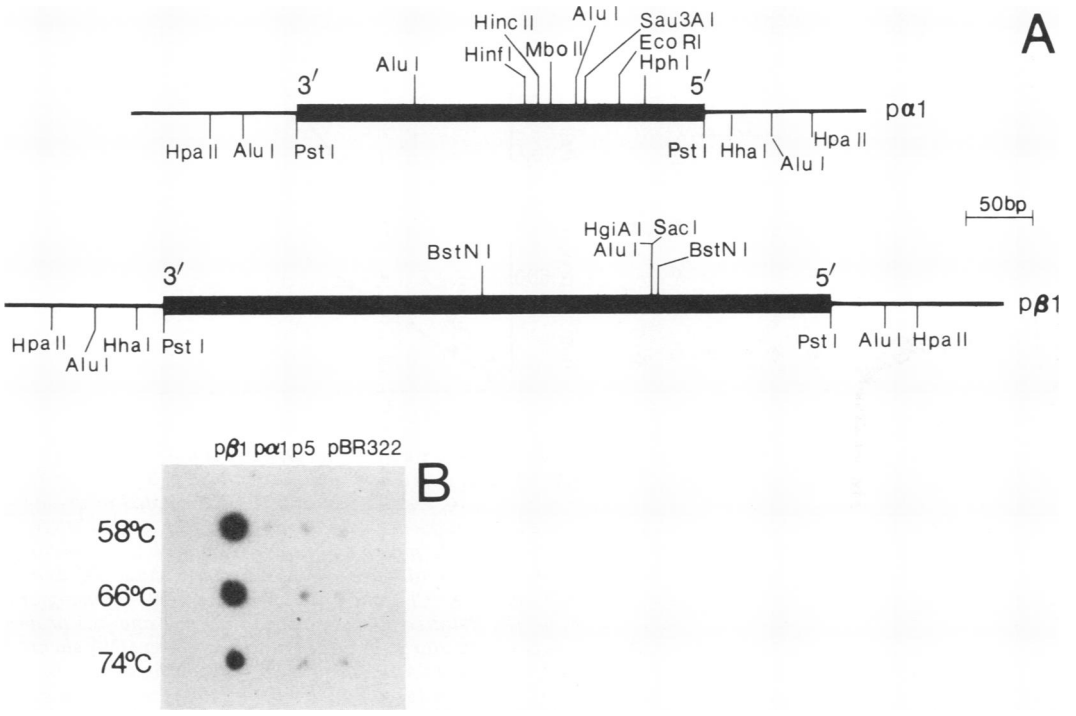


FIG. 4. *(A) Restriction endonuclease maps of pα1 and pβ1. The restriction enzymes indicated were the only enzymes of 26 tested that cleaved the inserted pα1 (300-base pair [bp]) sequence. The restriction sites for the pβ1 insertion (450 base pairs) tested with the same set of enzymes are shown; pβ1 also contains three MboII and four HphI sites, which are not shown. (B) Cross-hybridization analysis of pα1 and pβ1 sequences by dot hybridization at three different temperatures. Equivalent amounts of the following plasmid DNAs were spotted onto nitrocellulose filters: pα1, pβ1, p5 (a recombinant plasmid containing a nontubulin insertion sequence), and pBR322. The filters were hybridized with $^{32}$P-labeled pβ1 insertion DNA that was labeled after excision by nick-translation. A total of 20 μg of polycytidylic acid per ml was present during the prehybridization and hybridization reactions. Three different temperatures of hybridization in 0.75 M NaCl were used (58, 66, and 74°C).*

TABLE 1. *Tubulin gene copy number in the sea urchin genome*[a]

| Expt[b] | Gene copy no. as determined by autoradiography | | | | Gene copy no. as determined by liquid scintillation counting | | | |
|---|---|---|---|---|---|---|---|---|
| | α Tubulin | | β Tubulin | | α Tubulin | | β Tubulin | |
| | *L. pictus* DNA | *Pvu*II-digested DNA | *L. pictus* DNA | *Pvu*II-digested DNA | *L. pictus* DNA | *Pvu*II-digested DNA | *L. pictus* DNA | *Pvu*II-digested DNA |
| 1 | | | | | 9.5 | | 12 | |
| 2 | | | | | 10 | 9.5 | 12 | 12.5 |
| 3 | 10.5 | | 13 | | | | | |
| 4 | 13 | 16 | 14 | 15 | | | | |
| 5 | 11.5 | 12.5 | 12 | 16 | 9 | 9.5 | 11 | 15 |

[a] Average estimates of α- and β-tubulin gene copy numbers were obtained by dot hybridizations (experiments 1 through 3) or gel blot hybridizations (experiments 4 and 5) of *L. pictus* germline DNA and increasing amounts of pα1 and pβ1 DNAs with pα1 and pβ1 probes. The relative hybridizations were analyzed by microdensitometric tracings of the autoradiograms or by liquid scintillation counting of the nitrocellulose filters.

