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Multiple lobster tubulin isoforms are encoded by a simple gene family

(Microtubules; *Homarus americanus*; Crustacea; tegumental glands; phylogenetics; chaperonins)

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SUMMARY

Microtubule proteins isolated from pleopod tegumental gland (PTG) tissue of the American lobster, *Homarus americanus*, reveal a complex tubulin (Tub) profile. To determine whether Tub heterogeneity in PTG is due to expression of a large *tub* gene family or the result of post-translational modification, a PTG cDNA library was constructed. Clones coding for both α- and β-Tub were isolated, sequenced and found to contain open reading frames (ORFs) of 451 amino acids (aa). Alignments reveal phylogenetic clustering with other arthropods and identify unique changes in primary structure which may have functional significance. These clones, when used to probe restriction enzyme-digested lobster genomic DNA in transfer-hybridization experiments, revealed a simple banding pattern indicating a lobster *tub* gene family of limited complexity. Lobsters appear to make use of a small *tub* gene family and fulfill the varied functional requirements imposed upon cellular microtubules through post-translational modifications of relatively few gene products.

INTRODUCTION

Microtubules are cytoskeletal elements found in the cytoplasm of eukaryotic cells, mediating numerous processes including cell division, intracellular chromosomal and vesicular trafficking, ciliary and flagellar beating and maintenance of cellular architecture. Microtubules are primarily composed of three different classes of Tub proteins: the α- and β-Tub form heterodimers which assemble into functional microtubules, while γ-Tub are involved with microtubule nucleation (Stearns et al., 1991) and possibly the formation of unique 'γ-tubules' (Shu and Joshi, 1995). The α- and β-Tub are encoded by two distinct gene families, each elaborating a set of α- or β-Tub isoforms.

Despite the rapid accumulation of nt and aa sequence data for Tub from higher eukaryotes, few studies have involved the invertebrate *tub* gene families, including arthropods. We consider this a striking omission given the widespread distribution of the arthropods and their adaptation to virtually every ecological habitat. Included in this category are the crustacean members of this phylum. Crustaceans are unusual due to their complex life histories and adaptation to varied, often harsh, environmental conditions. To this end, we have initiated a study designed to present a more complete picture of *tub* gene structure and function within this intriguing group of organisms. Here we report the first available information on crustacean *tub* genes, represented by the American lobster, *Homarus americanus*.

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); BSA, bovine serum albumin; 2-D, 2-dimensional; kb, kilobase(s) or 1000 bp; mAb, monoclonal Ab; MAP, microtubule-associated protein; nt, nucleotide(s); ORF, open reading frame; PTG, pleopod tegumental gland(s); TBS, tris-buffered saline; Tub, tubulin(s); *tub*, gene (DNA, RNA) encoding Tub.

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RESULTS AND DISCUSSION

(a) Lobsters contain numerous isoforms of Tub

When microtubule proteins purified from PTG tissue were resolved by 2-D gel electrophoresis (Fig. 1A), nine distinct α-Tub and nine β-Tub isoelectric variants migrating at 57 and 54 kDa, respectively, were observed. The identification of α-Tub within the 2-D gel profile was confirmed using Western blot analysis with the monoclonal anti α-Tub Ab, DM1A (Fig. 1B). This complex arrangement of Tub isoforms was intriguing given the low number of iso-Tub observed in a related crustacean, the brine shrimp *Artemia salina* (Langdon et al., 1990). The unexpected heterogeneity of the lobster Tub population could have arisen either as products of large *tub* gene families or through extensive post-translational modification of polypeptide products expressed from a limited *tub* gene family. In order to distinguish between these possibilities, isolation and characterization of lobster *tub* genes by molecular cloning was undertaken.

