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The marine red alga *Chondrus crispus* has a highly divergent β-tubulin gene with a characteristic 5′ intron: functional and evolutionary implications

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Abstract

We characterized a nuclear gene and its corresponding cDNA encoding β-tubulin (gene *TubB1*) of the marine red alga *Chondrus crispus*. The deduced TubB1 protein is the most divergent β-tubulin so far reported with only 64 to 69% amino acid identity relative to other β-tubulins from higher and lower eukaryotes. Our analysis reveals that *TubB1* has an accelerated evolutionary rate probably due to a release of functional constraints in connexion with a specialization of microtubular structures in rhodophytes. It further indicates that isoform diversity and functional differentiation of tubulins in eukaryotic cells may be controlled by independent selective constraints. *TubB1* has a short spliceosomal intron at its 5′ end which seems to be a characteristic feature of nuclear protein-coding genes from rhodophytes. The splice junctions of the four known rhodophyte introns comply well with the corresponding consensus sequences of higher plants in agreement with previous suggestions from phylogenetic inference that red algae and green plants may be sister groups. The paucity and asymmetrical location of introns in rhodophyte genes can be explained by differential intron loss due to conversion of genes by homologous recombination with cDNAs corresponding to reverse transcribed mRNAs or partially spliced pre-mRNAs, respectively. The identification of an intron containing *TubB1* cDNA in *C. crispus* confirms that pre-mRNAs can escape both splicing and degradation in the nucleus prior to transport into the cytoplasm. Differential Southern hybridizations under non-stringent conditions with homologous and heterologous probes suggest that *C. crispus* contains a second degenerate β-tubulin gene (or pseudogene?) which, however, is only distantly related to *TubB1* as it is to the more conserved homologues of other organisms.

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X71784 (cDNA sequence) and X71785 (genomic sequence).

Introduction

Tubulin, a heterodimer of two distinct polypeptides α and β, is the most abundant protein of microtubules. In most eukaryotes α- and β-tubulins are encoded by multiple genes mostly unlinked to each other and dispersed in the genome [9, 22]. The size of the tubulin gene families seems relatively independent of genome size, since the smallest genome of higher plants, that of *Arabidopsis thaliana*, harbors at least 15 expressed tubulin genes encoding 6 α- and 9 β-tubulins, representing more active tubulin genes than are present in the human genome which is about 30 times larger. It was proposed that the number of tubulin genes may be correlated with the developmental complexity of the organism [41, 52]. In plants and animals, tubulin isotypes, whether functionally specialized or not, are often expressed differentially in a tissue-specific or life cycle stage-specific manner [7, 39].

Lower eukaryotes have little tubulin diversity although small gene families have been reported for several fungi (see [46] and references therein) including *Colletotrichum graminicola* (two β), *Saccharomyces cerevisiae* (two α), *Schizosaccharomyces pombe* (two α) and *Aspergillus nidulans* (two α and two β) and for the protozoan *Plasmodium falciparum* (two α [53]). The myxomycete *Physarum polycephalum* with its complex life cycle is an interesting exception. It has at least five α-tubulin and three β-tubulin genes and, hence, a tubulin gene family comparable in complexity to that of *Drosophila* [47]. Single-copy genes for β-tubulin have been found in *S. cerevisiae*, *S. pombe*, *Candida albicans* and *Neurospora crassa* (see [46] and references therein) and for α- and β-tubulin in *Toxoplasma gondii* [45]. The unicellular green alga *Chlamydomonas reinhardtii* [62], the ciliates *Stylonychia lemnae* [10] and *Tetrahymena thermophila* [19] have each two β-tubulin genes encoding the same protein.

Little is known about tubulin gene families in macroalgae, organism showing a developmental complexity intermediate between protists and higher eukaryotes (plants and animals). The recent finding [40] of four different β-tubulin genes in *Ectocarpus variabilis*, a primitive brown alga of simple morphology, seems to support the proposed correlation between gene family size and developmental complexity. Here we report the structure and evolution of the first tubulin gene of red algae, that encoding beta-tubulin of *Chondrus crispus* (gene *TubB1*). Surprisingly, *TubB1* evolves extremely rapidly suggesting that microtubular structures of red algae may be functionally specialized. Like other rhodophyte genes *TubB1* has a short spliceosomal intron at its 5′ end with splice junctions resembling those of green plants. The functional and evolutionary implications of these findings are discussed.

Material and methods

The cDNA and genomic libraries used in this study were constructed from poly(A)$^+$-mRNA and genomic DNA, respectively, isolated from protoplasts prepared from apical tips of *Chondrus crispus* gametophytes as described in detail previously [36, 37].

Isolation of cDNA and genomic clones

The cDNA library was screened by hybridization with the random-prime-labelled [14] cDNA encoding *TubB1* of pea [34]. The hybridization was performed at 55 °C overnight in 6 × SSPE, 0.2% PVP, 0.2% Ficoll, 0.1% SDS, 0.5 μg/ml denatured salmon sperm DNA and the labelled probe. Filters were washed twice in 2 × SSPE, 0.1% SDS at 55 °C for 20 min. cDNA insertions obtained from the purified lambda phage after digestion were subcloned into the *Eco* RI site of the phagemid pBlueScript-SK (Stratagene). The cDNA library was screened once more under non-stringent conditions with the *TubB1* cDNA found in *C. crispus*. To identify divergent *TubB* cDNAs the positive clones were rehybridized under high stringency (68 °C) with a 3′-specific probe (fragment *EcoRI-Sty* I, 230 bp) of the *TubB1* cDNA of *C. crispus*. Filters were washed twice for 20 min at 68 °C, once in 2 × SSPE,

0.1% SDS and once in 0.2× SSPE, 0.1% SDS. The *TubB* clones which did not hybridize with the *TubB1*-specific probe were subcloned into pBlueScript-SK.