[b] In experiment 1, 16 μg of unsheared sea urchin DNA and copy number standard DNAs (see text) were bound to nitrocellulose filters. Each DNA sample was loaded as eight dots of equal size. In experiment 2, we used the same procedure as in experiment 1, except that randomly sheared (~20-kb) or *Pvu*II-digested sea urchin DNA was used. In experiment 3, 2 μg of randomly sheared (~20-kb) sea urchin DNA and the appropriate copy number standards were bound to nitrocellulose, each in one dot. In experiments 4 and 5, 15 μg of randomly sheared genomic DNA or genomic DNA digested with *Pvu*II and copy number standard DNAs were run in adjacent lanes on agarose gels and transferred to nitrocellulose filters. In experiments 1, 3, and 4, *Hin*dIII-digested λ phage DNA was included as a carrier in the plasmid standard DNAs. The amounts of λ DNA used were the same as the amounts of sea urchin DNA loaded.

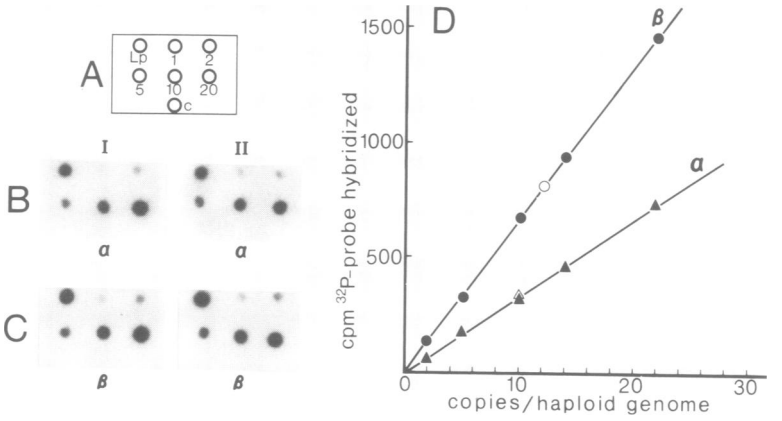


FIG. 5. *Quantification of tubulin gene copy number in sea urchin germline DNA by dot hybridization. (A) Diagrammatic representation of the pattern of DNA dots bound to a nitrocellulose filter. Lp, 2 μg of L. pictus sperm DNA randomly sheared to a length of ~20 kb; 1, 2, 5, 10, and 20, pα1 or pβ1 DNA loaded in amounts equivalent to 1, 2, 5, 10, and 20 copies, respectively, of tubulin sequence contained in 2 μg of L. pictus DNA; c, 2 μg of HindIII-digested λ phage DNA. (B) Autoradiograms of L. pictus and pα1 DNAs which were bound to nitrocellulose filters as described above in the presence (I) or absence (II) of carrier λ phage DNA and hybridized with $^{32}$P-labeled nick-translated pα1 insertion DNA. (C) Same experiment as in (B), but using pβ1 DNA standards and pβ1 insertiion DNA as the probe. (D) Relative hybridization of the pα1 and pβ1 probes to increasing amounts of pα1 (▲) and pβ1 (●) copy number standards. A total of 16 μg of sperm DNA sheared to a length of ~20 kb or digested with PvuII and pα1 (or pβ1) DNA in amounts corresponding to 2, 5, 10, 14, and 22 copies of tubulin gene sequence contained in 16 μg of L. pictus DNA were bound to nitrocellulose, each as eight dots of equal size. The filters were hybridized with $^{32}$P-labeled pα1 (or pβ1) insertion DNA. The amounts of hybridization were measured by liquid scintillation counting. The relative hybridization values of L. pictus DNA indicated the numbers of α-tubulin genes (△) and β-tubulin genes (○) within the haploid L. pictus genome.*