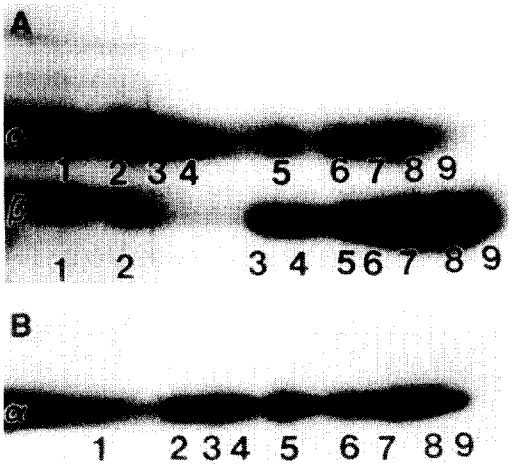


Fig. 1. 2-D electrophoretic (**A**) and Western blot (**B**) analysis of PTG Tub. α, α-tubulin; β, β-tubulin. Numbers refer to individual isoforms. **Methods**: PTG microtubules were purified in a taxol-dependent manner according to the method of Vallee (1982). Taxol was a gift from Dr. M. Suffness, N.C.I., Bethesda, MD. 10 μl of microtubule protein sample (1 mg/ml) in 2-D sample buffer (9.5 M urea/2% Triton X-100/5% 2-mercaptoethanol/2.4% Bio-Lyte (Bio Rad Laboratories, Richmond, CA) 4–6/0.8% Bio-Lyte 3–10) was applied to 4% polyacrylamide minitube gels. *1st dimension*: Isoelectric focusing was performed at 500 V for 30 min and 750 V for 4 h. *2nd dimension*: Tube gels were subsequently placed onto 8% polyacrylamide slab gels (Laemmli, 1970) and electrophoresed at 30 mA for 1.5 h. Proteins were identified by silver staining (Wray et al., 1981). *Western blotting*: Slab gels were electro-transferred to nitrocellulose. Filters were washed in 0.1% BSA in TBS (25 mM Tris, pH 8.0/150 mM NaCl) at 25°C for 20 min followed by blocking in 1% nonfat dry milk in TBS at 25°C for 2 h. Blots were incubated in a 1:1000 dilution of anti α-Tub mAb DM1A (Sigma Immunochemicals, St. Louis, MO) in TBS containing 0.05% Tween-20 at 37°C for 12–18 h. Blots were then washed three times in 0.1% BSA/TBS followed by incubation in a 1:5000 dilution of horse radish peroxidase conjugated goat anti-mouse IgG (Amersham, Arlington Heights, IL) in TBS/Tween-20 for 2 h at 25°C. Blots were washed in 0.1% BSA/TBS and developed using the Enhanced Chemiluminescence Detection Kit (Amersham).

(b) Isolation and sequencing of lobster *tub* cDNAs

Because PTG synthesize large amounts of Tub at times of molting and spawning (Talbot and Zao, 1991), a cDNA library was prepared from poly(A)$^+$mRNA isolated from this tissue during these times. Genomic and cDNA *tub* clones from the arthropods *Artemia* and *Drosophila* were used to screen the library for lobster *tub* clones. Three potential α-*tub* and two potential β-*tub* cDNAs were identified. DNA sequence analysis (Fig. 2) revealed that all three α-*tub* clones and both β-*tub* clones were identical to each other. ORFs of 451 aa were found in both the α-*tub* (Fig. 2A) and β-*tub* (Fig. 2B) clones.