The genomic library of *C. crispus* was screened with the homologous *TubB1* cDNA identified as described above and labelled by random priming. The hybridization was performed at 65 °C overnight in the same buffer as specified above. Filters were washed twice for 20 min at 60 °C, once in 2× SSPE, 0.1% SDS and once in 0.2× SSPE, 0.1% SDS. Positive plaques were rescreened and purified to single plaques by standard procedures. Genomic fragments digested with *Eco* RI and *Cla* I were cloned into pBlueScript-SK.

DNA sequence analysis

An ordered set of deletion clones was prepared for each clone by using exonuclease III following the Stratagene protocol. The deletion clones were sequenced by the dideoxy chain termination method using the ALF DNA Sequencer from Pharmacia. When required, oligonucleotides (17-mers) were synthesized according to sequence information and used directly as primers for further sequencing.

PCR amplification

PCR amplification of a homologous *TubB1* probe used for Southern hybridization was performed in the DNA Thermal Cycler (Perkin-Elmer Cetus). The reaction mixture contained 1 ng of template DNA (*Chondrus TubB1* cDNA), 25 ng of each primer (CCGAATTCTGCGACGTGCTTCAAG and CCGAATTCATCATGCGATCGGGAT), 0.4 mM of each dNTP, 0.1 units of *Taq* DNA polymerase (Appligene) and *Taq* DNA polymerase reaction buffer (40 mM Tris-HCl pH 8.3, 47.5 mM KCl, 2.2 mM $MgCl_2$). Denaturation of DNA was performed at 93 °C for 1 min. Primer annealing and primer extension reactions were carried out at 55 °C for 1 min and at 72 °C for 2 min, respectively. After 35 cycles of amplification, the PCR products were digested with *Eco* RI and cloned into the *Eco* RI site of pBlueScript-SK.

Southern hybridization

C. crispus DNA (8 μg) was digested to completion with 15 U of the respective restriction enzyme, electrophoresed on a 0.7% agarose gel, capillary transfered and UV coupled to a Hybond N (Amersham) nylon membrane. Filters were hybridized for 20 h at 55 °C in 6× SSPE, 0.1% SDS, 0.2% PVP and 0.2% Ficoll containing 100 ng of random-prime-labelled DNA probes. Filters were washed twice for 10 min at 55 °C, once in 6× SSPE, 0.1% SDS and once in 2× SSPE, 0.1% SDS. Autoradiograms were exposed for 20 h at −80 °C.

Phylogenetic data analysis

Beta-tubulin peptide sequences were obtained from GenBank. The phylogenetic tree was constructed with the Neighbor-Joining method [54] from a matrix of amino acid distances based on an alignment of the first 434 amino acids. The alignment was done with the program CLUSTAL V [27]. The calculation of the distance matrix and the phylogenetic analyses including bootstrapping (100 cycles) were performed with the programs PROTDIST (Dayhoff PAM 001 matrix), NEIGHBOR and SEQBOOT, respectively, of the program package PHYLIP 3.5C (distributed by the editor, see [16] for version 3.2).

Results

*Isolation and characterization of cDNAs encoding β-tubulin (*TubB1*) from* Chondrus crispus

An amplified cDNA library (based on 2×10^6 original recombinants cloned into the vector λgt11 [37]) was screened at low stringency with the heterologous cDNA encoding *TubB1* of pea [34].

The longest positive clone was submitted to sequence analysis. The cDNA of *Chondrus crispus TubB1* is 1808 bp long, contains the entire coding region (1362 bp), a 5′ leader of 93 bp, a 3′ trailer of 353 bp and no poly(A) tail. In order to identify divergent β-tubulin cDNAs, 100000 phage of the same library were screened at low stringency using the homologous full-length *TubB1* clone. 24 positive clones were identified corresponding to a *TubB1* transcript frequency of about 0.02% in *C. crispus* protoplasts. Only two of these 24 positive clones did not hybridize under stringent conditions with a 3′-specific probe of *TubB1*. Sequence analysis showed that both non-hybridizing clones were identical in sequence relative to one another and relative to *TubB1*, but they had shorter 3′ trailors (27 bp). In addition and surprisingly, both cDNAs, like the genomic sequence from which they were transcribed (see below), contained a short phase-1 intron located 16 bp downstream of the AUG codon, suggesting that they correspond to a cytosolic pre-transcript which escaped both splicing and degradation in the nucleus. Stop codons in all three reading frames of the intron sequence make it seem unlikely that this *TubB1* pre-transcript can be translated.

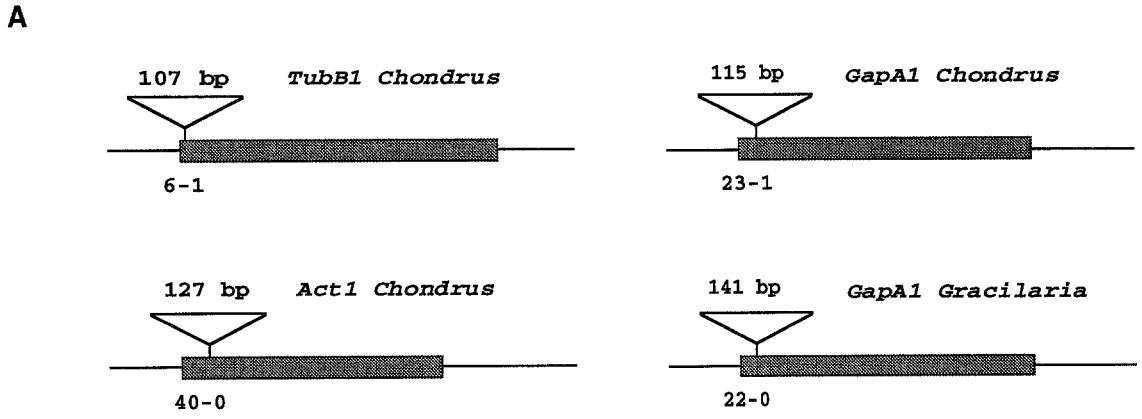
Isolation and characterization of a genomic clone encoding β-tubulin from Chondrus crispus *(gene* TubB1*)*