patterns generated by pα1 and pβ1 probes revealed that in all cases the two probes hybridized to different sets of restriction fragments. This suggested that the α- and β-tubulin genes are not linked closely as αβ gene pairs in the germline DNA of sea urchins. Preliminary analyses of cloned genomic tubulin sequences supported this interpretation and indicated that some genes within the same family are clustered. Clones containing α- or β-tubulin sequences were selected from a recombinant Charon 4 λ-*L. pictus* genomic DNA library by using $^{32}$P-labeled pα1 or pβ1 DNA as a probe (2). Three different clones that hybridized to the pα1 probe and nine different clones that hybridized to the pβ1 probe were identified by gel electrophoresis, blotting, and hybridization of DNA restriction fragments to labeled pα1 and pβ1 DNAs. The restriction endonuclease digestion and hybridization patterns of some of these genomic clones are shown in Fig. 7A and B. In all cases the DNAs from individual clones hybridized to either the pα1 probe or the pβ1 probe but not to both. However, we could not eliminate the possibility that there was an unidentified rare clone that contained both α- and β-tubulin sequences. Nevertheless, considering the number of different clones identified (12 clones), the lengths of the cloned genomic DNA fragments examined (12 to 20 kb), and the different pα1 and pβ1 hybridization patterns to restricted total genomic DNA (Fig. 6), our results suggested that most of the α- and β-tubulin genes are not organized as closely linked αβ gene pairs.

In contrast, some clustering of tubulin genes within the same family was indicated by the following results. Digestions of some of the genomic clones with certain restriction enzymes which do not cut inside the pα1 (or pβ1) probe yielded two fragments that hybridized to the pα1 (or pβ1) probe. The most straightforward explanations for such results were that (i) the two fragments were portions of a single gene that had an intron which was within the region spanned by the probe and contained that particular restriction site, and (ii) there was more than one tubulin gene in the cloned DNA insertion and the restriction site lay between two genes or in an intron of one of the genes. To distinguish between these two possibilities, we hybridized the 5′ and 3′ halves of the cDNA probes to the restriction fragments of the genomic clones. In two of the three α genomic clones and in four of the nine β genomic clones, the same sets of restriction fragments hybridized to both the 5′ half and the 3′ half probes, suggesting that each of these clones contained at least two genes belonging to the same family. Examples from

this experiment are shown in Fig. 7C. Clones λLp23 (α), λLp21 (α), λLp35 (β), and λLp6 (β) each contain at least two tubulin genes. λLp12 (β), which contains only one tubulin gene, is shown for comparison.

## DISCUSSION

Considerable evidence from developmental, biochemical, and morphological studies of tubulin carried out in many systems, including sea urchins, has suggested that there are several heterogeneous α and β tubulins, which are specific for different types of microtubules. To test this hypothesis and ultimately to determine how the synthesis of microtubule-specific αβ tubulin heterodimers is regulated developmentally, we isolated cDNA and genomic clones that encode α and β tubulins in the sea urchin, an organism that is especially suitable for such studies.

**Tubulin gene multiplicity and heterogeneity.** Estimates of the total number of α- and β-tubulin genes per haploid germline genome