(c) Assignment of lobster *tub* sequences

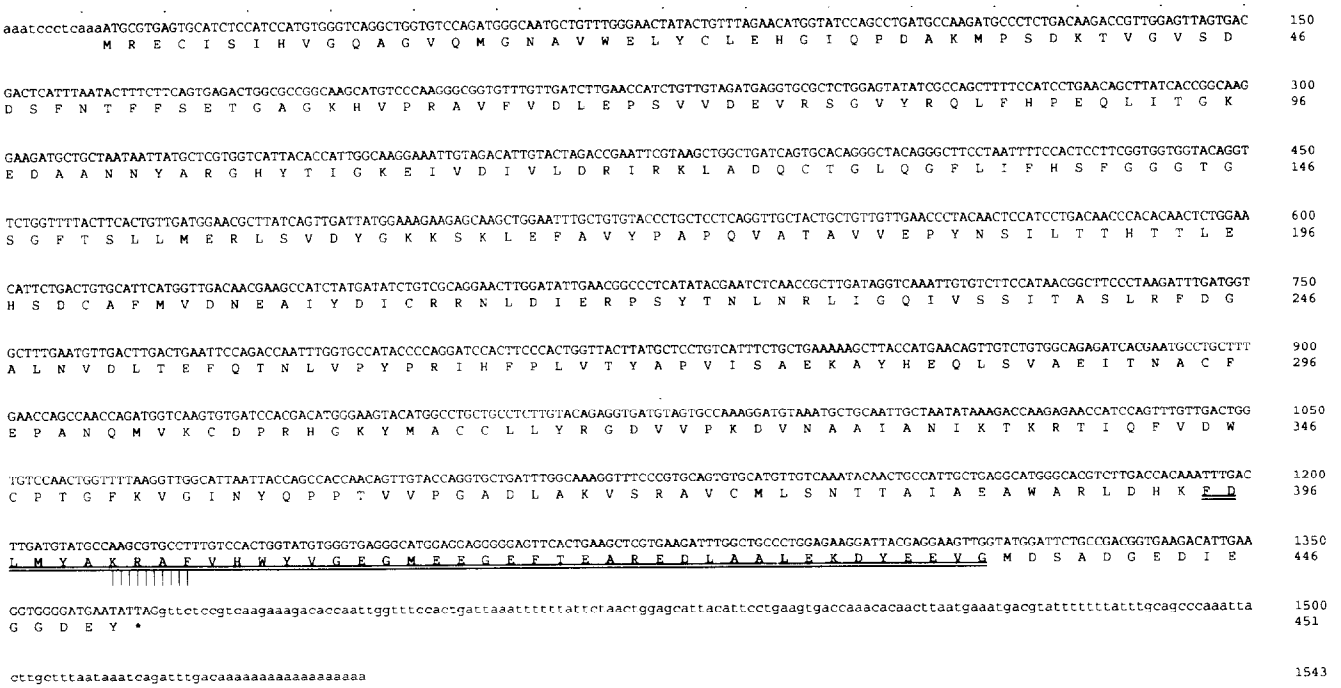
We can unambiguously catalogue the lobster cDNAs as encoding either α- or β-Tub based on the high degree of similarity shared with known *tub* sequences. The α-*tub* clone shared 95% aa identity with the α-I *tub* of *D. melanogaster* (Theurkauf et al., 1986) and 96% aa identity to an α-*tub* from *Artemia* (Dr. T. MacRae, personal communication) while the lobster β-*tub* clone had 95% aa identity with the β-II *tub* from *D. melanogaster* (Rudolph et al., 1987). In addition, highly conserved sequences diagnostic for α-and β-Tub further classified the lobster clones. For the α-*tub* clone, aa 394–436 were nearly identical to highly conserved residues found in other metazoan α-*tub* sequences (Fig. 2A; Burns and Surridge, 1994a). Among known β-Tub, a characteristic region of 34 aa considered to be invariant is present near the C-terminus, and was found at aa 387–420 encoded by the lobster β-*tub* clone (Fig. 2B). In addition, aa 1–4 and 101–111, recognized as highly conserved among β-*tub* sequences, were also found in the lobster β-*tub* coding domain (Fig. 2B). Additional sequences (KRAF at α: aa 401–404; RKAF at β: aa 395–398), implicated in chaperonin mediated folding of the α- and β-Tub polypeptides (Burns and Surridge, 1994b), were present in the lobster clones.

(d) Distinguishing characteristics of β-*tub*

Prominent among the distinguishing features of the β-Tub sequence was the presence of aa insertions. The in-frame Ala-Asp-Lys-Asp insertion at aa 39 near the N terminus of the molecule was similar in location to the unique insertion found in *Dictyostelium* β-Tub (Trivnos-Lagos et al., 1993). The two insertions vary in both aa content and length, however. This region could provide additional recognition sites for species or tissue-specific microtubule-associated proteins (MAPs) on the molecule.