The genomic library was screened with the homologous cDNA encoding *TubB1* from *C. crispus* identified as described above. One of the positive genomic clones was purified and submitted to sequence analysis. The structural features of the gene *TubB1* from *C. crispus* (3367 bp) were compared with those of the previously characterized genes *GapC1* and *GapA1* of *C. crispus* encoding cytosolic and chloroplast glyceraldehyde-3-phosphate dehydrogenases [36]. *TubB1* resembles *GapC1* in that it has two potential CAAT and TATA boxes at positions −209/−190 and positions −152/−137 relative to AUG, respectively. It also carries an imperfect G-box motif (GCCACGTCTC rather than GCCACGTGGC) at position −431. In higher plants this palindromic element and related variations are found in several classes of promotors (e.g. *rbcS*, chalcone synthase, histone genes) where they interact with a family of DNA-binding proteins such as TAF-1, GBF and CG-1 [29]. Further similarities between *TubB1* and *GapC1* concern the corresponding leader regions which are characterized in both cases by a relatively high C-content (50 and 60% C, respectively) and by three tandem repeats of two related motifs, $[CCACCC]_3$ (*TubB1*, position −128) and $[ACCCCGAT/CCG]_3$ (*GapC1*, position −66), respectively.

The *TubB1* gene, like most other rhodophyte genes known, contains at its 5′ end a short spliceosomal intron (Fig. 1A). It is 107 bp long and interrupts the coding sequence in phase one 16 bp downstream the initiation codon AUG. Relative to the intron positions in other β-tubulin genes and based on the sequence alignment in Fig. 2 the *TubB1* intron of *C. crispus* occupies position 6-1, which is one nucleotide apart from intron 1 of fungal β-tubulin genes at position 6-0. The codon positions are defined by codon number and phase and correspond to positions 5-1 and 5-0 in our previous alignment [34] not taking into account the *Chondrus*-specific aminoterminal insertion of a threonine (see below).

Intron junctions of rhodophytes resemble those of higher plants

In Fig. 1B and C, the border junctions and the presumptive branch point of *TubB1* and three other rhodophyte introns from *C. crispus* (*GapA1* and *Act1* encoding actin [6, 36]) and *Gracilaria verrucosa* (*GapA1* [64]) are compared to the corresponding consensus sequences from plants, animals and yeast. Although four rhodophyte introns are probably not enough to draw definite conclusions, it can be clearly seen that their splice junctions are relatively degenerate and more similar to the corresponding consensus sequences of higher eukaryotes (plants and animals) than to the strongly conserved splice junctions of yeast.



B

	5'-Donor	Branch site	3'-Acceptor
TubB1 Chondrus	T T G : **G T A** C G **T**.....45 bp.....A	T **C T** A **A C**	G.....43 bp.....C T T **A G** : T (72% T+C)
GapA1 Chondrus	C A G : **G T A** C G **T**.....64 bp.....T	G **C T** A **A C**	G.....32 bp.....C G C **A G** : G (56 % T+C)
Act1 Chondrus	A A G : **G T A** A C **T**.....65 bp.....T	A **C T** A **A C**	A.....43 bp.....A A C **A G** : G (60 % T+C)
GapA1 Gracilaria	A G C : **G T A** A G **T**.....99 bp.....T	A **C T** G **A C**	T.....23 bp.....T A C **A G** : G (79 % T+C)
Plant consensus	$C_{33}A_{55}G_{72}$: **G T** $A_{70}A_{55}G_{65}T_{49}$.....	$T_{40}T_{33}C_{69}$ R$_{45}$R$_{54}$T$_{24}$ **T** R$_{74}$ **A**	Y$_{71}$.....$T_{68}G_{50}C_{67}$ **A G** : G_{60}
Vertebrate consensus	$C_{38}A_{62}G_{77}$: **G T** $A_{60}A_{74}G_{84}T_{50}$..... A$_{39}$	.....C$_{76}$T$_{95}$G$_{51}$ Y$_{97}$ R$_{80}$ **A**	C$_{51}$..... (Y)$_n$NC$_{65}$ Y$_{80}$ **A G** : G_{52}
Yeast consensus	G : **G T A** T **G T**.....	**T A C T A A C**	A.....**Y A G** :

C

Pyrimidine box (-5 to -15)

	-15	-14	-13	-12	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	:	1
TubB1 Chondrus	T	T	T	T	T	T	A	T	C	G	C	T	T	**A**	**G**	:	T
GapA1 Chondrus	G	A	T	C	T	A	A	T	C	T	C	G	C	**A**	**G**	:	G
Act1 Chondrus	G	A	T	G	A	T	A	C	G	C	A	A	C	**A**	**G**	:	G
GapA1 Gracilaria	G	C	A	T	T	A	T	A	T	C	T	A	C	**A**	**G**	:	G
Plant consensus	T_{47}	T_{53}	T_{51}	T_{44} R$_{44}$	T_{53}	T_{47}	T_{42} R$_{39}$	T_{38} R$_{47}$	T_{41} R$_{44}$	T_{44} R$_{43}$	T_{68}	G_{50}	C_{67}	**A**	**G**	:	G_{60}
Vertebrate consensus	T_{51} Y$_{70}$	T_{44} Y$_{69}$	T_{50} Y$_{71}$	T_{53} Y$_{74}$	T_{60} Y$_{84}$	T_{49} Y$_{79}$	T_{49} Y$_{82}$	T_{45} Y$_{72}$	T_{45} Y$_{81}$	T_{57} Y$_{93}$	T_{58} Y$_{85}$	N	C_{65}	**A**	**G**	:	G_{52}

Fig. 1. Nuclear protein coding genes of the red algae *Chondrus crispus* and *Gracilaria verrucosa* (A) contain single introns at their 5′ ends resembling those of higher plants. Intron sizes and positions (codon number and phase) are indicated for each gene. Border junctions, putative branch sites (B) and pyrimidine boxes (C) of rhodophyte genes were compared to the corresponding consensus sequences from plants, animals and yeast [31, 57]. Percentage values of nucleotide conservation are indicated as subscripts. Strongly conserved nucleotides (ca. 100%) are shown in bold. Letters R and Y in sequences correspond to purines (A + G) and pyrimidines (C + T), respectively. The T + C values (% T + C) given in (B) apply to the sequence between the branch site adenosine and the 3′-splice site AG. *Act1* and *GapA1* symbolize genes encoding actin and chloroplast glyceraldehyde-3-phosphate dehydrogenase, respectively.