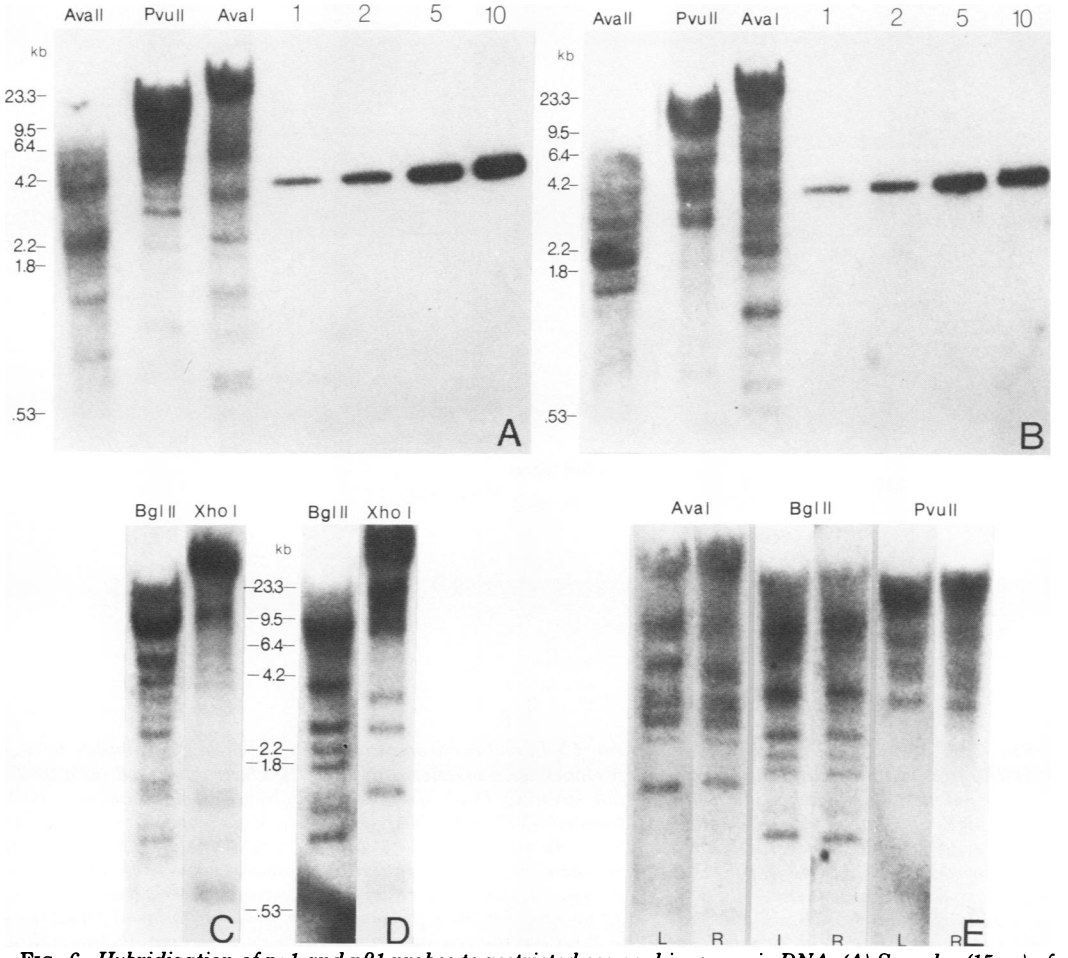


FIG. 6. *Hybridization of pα1 and pβ1 probes to restricted sea urchin genomic DNA. (A) Samples (15 μg) of sea urchin germline DNA were restricted with various endonucleases and analyzed on 0.8% agarose gels. In adjacent lanes, SalI-linearized pα1 DNAs were loaded in insertion amounts equivalent to 1, 2, 5, and 10 copies per haploid genome (9.7 × $10^8$ base pairs) (lanes 1, 2, 5, and 10, respectively). Then DNAs were transferred to nitrocellulose and hybridized with excised $^{32}$P-labeled nick-translated pα1 insertion. (B) Same experiment as in (A), but using pβ1 DNA standards and the pβ1 insertion as a probe. (C) Restricted sea urchin DNA (15 μg) was electrophoresed, transferred to nitrocellulose, and hybridized with pα1 insertion probe. (D) Restricted DNA (15 μg) was electrophoresed in the same gel that was used for (C), transferred to nitrocellulose, and hybridized with pβ1 insertion probe. (E) Germline DNA (15 μg) was restricted, electrophoresed, transferred to nitrocellulose, and hybridized with the left (lanes L) or right (lanes R) half of the pβ1 probe. (pβ1 was digested with SacI and SalI restriction enzymes. The 1.43-kb fragment including 120 base pairs of insertion sequence was the left half [5'] probe. The 3.11-kb fragment including 330 base pairs of insertion sequence was the right half [3'] probe. The two probes did not cross-hybridize, as shown in Fig. 7C.)*