Among the most conserved aa positions within Tub

A



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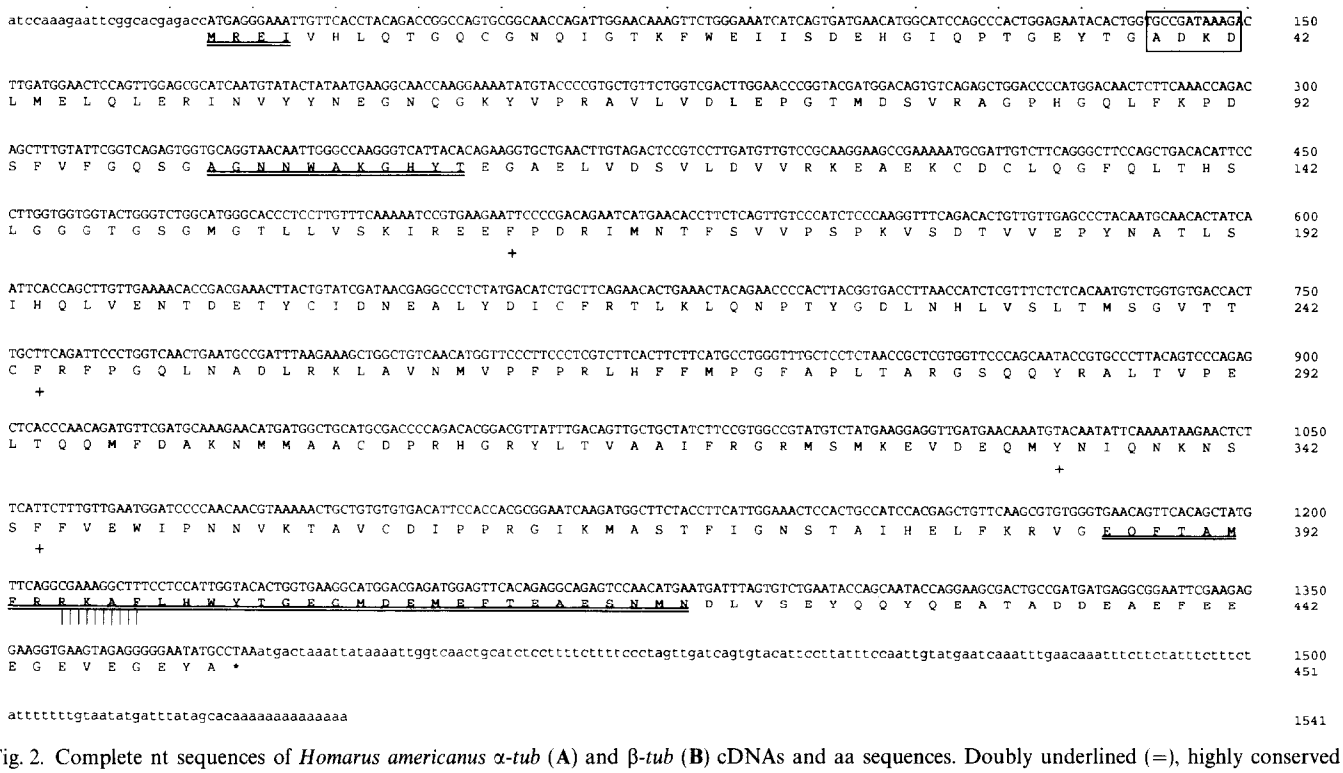