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          10        20        30        40        50        60        70        80        90       100
 1 M-RQILHIQGGQCGNQIGAKFWEVVCAEHGIDPTGRYTGDS-DLQLERIDVYYNEASGGRFVPRAVLMDLEPGTMDSIRSGPYGQIFRPDNFVFGQSGAG
 2 M-.E.....................I.D.....H..Q.V...-P.........F......KY.................L....F...............
 3 M-.E...V.A........G.....M.D.....A..N.V.NF-H.....VN..........Y.................L....F.K...........N...
 4 M-.E.V.....................SD........T.H...-.......N..F...T...Y....I...........V...................T...
 5 M-.E.V.....................ISD.......T.H...-.......N......T...Y....I...........V.A..F..L..........T...
 6 M-.E.V...A.................ISD........S.H.E.-.......N......T..KY......V.........V.A..F.............T...
 7 M-.E.V...A.................IISD.....A..T.H...-.......N.........KY......V.........V....F...............
 8 M-.E.V...A.................ISD...V..S.E.R...-E..I...N..F...A...Y....I.V.........V.A..F...............
 9 M-.E.V.V.A........S.....ISD.........S.H...-.......NC.F...T...Y....I...........V.A..F..L..........T...
10 M-.E.V...A........S.....ISD.Q.V..PDIP-...-.......N......T...Y..-.I...........V.A.................T...
11 M-.E.V.L.T......V.SA..QTISG...L.AS.I.....-......MN..F...G.NKY......I.........AL....N.ALY.....IY...S..
12 M-.E.I..SA..Y......A...TI.G...L.FN.T.H.HD-.I.K..LN..F....S.KW...SINV....W.I.AV.NSAI.NL.....YI....S...
13 M-.E.I.L.T......V.TA..QTIHG...L.QD.VFR.SD.EQ.S..LS..FT..AKQKY......V....A...A.....L.DF.....M.Y......
14 MT.S.VSL.V......V.L....GIS.....VD.K.I..RP.QE.H..G.....S.S.SY....AML.....VLMA.KNSKR..L.H....AY......

         110       120       130       140       150       160       170       180       190       200
 1 NNWAKGHYTEGAELIDSVLDVVRKEAENCDCLQGFQVCHSLGGGTGSGMGTLLISKIREEYPDRMMLTFSVFPSPKVSDTVVEPYNATLSVHQLVENADE
 2 ..........................S.....................................M.....................................
 3 ..................................I.....................................AV.EG.....................
 4 ........................S.....................................V.....................................
 5 ........................G.........IT.................V.......I.E....V.....................................
 6 ........................S.........IA.....................C....V.....................................
 7 ............V............S.........LT.........................S.N.Y..V.....................T..
 8 ............V.A.......RS.A.........I.........A.......A..........C....V.....................H...
 9 ..........................S..A.....LT..M.....A.........V.......V.S.Y..I.....................Q
10 ..........P..............S.........IA.....................M....I.............T.................
11 ..C...........V.Q.I....R...S.....A...T.....................F.....A....M.....................HS..
12 .V............V...M..I.R...G..S.....IT..............F...K..L.....A....L....T.................HS..
13 ............V.Q......R...A..S.....IT..............A.V...F.....A....L..A...EV..........I......S..
14 ............VETA..II.R...T..V......T.............V...........C.Y..L.............C...I...I....C

         210       220       230       240       250       260       270       280       290       300
 1 VMVLDNEALYDICFRILKLSNPSFGDLNHLISATMSGVTCCLRFPGQLNSDLRKLAVNLIPFPRLHFFMVGFAPLTSRGSQQYRTLSVPELTQQMWDAKN
 2 C.............T...A..T.....................................................................SA.............
 3 C.............T...T...........T.....................................................S.TI.........R.
 4 C.............T...TT.T.........V...I.........A.....................T...........A.T.............
 5 C..I..........T...TT.TY......V..A.....................................I...........A.T........F....
 6 ..CI........S..T...TT.TY......V..V...I.....................................L...........VG..S.T........F....
 7 TYCI..........T...TT.TY......V.L......T.........A........MV.........P.............A.T........F....
 8 .FCI..........T...TC.TY......V.LV...C.S.........A.................L...........I..A.T....VS..F.N..
 9 CFT...........T...TT.TY......V..AIC.T..S........C........MV.........I.............A.T........CF.S..
10 ..CIG........LPT...TT.T..-HET.V..V.....................................L.............A.T........F....
11 TFC...D......I.T....S..Y......V..V...I.VS.................MV.................S.SSF..I.........F.SR.
12 TFCI.........Q.T...NQ..Y....N.V.SV.....TS..Y..............V...........Y....AI...SF.S.T........F....
13 TFCI.........R.T..QAH..Y.H....V.RV...L.TGF.......A.........V........T........SA.--FSN.GIA......F.P..
14 .FAI......N..YNT..IEQ..YDE..S...SV...I..S.......A.........V........A..H...AASN.AG..S......AG..F.RN.