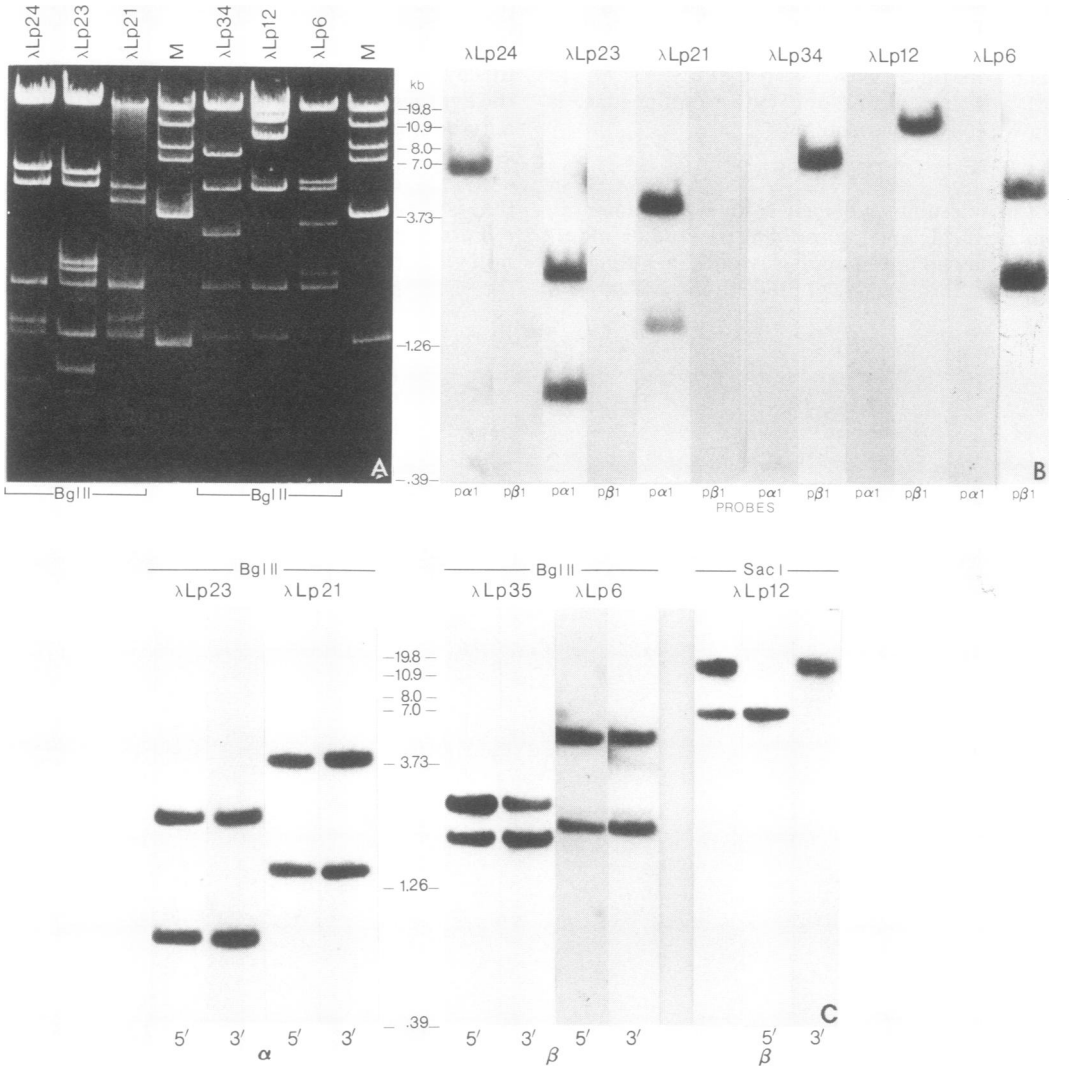


FIG. 7. *α- and β-tubulin sequences in Charon 4 λ-L. pictus chromosomal DNA clones. (A) Samples (0.5 μg) of DNAs from six λ-sea urchin genomic DNA clones were restricted with BglII, electrophoresed on a 0.85% agarose gel, and visualized by ethidium bromide staining. Lane M contained molecular weight markers (both EcoRI-digested Charon 4 λ DNA and HpaI-digested φX174 RF DNA). (B) The endonuclease-restricted DNAs shown in (A) were transferred to nitrocellulose and probed with $^{32}$P-labeled nick-translated pα1 and pβ1 DNAs in reciprocal reactions. (C) DNAs from λ genomic clones containing α-tubulin sequences (λLp23 and λLp21) or β-tubulin sequences (λLp35, λLp6, and λLp12) were restricted, electrophoresed, transferred to nitrocellulose, and hybridized with 5′ or 3′ half probes. For the α probes another α-tubulin plasmid (pα2) was used to obtain 5′ and 3′ half probes, since the 3′ half of the pα1 insertion (which contains mainly noncoding sequences) did not hybridize to λLp21 or λLp23 DNA. pα2 is a testis α-tubulin cDNA clone containing an 850-base pair insertion which bears the same restriction sites as pα1. pα2 insertion DNA was digested with SalI, a restriction site located approximately 180 base pairs beyond the 5′ end of the pα1 insertion. pα2 5′ was the 250-base pair fragment, and pα2 3′ was the 600-base pair fragment (which included all of the pα1 insertion sequence). The pβ1 5′ and 3′ probes are described in the legend to Fig. 6.*

were obtained by using the dot hybridization method of Kafatos et al. (20). Known amounts of genomic DNA and cloned α- or β-tubulin cDNA copy number standards were bound directly in dots of equal size on a nitrocellulose filter, and the filter was hybridized with a labeled pα1 or pβ1 cDNA probe. Hybridizations were performed at a moderately high criterion so that we could detect both identical and closely related sequences. Comparisons of the amounts of probe bound by the genomic DNA and the cloned cDNA standards indicated the presence

of 9 to 11 α-tubulin genes and 12 or 13 β-tubulin genes. Slightly higher copy numbers were obtained by using the "reconstruction" method of Lis et al. (26). We found that the minimum number of tubulin sequences (9 to 13 sequences) in the sea urchin genome was significantly higher than the estimates of four genes in both *Drosophila* (37) and chickens (10). We do not know whether this difference has any functional significance since the gene copy number experiments did not distinguish among identical repeats, closely related variant genes, and pseudogenes.