Fig. 2. Complete nt sequences of *Homarus americanus* α-*tub* (**A**) and β-*tub* (**B**) cDNAs and aa sequences. Doubly underlined (=), highly conserved aa regions; (|||), sequences implicated in chaperonin mediated polypeptide folding; boxed (□), regions of unique aa insertion; (+), aromatic aa substitution; (*), stop codon. **Methods**: Poly(A)$^+$mRNA was extracted from PTG using the FastTrack (Invitrogen Corp., San Diego, CA) mRNA isolation kit. A cDNA library was constructed using the λ-ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA). In vitro packaging was accomplished using the Gigapack II Gold packaging extract (Stratagene) followed by amplification in an *E.coli* XL1-Blue MRF host strain. A 1200-bp α-*tub* fragment (αL1) isolated from *Artemia* (Dr. T. MacRae, Dalhousie University, Halifax, N.S.) and a 6.5-kb genomic subclone of the *Drosophila* β-I *tub* gene (Dr. Henry Hoyle, Indiana University, IN) were employed to isolate *Homarus* α-*tub* and β-*tub* cDNAs, respectively. Gel purified α- and β-*tub* fragments were labeled in vitro with [^{32}P]dATP using the Prime-It II random priming kit (Stratagene). Plaque hybridization was conducted on duplicate filters according to standard methods (Stratagene; Clonetech, Palo Alto, CA). Autoradiograms from duplicate filters were aligned and candidate plaques were removed, eluted in 1 ml SM buffer (100 mM NaCl/1 mM $MgSO_4$/5 mM Tris·HCl (pH 7.5)/0.01% gelatin), and titered. Secondary plaque lifts were performed as above. Candidate phagemid molecules were rescued using established procedures (Stratagene). Phagemid molecules were purified by alkaline mini-lysate preparations (Maniatis et al., 1982). Nucleotide sequence was determined using double stranded dideoxy strand termination reactions (Sanger et al., 1977). These sequences can be obtained from GenBank with accession Nos. U41810 (α) and U41811 (β).

are those which contain aromatic residues (Burns and Surridge, 1994a). These large functional groups are implicated in polypeptide folding and inter-molecular interactions necessary for proper microtubule polymerization. The lobster β-*tub* clone was unique among metazoan sequences in that substitutions of aromatic residues for non-aromatic aa were found at positions β: aa 244 (Leu→Phe) and β: aa 335 (Leu→Tyr) (Fig. 2B). Conservative Tyr→Phe conversions were found at positions β: aa 163 and β: aa 344 (Fig. 2B).

(e) Phylogenetic relationship of lobster Tub with other known Tub

When examined over wide phylogenetic distances, *tub* genes tend to evolve from simple gene sets encoding few, multifunctional gene products, as in fungi and protozoa, to complex families elaborating multiple products with highly specialized functions in the metazoans. Further functionality was assigned to the lobster *tub* clones by examining phylogenetic relationships between lobster sequences and other known Tub. The hypervariable C-terminal domain, used to define isotype-specific differences between *tub* (Cleveland, 1987; Joshi and Cleveland, 1990), third position codon substitutions, and two areas of alignment ambiguity within the α-*tub* sequences introduced by insertions found within the *Drosophila* α4 (Theurkauf et al., 1986) and *Dictyostelium* (Trivinos-Lagos et al., 1993) were omitted to best reveal phylogenetic affinities.

Fig. 3 shows a neighbor joining distance analysis of aligned aa sequences for α- and β-Tub. For α-Tub, three distinct clusters were generated among the aa sequences analyzed: (*1*) fungi, (*2*) protozoa and (*3*) metazoa (Fig. 3A). As expected, the lobster α-Tub coding sequence clustered within the metazoan clade, sharing the highest degree of similarity with another crustacean, *Artemia* (96% aa identity). Although *Homarus* is the only crustacean β-tub sequence available, a similar three component pattern was observed (Fig. 3B).

Two extreme examples of *tub* gene family organization are depicted in these trees. Representatives of the fungi and protozoa tend to contain one or few multifunctional *tub* genes. In contrast, the metazoans often maintain a complex set of functionally specialized *tub* coding sequences (Murphy, 1991; Raff, 1994). *Drosophila*, whose α- and β-*tub* gene families each consist of four members (Sanchez et al., 1980; Natzle and McCarthy, 1984), seems to have adopted both strategies for *tub* gene family organization as ubiquitous expression of a limited number of multifunctional *tub* genes occurs in most tissues while expression of functionally specialized *tub* genes is restricted to specific tissues (Theurkauf et al., 1986; Raff, 1994). Since *Drosophila* contains four α-*tub* genes while *Artemia* is believed to contain one or very few (Langdon et al., 1990), these observations are consistent with a common arthropod ancestor maintaining a relatively small number of *tub* coding sequences.