         310       320       330       340       350       360       370       380       390       400
 1 MMCAADPRHGRYLTASAMFRGKMSTKEVDEQMMNVQNKNSSYFVEWIPNNVKSTVCDIPPTGLKMASTFIGNSTSIQEMFRRVSEQFTAMFRRKAFLHWY
 2 ..............R..V....L..........I.....................S....A.K.....................................
 3 ............................Q..I.....................S.........S.S...M.....................V..........
 4 ..................L...R..........L.....................S......K....SA.......A.....K.................
 5 ..................L...R..........L.....................I..SI.....K.....V..V....A.....K..A.............
 6 ..A.S.............R..........L.....................I..S......K.....V.......A...L.K.................
 7 ..A.C.........VA.I...R..M.......L.I.................TA......R....SA.......A...L.K.I...............
 8 ..A.S..........A.....R..........L.I.................M.VS......R.....A.......C...L.K..G...S...........
 9 ..............CAV....R..........L..V.................ASI.....K....ST..V..T.A...VWK..A....S...........
10 ..A.S..A.............R..........L.....................I..S......K....SA.....N.A.....K.................
11 ..T..NYQN..F..C.TL....VAM...ED..R.M...Y.............QTAL.SM..K.....A..V.....V..L.N...N.............
12 ..A.....N.....VA.F....V.V...EDE.HK..S...D..........QTA..SVA.Q..D..A...A.......L.K..GD..S...K........
13 V.L.S.F.D..F..C.T.....V.M.Q.E..IQAIK....AN.........IQTAH.SV..K..DVS........A..NS....GD..SL.........
14 ..AEI...E......AVY....V.....EDE.TLM.T...A.......H.I.TS.....AA.E.IS.A....T.A.EAT.K.FGN..RS..........