The existence of heterogeneous α- and β-tubulin mRNA's was indicated by the finding that testis mRNA directed the synthesis in vitro of several distinct α and β tubulins that could be resolved by isoelectric focusing. Different patterns of translation products were obtained with mRNA's hybridized to the pα1 and pβ1 DNAs at different temperatures, indicating that heterogeneity cannot be due solely to post-translational modifications of a single polypeptide. The existence of tubulin protein heterogeneity in sea urchins was suggested by Stephens (44), but in those experiments the possibility of post-translational modifications cannot be ruled out unequivocally. Recently, we isolated several additional α- and β-tubulin cDNA clones which bear overlapping as well as different restriction sites compared with pα1 and pβ1, respectively. This finding directly confirms the existence of multiple, different tubulin mRNA's (Alexandraki and Ruderman, unpublished data).

The presence of several different α- and β-tubulin mRNA's is explained most easily by the existence of several different α- and β-tubulin genes. Gene heterogeneity can explain the differential hybridization intensities of the total genomic DNA restriction fragments. It is also consistent with the finding that individual cloned genomic fragments containing α (or β) sequences produce different restriction patterns. Of course, such differences could also be due to heterogeneity in adjacent noncoding DNA regions. As the genomic library was constructed by using DNA from more than one individual, some variation could be due to DNA polymorphism in different individuals or to allelic polymorphism in diploid genomes. However, other data argue that this source of variation is probably not significant; DNAs isolated from individual animals and tested by restriction endonuclease analysis and blot hybridization produce identical patterns. Furthermore, the heterogeneity of the genomic DNA clones was not generated artifactually in the cloning procedure; the restriction fragments from cloned DNAs that contain tubulin sequences correspond in size to fragments containing tubulin sequences present in sea urchin total genomic DNA digested with the same enzymes (Alexandraki, unpublished data).

**Genomic organization of the α- and β-tubulin gene families.** An examination of the hybridization patterns of pα1 and pβ1 tubulin probes to total genomic DNA restriction fragments (Fig. 6) indicated that the multiple α- and β-tubulin genes are not organized as tandemly repeated identical units. In *Drosophila*, genetic and chromosomal in situ hybridization experiments also have indicated that different α- and β-tubulin genes are dispersed throughout the genome (21, 22, 37). In chickens, chromosomal in situ hybridizations have shown that α- and β-tubulin genes are dispersed and unlinked (10). The possibility that some genes of the same family are clustered in the sea urchin genome is supported by two findings. First, the presence of high-molecular-weight DNA restriction fragments which show hybridization intensities higher than one copy equivalent suggests local clustering, although such a result could be explained equally well by comigration of several different fragments of the same size, each containing a single gene sequence. Second and more directly, a preliminary analysis of genomic clones has shown that several of these clones contain more than one gene of the same family. In *Drosophila*, such clustering is not obvious but cannot be ruled out by the previously published data (37).

Two different lines of evidence suggest that most of the α- and β-tubulin genes in the sea urchin genome are not organized as linearly contiguous αβ gene pairs. First, in all cases, the hybridization patterns of pα1 probe to total genomic DNA restricted separately by several enzymes are different from those generated by the pβ1 probe. Second, 12 different genomic clones which carry 12 to 20 kb of sea urchin DNA containing tubulin gene sequences hybridize uniquely to either the pα1 probe or the pβ1 probe; no genomic clones which hybridize to both have been found. The possibility of an undetected αβ gene pair still remains and could be confirmed by in situ hybridization to sea urchin chromosomes or by identification of a cloned fragment containing both genes. Even if α- and β-tubulin genes are not linked closely at the level of DNA, the coordinated expression of these genes may be modulated by distant regulatory sequences. A detailed analysis of the genomic clones may reveal such common regulatory regions for the two families.