(f) Estimation of the number of *tub* genes present within the lobster genome

The complex assortment of Tub proteins present in PTG tissue (Fig. 1) suggested that either Tub were encoded by a large *tub* gene family not anticipated from our phylogenetic analysis, or they were elaborated from a small gene family, the products of which were subject to post-translational modification. To discriminate between these possibilities, we estimated the number of *tub* genes present within the lobster genome.

The lobster α- and β-*tub* cDNA clones were ^{32}P-labeled and hybridized to restriction enzyme-cleaved lobster genomic DNA immobilized on nylon membranes. A limited number of discrete restriction fragments were detected (Fig. 4). *Pst*I does not cleave internal to our α-*tub* probe while a single *Hin*dIII site is present. When these two enzymes were used to digest lobster genomic DNA (lanes 1 and 2, respectively), 5 bands were detected when probed with the labeled α-*tub* probe, suggesting a total of 4–5 α-*tub* genes. We conclude this estimate to be an upper limit on the number of lobster α-*tub* genes in that these patterns may still be complicated by the presence of introns. When *Xba*I, which also does not cleave the α-*tub* clone, was used to digest genomic DNA (lane 3), 4 bands were visualized. This observation was consistent with the estimation of 4 or 5 α-tub genes present within the lobster genome.

Neither *Pst*I nor *Hin*dIII cleave the β-*tub* clone. When DNA was digested with *Pst*I (lane 4), 4 distinct bands resulted; 5 bands were visualized when the β-*tub* probe was hybridized to *Hin*dIII-digested DNA (lane 5). These results suggest an upper limit of 5 β-*tub* genes in the lobster genome. The possibility of a smaller β-*tub* gene family is supported by the *Xba*I digest (lane 6) where only three bands were detected. We conclude that 3–5 β-*tub* genes are carried within the lobster genome.

(g) Potential post-translational modifications of lobster α-Tub and β-Tub

Sequence analysis has revealed potential aa residues for known post-translational modifications which occur on Tub molecules. α:Lys^{40} (Fig. 2A) has been identified as a conserved site for acetylation of α-Tub (LeDizet and Piperno, 1987). Other potential sequence-dependent post-translational modifications include poly-glutamylation (Edde et al., 1990), poly-glycylation (Redeker et al., 1994) and detyrosination of the α-Tub C-terminal Tyr residue (Argarana et al., 1980; Wehland