         410       420       430       440       450
 1 TGEGMDEMEFTEAESNMNDLVAEYQQYQDATADEDEYGEEEGDEEEYGQHDI*
 2 ..............................G.EEYEEEEEEYET*
 3 ............VRA.................VDDHEDEDEDEAMAA*
 4 ......................S........S.E.EGEFEGEEEEA*
 5 ......................S..........E.EGEFEEEEGEN*
 6 ......................S.........I.DEEGGEEEEGGAEEEARQRKHYVIDY<
 7 ......................S......E.....DAEFEEEQEAEVDEN*
 8 ......................S......E.GV..GEEFEEEED*
 9 ......................S..........E.EGEFDEDEELDDAMG*
10 ......................S.........VE.EGEFDEEEDVEQY*
11 ....................MS......E..VSDGEGAYDAEEGEAYEQEE*
12 .S.....L..S..........S......E..VEDDEEVDENGDFGAPQNQDEPITENFE*
13 ......................S.........GM.DEYGEEYEDEAPAEEE*
14 KS.....L..S.....LA...S.....GE....GVEGYEEEGYENDHPEDDEE*
```

			% identities	
1.	Pea ß1	(X54844)	100.0	67.8
2.	Arabidopsis ß4	(M21415)	93.5	66.2
3.	Soybean ß1	(M21296)	89.6	63.9
4.	Chlamydomonas ß1	(M10064)	91.2	67.6
5.	Tetrahymena ß1	(L01415)	88.4	67.3
6.	Physarum ß1	(M58521)	87.5	69.2
7.	Drosophila ß1	(M20419)	85.2	69.0
8.	Giardia ß1	(X06748)	82.9	67.6
9.	Ectocarpus ß5	(M32875)	81.9	66.4
10.	Euglena ß1	(X15797)	85.8	66.7
11.	Aspergillus tubc	(M17520)	75.7	64.3
12.	Yeast ß1.	(V01296)	72.0	66.9
13.	Colletotrichum ß1	(M34491)	69.7	63.9
14.	Chondrus crispus ß1		67.8	100.0

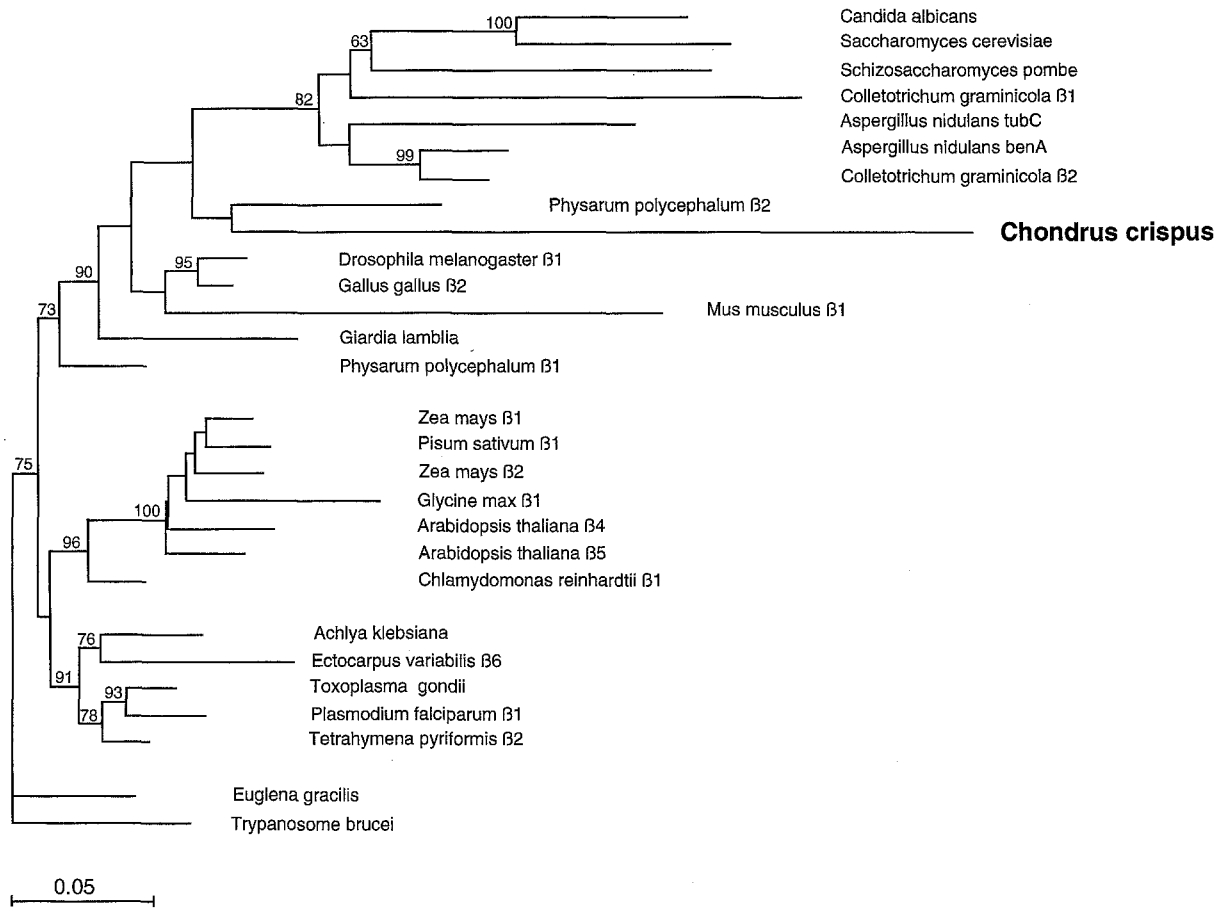

Fig. 3. Phylogenetic tree of beta-tubulins constructed by the Neighbor-Joining method [54] from a matrix of amino acid distances based on an alignment of the first 434 amino acids (see Fig. 2). For technical details see Material and methods. The tree was rooted using *Trypanosoma brucei* as outgroup. Boostrap values above 60% are indicated at internal nodes. Scale bar indicates amino acid substitutions per site. Accession numbers of sequences not shown in Fig. 2: *Achlya klebsiana*, J05597; *Arabidopsis thaliana* β5, M84702; *Aspergillus nidulans benA*, M17519; *Mus musculus* β1, A25437; *Candida albicans*, M19398; *Colletotrichum graminicola* β2, M34492; *Gallus gallus*, V00389; *Physarum polycephalum*, M20191; *Plasmodium falciparum* βa, M28398; *Schizosaccharomyces pombe*, M10347; *Tetrahymena pyriformis* βTT2, X12769; *Toxoplasma gondii*, M20025; *Trypanosoma brucei*, K02836; *Zea mays* β1, X52878; *Zea mays* β2, X52879. All accession numbers refer to GenBank except for *M. musculus* β1 which comes from the database PIR.

Within higher eukaryotes there is a better fit with plants than with vertebrates especially with respect to the 'pyrimidine box' at position −5 to −15 relative to the 3′ splice site. This element, which is absent in yeast, is more strongly conserved in vertebrates than in plants [57]. As shown in Fig. 1C, rhodophyte introns, like those of plants, seem to have a relatively elevated purine content in this region.

Fig. 2. Amino acid sequence alignment of β-tubulins from 14 different species representing higher plants, animals, fungi, protists and algae (green, brown and red algae) as specified at the bottom of the figure. After each species name GenBank accession number and percentage sequence identities relative to pea and *Chondrus crispus*, respectively, are given. Residues identical to sequence 1 for pea are indicated by dots. Stop codons are symbolized by asterisks (*). The arrowhead (<) at the end of sequence 6 designates a C-terminal extension (-VPSVCDILIR*) present in *Physarum* beta-1.

Phylogentic analysis of the degenerate TubB1 *gene from* Chondrus crispus

In Fig. 2 the deduced polypeptide sequence of *C. crispus* β-tubulin was aligned (sequence 14) together with a representative set of β-tubulin sequences from plants, algae, animals, fungi and protists. This comparison shows that the *C. crispus* TubB1 polypeptide is the most divergent β-tubulin so far reported showing only 64 to 69% amino acid sequence identity relative to other eukaryotic β-tubulins. In particular, *C. crispus* β-tubulin has the unusual amino terminus MTRSI, while most other sequences begin with MREI. In animals this peptide seems to be implicated in the selective degradation of β-tubulin mRNA in response to changes in the level of free tubulin subunits [20, 59].

In Fig. 3 a phylogenetic tree has been constructed on the basis of 28 amino acid sequences from 23 separate species using the neighbor-joining method [54]. The most striking feature of this tree is the extremely long branch of *C. crispus* β-tubulin exceeding those of the highly divergent fungal β-tubulins and corresponding to an evolutionary rate which is at least two to three times faster than that of the conserved β-tubulins from protists, animals, plants and algae including the relatively primitive brown alga *Ectocarpus variabilis*. However, the position of *C. crispus* in this tree close to fungi is unusual and may reflect a 'long branches attract' artifact rather than a true phylogenetic relationship (see Discussion).

Chondrus crispus *contains two degenerate and distantly related β-tubulin genes*

Southern hybridization experiments were performed with a heterologous (Fig. 4A) and ho-

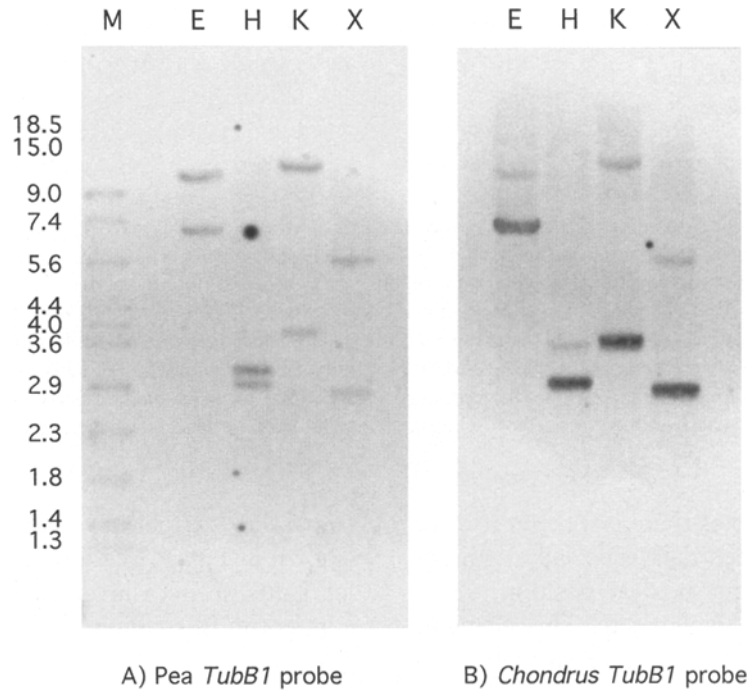

Fig. 4. Counting of beta-tubulin genes in *Chondrus crispus* by genomic Southern blotting. Lanes E, H, K and X in panels A and B contain each 8 μg of genomic DNA digested with *Eco* RI, *Hin*d III, *Kpn* I and *Xho* I, respectively. The digests were electrophoresed on a 0,7% agarose gel and the separated fragments were blotted onto a nylon membrane. Hybridization was performed under low stringency (55 °C) with two separate probes: a heterologous probe (panel A) consisting of a 470 bp *Exo* III-*Hin*d III genomic fragment spanning codons 253 to 410 of pea *TubB1* and a homologous probe (panel B) representing a 119 bp PCR fragment spanning codons 129 to 166 of *TubB1* from *Chondrus crispus*. Sizes in kb of molecular weight markers (lane M) are indicated in panel A.