ACKNOWLEDGMENTS

We thank M. Schmidt for technical assistance with some of the experiments. We are grateful to A. Efstratiadis, L. Villa-Komaroff, T. Eickbush, and G.-K. Sim for advice, to G.

Thireos for helpful discussions, and to G. Moore and E. H. Davidson for providing the λ library. We thank F. C. Kafatos for making his P2 facilities available during the initial experiments and B. Roberts for communicating his hybrid selection translation protocol before publication.

This work was supported by Public Health Service research grant HD 10994 from the National Institute of Child Health and Human Development, by grant PCM 7923046 from the National Science Foundation, and by Public Health Service Career Development Award NIH HD 00349 from the National Institutes of Health (all to J.V.R.).

## LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408-1413.
2. **Benton, W. D., and R. W. Davis.** 1977. Screening of λgt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180-182.
3. **Bibring, T., and J. Baxandall.** 1977. Tubulin synthesis in sea urchin embryos: almost all tubulin of the first cleavage mitotic apparatus derives from the unfertilized egg. Dev. Biol. **55**:191-195.
4. **Bibring, T., and J. Baxandall, S. Denslow, and B. Walker.** 1976. Heterogeneity of the alpha subunit of tubulin and the variability of tubulin within a single organism. J. Cell Biol. **69**:301-312.
5. **Blattner, F. R., B. G. Williams, A. E. Blechl, K. Denniston-Thompson, H. E. Faber, L. A. Furlong, D. J. Grunwald, D. O. Kiefer, D. D. Moore, J. W. Schumm, E. L. Sheldon, and O. Smithies.** 1977. Charon phages: safer derivatives of bacteriophage lamba for DNA cloning. Science **196**:161-169.
6. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.
7. **Boyer, H. W., and D. Rouland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *E. coli*. J. Mol. Biol. **41**:459-472.
8. **Britten, R. J., D. E. Graham, and B. R. Neufeld.** 1974. Analysis of repeating DNA sequences by reassociation. Methods Enzymol. **29**:363-418.
9. **Bryan, R. N., G. A. Cutter, and M. Hayashi.** 1978. Separate mRNAs code for tubulin subunits. Nature (London) **272**:81-83.
10. **Cleveland, D. W., S. H. Hughes, E. Stubblefield, M. W. Kirschner, and H. E. Varmus.** 1981. Multiple α and β tubulin genes represent unlinked and dispersed gene families. J. Biol. Chem. **256**:3130-3134.
11. **Cleveland, D. W., M. W. Kirschner, and N. J. Cowan.** 1978. Isolation of separate mRNAs for α- and β-tubulin and characterization of the corresponding in vitro translation products. Cell **15**:1021-1031.
12. **Cleveland, D. W., M.A. Lopata, R. J. MacDonald, N. J. Cowan, W. J. Rutter, and M. W. Kirschner.** 1980. Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. Cell **20**:95-105.
13. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication of *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667-676.
14. **Efstratiadis, A., F. C. Kafatos, A. M. Maxam, and T. Maniatis.** 1976. Enzymatic in vitro synthesis of globin genes. Cell **7**:279-288.
15. **Feit, H., U. Neudeck, and F. Baskin.** 1977. Comparison of the isoelectric and molecular weight properties of tubulin subunits. J. Neurochem. **28**:697-706.
16. **Fujiwara, K., and T. D. Pollard.** 1978. Simultaneous localization of myosin and tubulin in human tissue culture cells by double antibody staining. J. Cell Biol. **77**:182-195.
17. **Fulton, C., and J. D. Kowit.** 1975. Programmed synthesis of flagellar tubulin during cell differentiation in *Naegleria*. Ann. N.Y. Acad. Sci. **253**:318-332.
18. **Gilbert, W., and A. Maxam.** 1973. The nucleotide sequence of the lac operator. Proc. Natl. Acad. Sci. U.S.A. **70**:3581-3584.
19. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. **72**:3961-3965.
20. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. **7**:1541-1552.
21. **Kemphues, D. J., R. A. Raff, T. C. Kaufman, and E. C. Raff.** 1979. Mutation in a structural gene for a β tubulin specific to testis in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. **76**:3991-3995.
22. **Kemphues, K. J., E. C. Raff, R. A. Raff, and T. C. Kaufman.** 1980. Mutation in a testis-specific β-tubulin in *Drosophila*: analysis of its effects on meiosis and map location of the gene. Cell **21**:445-451.
23. **Kessler, S. W.** 1976. Cell membrane antigen isolation with the staphylococcal protein A-antibody adsorbent. J. Immunol. **117**:1482-1490.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
25. **Laskey, R. A., and A. D. Mills.** 1975. Quantitative film detection of $^{3}H$ and $^{14}C$ in polyacrylamide gels by fluorography. Eur. J. Biochem. **56**:335-341.
26. **Lis, J. T., L. Prestige, and D. S. Hogness.** 1978. A novel arrangement of tandemly repeated genes at a major heat shock site in *D. melanogaster*. Cell **14**:901-919.
27. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G.-K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687-701.
28. **Maniatis, T., A. Jeffrey, and H. van de Sande.** 1975. Chain length determination of small double- and single-stranded DNA molecules in polyacrylamide gel electrophoresis. Biochemistry **14**:3787-3794.
29. **Marotta, C. A., P. Strocchi, and J. M. Gilbert.** 1979. Biosynthesis of heterogeneous forms of mammalian brain tubulin subunits by multiple messenger RNAs. J. Neurochem. **33**:231-246.
30. **McDonnel, M., M. N. Simon, and W. F. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119-146.
31. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007-4021.
32. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247-256.
33. **Ricciardi, R. P., J. S. Miller, and B. E. Roberts.** 1979. Purification and mapping of specific mRNAs by hybridization-selection and cell-free translation. Proc. Natl. Acad. Sci. U.S.A. **76**:4927-4931.
34. **Roberts, K., and J. S. Hyams (ed.).** 1979. Microtubules. Academic Press, Inc., London.
35. **Roychoudhury, R., E. Jay, and R. Wu.** 1976. Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. Nucleic Acids Res. **3**:101-116.
36. **Ruderman, J. V., and M. L. Pardue.** 1978. A portion of all major classes of histone messenger RNA in amphibian oocytes is polyadenylated. J. Biol. Chem. **253**:2018-2025.
37. **Sanchez, F., J. E. Natzle, D. W. Cleveland, M. W. Kirschner, and B. J. McCarthy.** 1980. A dispersed