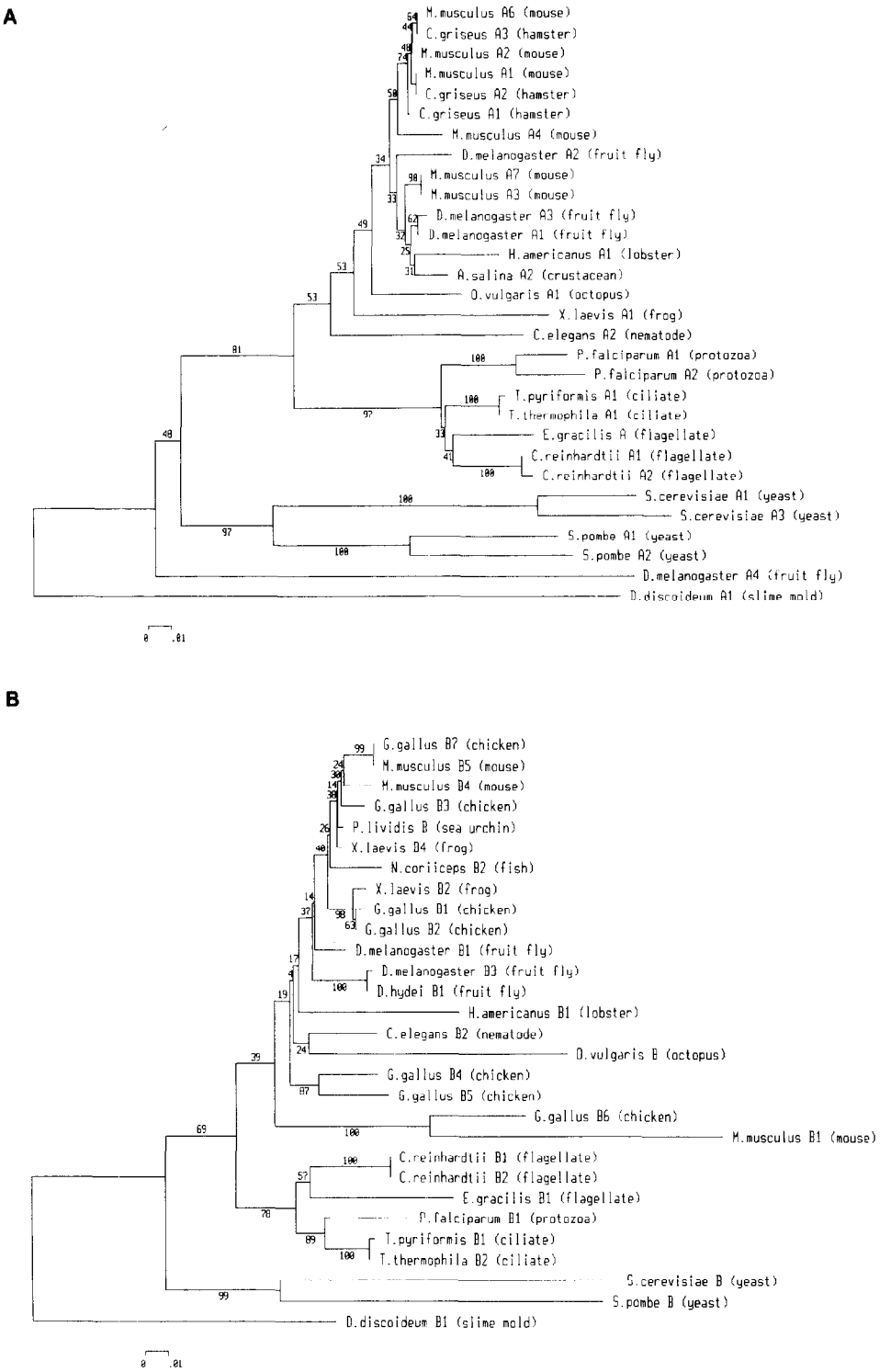

Fig. 3. Phylogenetic trees of (**A**) α-Tub and (**B**) β-Tub sequences. **Methods**: Alignments of 30 α-Tub and 29 β-Tub aa sequences were performed in CLUSTAL V (Higgins et al., 1991), imported into MacClade (Maddison and Maddison, 1992), and exported in Mega format for neighbor joining analysis (Saitou and Nei, 1987). Substitutions per site was estimated assuming a gamma distribution with parameter (a)=2. Reliability of the trees was tested with a bootstrap test (1000 replications; Efron, 1982). Values of bootstrap confidence levels are given on each interior branch. (scale bar), branch length of 0.01 substitutions per position following gamma distance corrections for site variation of aa substitution rate. Trees were rooted with *Dictyostelium discoideum* Tub. Sequences used for α-Tub analysis and their GenBank accession Nos. included *Artemia* (Dr. T. MacRae, personal communication), *Caenorhabditis elegans* (D14965), *Chlamydomonas reinhardtii* (M11447, M25916), *D. discoideum* (L13999), *Drosophila melanogaster* (M14643, M14644, M14645, M14646), *Euglena gracilis* (Z22877), *Cricetulus griseus* (M12329, M12252, M12253), *Mus musculus* (M13445, M13446, M13442, M13444, M13441, M13443), *Octopus vulgaris* (X15845), *Paracentrotus lividis* (X53618), *Plasmodium falciparum* (M34390, X15979), *Saccharomyces cerevisiae* (M28429, M28428), *Schizosaccharomyces pombe* (K02841, K02842), *Tetrahymena pyriformis* (X12767), *T. thermophila* (M86723), *Xenopus laevis* (X07046). β-Tub analysis included *C. elegans* (X15242), *Gallus gallus* (M11442, M11443, M14228, M15052, M14681, J02828, X07011), *C. reinhardtii* (M10064, M25918), *D. melanogaster* (X69560, M16922), *Drosophila hydei* (M20421), *D. discoideum* (L14000), *E. gracilis* (X15797), *M. musculus* (X04663, M28730), *Notothenia coriiceps* (S57698), *Octopus dofleini* (L10111), *P. lividis* (X15389), *P. falciparum* (M31205, M28398), *S. cerevisiae* (J01384), *S. pombe* (M10347), *T. pyriformis* (X12768), *T. thermophila* (L01416), *X. laevis* (X15798, U15444).