mologous (Fig. 4B) probe to count the number of β-tubulin genes in *C. crispus*. A gel blot of *C. crispus* genomic DNA digested with *Eco* RI, *Hin*d III, *Kpn* I and *Xho* I was prepared and hybridized under non-stringent conditions (55 °C) with a heterologous probe consisting of a 470 bp *Exo* III-*Hin*d III fragment spanning codons 253 to 410 of pea *TubB1* [34]. Each of the four separate digests shows two distinct bands of similar intensity (panel A) suggesting that *C. crispus* contains two β-tubulin genes of similar divergence relative to the conserved pea *TubB1* gene.

In Fig. 4B a second gel blot prepared in the same way as that shown in panel A was hybridized under non-stringent conditions (55 °C) with a homologous probe, a 119 bp PCR fragment spanning codons 129 to 166 of *Chondrus TubB1*. For each digest, except *Hin*d III, the homologous probe detected the same two restriction fragments as did the heterologous probe. However, the two bands differ greatly in intensity as expected for two homologous genes of relatively distant relationship. When the washing temperature was raised by only three degrees to 58 °C the weak bands representing the second gene disappeared completely (data not shown). This additional *TubB2* gene is located on two separate *Hin*d III fragments of about 3.3 and 3.6 kb as indicated by their differential hybridization with the heterologous and homologous probe, respectively (compare lane H in panels A and B), suggesting that *TubB2* has a *Hin*d III site in the region between codons 167 to 252 separating the two probes. We deduce from these differential Southern hybridizations and from our finding that all 24 cDNA clones identified correspond to *TubB1* that *C. crispus* has two degenerate and distantly related β-tubulin genes, one of which, *TubB2*, may be non-functional or expressed very weakly in protoplasts of *C. crispus* gametophytes.

Discussion

β-tubulin as a phylogenetic marker

The significance of β-tubulin as a phylogenetic marker is limited due to gene paralogy and large differences in evolutionary rate. The fast evolving functional *TubB1* gene of the red alga *C. crispus* is a typical example. Probably because of a 'long branches attract' artifact [15, 32], *Chondrus* goes together with fungi and not with green plants as expected from GAPDH phylogenies [35, 37, 63], although it should be kept in mind that GAPDH genes are of endosymbiotic origin [43], while β-tubulin may represent a true marker of the eukaryotic host cell. In spite of these uncertainties, the β-tubulin tree in Fig. 3 agrees to some extent with the accepted topology of *major* eukaryotic lineages. Plants group together with green algae (*Chlamydomonas reinhardtii*) and animals with fungi [1]. *Euglena gracilis* associates with *Trypanosoma brucei* (75% bootstrap significance) in agreement with previous evidence based on cytological observations [60] and rRNA phylogenies [58]. A loose relationship is also observed between the β-tubulins of the brown alga *Ectocarpus variabilis* and the oomycete *Achlya klebsiana* (76% bootstrap significance) which is compatible with the proposed evolutionary relationship between the heterokont flagellated oomycetes and chromophytes [2, 4]. However, the present tree disagrees with rRNA topologies concerning the position of the diplomonad *Giardia lamblia*, a protozoan parasite which has two nuclei and eight flagella but lacks mitochondria and normal endoplasmic reticulum or Golgi [13]. In terms of beta-tubulin and also GAPDH (data not shown) *G. lamblia* is a relatively late branch and clearly not the first divergence from the eukaryotic line of descent as inferred from small subunit rRNA phylogenies [58].

Isoform diversity and functional differentiation of tubulins may be controlled by independent selective constraints

Although there is a second β-tubulin gene in *C. crispus* (Fig. 4), this gene (or pseudogene?) also seems to be degenerate and only distantly related to *TubB1* as it is to the more conserved homologues of other organisms. The accelerated evolutionary rate of the functional *TubB1* gene may

be caused by an increase in the basic mutation rate, by a decrease in selective pressure or by both. Previous analyses of rhodophyte nuclear genes [6, 36, 64] gave no indication for a particularly high mutation rate in the nuclear genome of red algae suggesting that we are dealing mainly with a decrease in selection pressure due to release of functional and/or structural constraints. To explain the large differences in evolutionary rate and isotype diversity among β-tubulins two hypotheses seem particularly relevant: 1) Multifunctional β-tubulins are under higher evolutionary constraints than β-tubulins with specific or very few functions leading to slow and rapid sequence evolution, respectively [19], and 2) tubulin heterogeneity is mainly a neutral consequence of genetic diversity required for the fine tuning of differential tubulin gene expression in specialized tissues and during development [52].

In terms of hypothesis 1 the present results suggest that β-tubulin of *C. crispus* and perhaps of red algae in general is functionally highly specialized, even more so than the fast evolving β-tubulins of fungi, organisms known to have a relatively limited repertoire of microtubular structures compared to higher eukaryotes or protozoa [28, 49]. Red algae, like fungi, have neither flagella nor centrioles and microtubule numbers seem to be low in the cytoplasm at any stage of the rhodophyte life cycle. They become apparent mainly in dividing nuclei as components of the spindle apparatus, which seems to develop totally within the nuclear envelope [55]. It may be concluded, therefore, that microtubules of spindle fibers, at least in red algae and fungi but possibly also in other organisms, are relatively little constrained with respect to structure and function.