multigene family encoding tubulin in *Drosophila melanogaster*. Cell **22**:845–854.

38. **Sharp, P. A., B. Sugden, and J. Sambrook.** 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. Biochemistry **12**: 3055–3063.
39. **Sheir-Neiss, G., M. H. Lai, and R. N. Morris.** 1978. Identification of a gene for β-tubulin in *Aspergillus nidulans*. Cell **15**:639–647.
40. **Snyder, J. A., and J. R. McIntosh.** 1976. Biochemistry and physiology of microtubules. Annu. Rev. Biochem. **45**:699–720.
41. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
42. **Stephens, R. E.** 1977. Differential protein synthesis and utilization during cilia formation in sea urchin embryos. Dev. Biol. **61**:311–329.
43. **Stephens, R. E.** 1977. Major membrane protein differences in cilia and flagella: evidence for a membrane-associated tubulin. Biochemistry **16**:2047–2058.
44. **Stephens, R. E.** 1978. Primary structural differences among tubulin subunits from flagella, cilia, and the cytoplasm. Biochemistry **17**:2882–2891.
45. **Stephens, R. E., and K. T. Edds.** 1976. Microtubules: structure, chemistry and function. Physiol. Rev. **56**: 709–777.
46. **Thireos, G., R. Griffin-Shea, and F. C. Kafatos.** 1979. Identification of chorion protein precursors and the mRNAs that encode them in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. **76**:6279–6283.
47. **Valenzuela, P., M. Quiroga, J. Zaldivar, W. J. Rutter, M. W. Kirschner, and D. W. Cleveland.** 1981. Nucleotide and corresponding amino acid sequences encoded by α and β tubulin mRNAs. Nature (London) **289**:650–655.
48. **Villa-Komaroff, L., A. Efstratiadis, S. Broome, P. Lomedico, R. Tizard, S. P. Naber, W. L. Chick, and W. Gilbert.** 1978. A bacterial clone synthesizing proinsulin. Proc. Natl. Acad. Sci. U.S.A. **75**:3727–3731.
49. **Wahl, G. M., and M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proc. Natl. Acad. Sci. U.S.A. **76**:3683–3687
50. **Wensink, P. C., D. J. Finnegan, J. E. Donelson, and D. S. Hogness.** 1974. A system for mapping DNA sequences in the chromosomes of *Drosophila melanogaster*. Cell **3**:315–325.
51. **Witman, G. B., K. Carlson, and J. L. Rosenbaum.** 1972. *Chlamydomonas* flagella. II. The distribution of tubulins 1 and 2 in the outer doublet microtubules. J. Cell Biol. **54**:540–555.