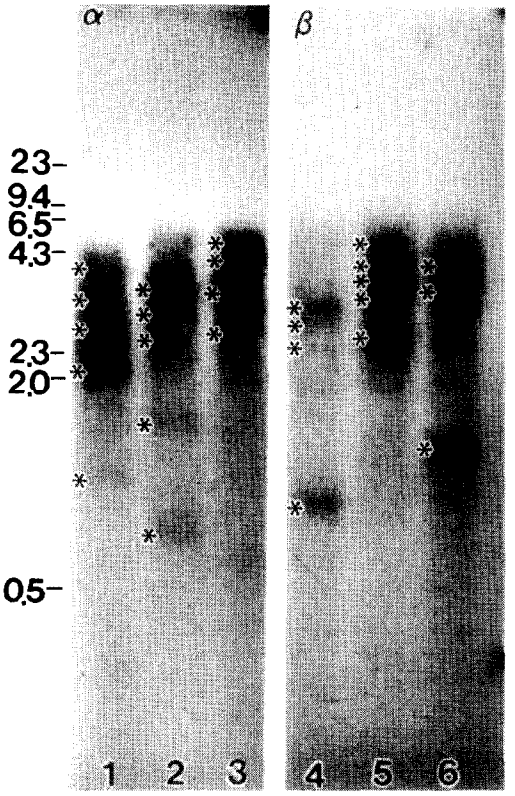

Fig. 4. Transfer-hybridization analysis of lobster genomic DNA. *Pst*I (lanes 1 and 4), *Hin*dIII (lanes 2 and 5), and *Xba*I (lanes 3 and 6) digested lobster DNA (25 μg) was fractionated by electrophoresis on 1% agarose gels, transferred to nylon filters and hybridized with ^{32}P-labeled lobster α-*tub* (α) or β-*tub* (β) cDNAs. The presence of bands not easily resolved in the higher MW range were verified using lower percentage (0.8%) agarose gels (not shown). λ/*Hin*dIII markers are provided in kb to the left of the figure. **Methods**: Genomic DNA was prepared according to Maniatis et al. (1982). DNA was digested overnight at 37°C with restriction enzymes according to manufacturers recommendations, and fractionated on 1% agarose gels. After denaturation, the DNA was transferred to nitrocellulose filters in 10 × SSC (1.5 M NaCl/0.1.5 M Na_3·citrate pH 7.5) and UV-cross linked. Hybridization was conducted according to the ExpressHyb hybridization buffer protocol (Clonetech, Palo Alto, CA). All enzymes were purchased from Promega Corp. (Madison, WI).

and Weber, 1987). With the exception of detyrosination, these post-translational modifications could account for the heterogeneity of iso-Tub elaborated from the simple *tub* gene family within the lobster genome.

(h) Conclusions

(*1*) Analysis of the number of iso-Tub present within the PTG of lobsters revealed a complex assortment of forms. We estimate that there are three to five copies of each gene class present in the lobster genome with our best approximation being four copies for each. This organization is similar to organisms that contain ubiquitous, multifunctional *tub* isoforms in addition to highly specialized gene products such as *Drosophila*.

(*2*) For lobsters, the diverse array of Tub isoforms is likely due to post-translational modification of a limited number of primary gene products. We postulate that this organism solves its complex microtubule needs by carrying a relatively small *tub* gene family, whose primary gene products are subject to extensive post-translational modifications, a strategy also postulated for *Artemia* (Langdon et al., 1990).

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