Concerning higher plants and animals hypothesis 1 carries the important corollary that the various conserved tubulin isoforms found in these organisms are multifunctional and, hence, are largely equivalent or neutral with respect to different microtubule structures in agreement with hypothesis 2. Indeed, coassembly of strikingly divergent tubulins in microtubule structures of transgenic animal cells has been observed repeatedly [5, 24, 33], supporting the idea that functional specialization among multiple genes occurs mainly at the level of gene expression rather than at the level of tubulin coding sequences. These results disagree with the original 'multitubulin hypothesis' proposed by Fulton and Simpson [18], although a number of cases have been reported where β-tubulin isotypes correlate with distinct functions or seem to be both functionally specialized and relatively conserved [12, 19]. In most cases, however, functional specialization seems to be correlated with a high evolutionary rate. Two typical examples, mouse β-1 and soybean β-1, have been included in the tree of Fig. 4 (see *Mus musculus* and *Glycine max*). Mouse β-1 is expressed solely in the marginal band microtubules of erythrocytes [61] and soybean β-1 exclusively in etiolated hypocotyls [25]. We suggested previously [34] that soybean β-1 may be a relatively dispensable protein due to mildly deleterious mutations which are tolerated in etiolated cells but not in differentiated light-grown tissue.

Intron loss in rhodophyte genes by cDNA mediated gene conversion?

Up to now five different nuclear protein-coding genes have been characterized in red algae: four genes of *C. crispus* encoding β-tubulin (*TubB1*, this paper), chloroplast and cytosolic GAPDH (genes *GapA1* and *GapC1* [36, 37]), and actin (gene *Act1* [6]), and one gene from *Gracilaria verrucosa* encoding chloroplast GAPDH (gene *GapA1* [64]). With the exception of *Chondrus crispus GapC1*, all genes are interrupted at their 5′ end by a short intron (Fig. 1A) suggesting that this may be a characteristic feature of nuclear protein-coding genes from red algae. The splice junctions of these rhodophyte introns are relatively degenerate and show the highest similarity with the corresponding consensus sequences of green plants in agreement with recent suggestions based on phylogenetic inference [37, 64] that red algae and green plants may be sister groups.

The paucity of spliceosomal introns in red algae is surprising, especially for the GAPDH genes which in higher eukaryotes (plants and verte-

brates) and green algae (*Chlamydomonas reinhardtii*) contain many introns: (3 to 5), 8 and (6 to 11) for genes *GapA*, *GapB* and *GapC*, respectively [30, 38]. There are two lines of evidence suggesting that the paucity and asymmetric location of rhodophyte introns are due to differential loss of introns rather than to their recent lineage specific acquisition, in agreement with the 'exon theory of genes' [21]. First, intron conservation patterns in several ancient duplicated genes (including those encoding GAPDH and tubulins) suggest that intervening sequences may be older than the separation of plants and animals [23, 34, 42] and even older than the divergence of prokaryotes and eukaryotes [30, 38, 51, 56], while red algae represent a relatively recent branch on the eukaryotic tree (see above and [3, 37, 48, 64]). Second, if the single introns found in rhodophyte genes were due to recent insertions, one would expect to find them anywhere along the coding sequence and not just at the extreme 5′ end of genes. Indeed, a similar situation is found in *Saccharomyces cerevisiae* where introns are rare and, when present, are almost always located at the 5′ end of genes. Both features, paucity and asymmetric location of introns, can be explained by the gene conversion model of Fink [17] suggesting a preferential loss of introns in the 3′ part of the genes due to homologous recombination of reverse transcribed mRNAs (cDNAs) with their corresponding genes. For several genes a 5′ to 3′ polarity of intron conservation with respect to position [34, 38, 44] and sequence [50] has been observed. This suggests that the Fink model may be a special case of a more general phenomenon of both intron loss and 'intron slippage' occuring in all eukaryotic genes due to occasional (more or less frequent) conversions of genes by homologous recombination with cDNAs corresponding to either reverse transcribed mRNAs [17] or to modified (incorrectly spliced) and reverse transcribed pre-mRNAs [8, 34, 44]. The present identification of an intron containing *TubB1* cDNA (see Results) is a direct confirmation that pre-mRNAs can escape both splicing and degradation in the nucleus prior to transport into the cytoplasm. Although reverse transcription has not yet been demonstrated directly in red algae, its occurence seems very probable given the ubiquitous distribution of retroelements in the genomes of higher and lower eukaryotes.

While intron loss by gene conversion may explain the paucity and polar distribution of introns, it does not provide an answer to the question why 5′ introns should be retained at all in rhodophyte and fungal genes. For yeast it has been suggested that 5′ introns may be implicated in the control of pre-mRNA abundance via the nonsense-mediated mRNA decay pathway [26]. Another possibility may be the implication of 5′ introns in transcriptional control of gene expression for which some evidence has been obtained with intron 1 of gene *GapA1* from maize [11].

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