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Molecular cloning and expression studies of two divergent α-tubulin genes in *Neurospora crassa*

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Abstract

Three α-tubulin isoforms were previously detected in *Neurospora crassa*. We have cloned and analysed two α-tubulin cDNAs, Tub αA and Tub αB that encode polypeptides of 453 and 451 amino acids, respectively. The encoded amino acids exhibit an unusual divergence of 35%. This is the highest divergence ever observed between α-tubulins from the same species. The expression of the two genes is developmentally regulated. We did not detect any transcription of the *Tub αA* gene in dormant macroconidia and during the first 30 min of development even though the α-tub A protein is already present in the early stage of germination. In contrast, the *Tub αB* gene is continuously transcribed during the vegetative cycle and the expression profile of the protein follows the ones of its mRNA.

Keywords: *Neurospora crassa*; α-Tubulin; mRNA transcription

1. Introduction

Microtubules are dynamic polymers composed of α- and β-subunits of approximately 50 kDa each and are involved in all eukaryotic cells in a large array of processes including cell division, chromosomes segregation, nuclear and mitochondrial movements. Most species express multiple isoforms of α- and β-tubulins that are encoded by multigene families. The size of the families ranges from one or two genes in fungi, to 5 or 7 in vertebrates or to as many as 6 α and 9 β genes in plants. In *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Aspergillus nidulans*, two α-tubulin genes have been identified. In both yeast cells, one of the two α-tubulin genes is essential for cell survival whereas the other is not. In *A. nidulans*, the α-*tubA* gene is required during vegetative growth for mitosis and nuclear migration, whereas α-*tubB* gene is essential for sexual development. In these organisms, it has been shown that the different isoforms are functionally interchangeable. In con-

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Abbreviations: DTT, 1,4-dithio-DL-threitol; DAB, 3,3′-diaminobenzamidine tetrahydrochloride; EGTA, ethyleneglycol-bis (β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline

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trast, in *Drosophila melanogaster*, certain highly divergent α-tubulin genes have been found to be required for very specific function. The *84B α-tubulin* gene is expressed constitutively and the 84B protein is highly similar in sequence to the major α-tubulins in many other species. 84B constitutes the majority of α-tubulin present in cells of all tissues and during every stage of development. The highly divergent *67C α-tubulin* gene is co-expressed with the *84B α-tubulin* gene but only during oogenesis [reviewed in [1]]

The expression of α- and β-tubulins is regulated at the transcriptional and post-transcriptional levels. In *Tetrahymena thermophila*, the depolymerization of microtubules by colchicine or their stabilisation by taxol result in an increase of tubulin gene transcription [2,3]. In animal cells, a post-transcriptional mechanism controls the rate of tubulin synthesis. An increase of tubulin subunit concentration, induced by colchicine or nocodazole treatment or by microinjection of unassembled tubulin, produces a decrease of both α- and β-tubulin mRNA concentrations. The mRNA stability is regulated and dependent on continued translation elongation, since the treatment of cells with puromycin, which dissociates mRNAs from ribosomes, prevents autoregulation. The selective β-tubulin mRNA degradation involves binding of unknown cellular factors to the nascent amino-terminal β-tubulin tetrapeptide MREI as it emerges from the ribosome [reviewed in [4]]. Surprisingly, the α-tubulin tetrapeptide does not seem to be involved in the cotranslational degradation of mRNA [5].

In *N. crassa*, one β-tubulin gene [6] and three α-tubulin protein isoforms, α1′, α1″ of 55 kDa and α2 of 52 kDa have been previously identified by bidimensional gel analysis [7]. We report the isolation and characterisation of two distinct α-tubulin cDNAs of *N. crassa*, which we support to encode the two main isoforms, the third one rising as the result of a post-translational modification. Their distinct patterns of expression during the macroconidial germination together with their unusually high divergent primary structures suggest that the differential α-tubulin isoforms could be involved in specific functions during the development of *N. crassa*.

2. Materials and methods

2.1. Strains and media

Neurospora crassa St. Lawrence 74A (FGSC 262) was obtained from the Genetics Stock Center (University of Kansas Medical Center, Kansas City, Kansas) and was cultivated in liquid Vogel's medium. *E. coli* XL1-Blue (Stratagene) was used to propagate plasmids and for propagation of lambda phages. *E. coli* Bl 21 was used for the overexpression of the recombinant protein.

2.2. Isolation of cDNA clones

We screened a *N. crassa* cDNA library in the vector λ Zap II constructed with mRNAs extracted from 19-h-old mycelium [8]. One incomplete clone pM5.1 designed Tub αA was isolated by immunoscreening with a rat IgG antibody against tubulin (Yol 1/34). The partial clone pM5.1 was completed with the 5′ RACE system (Gibco, BRL), using the gene-specific primer 5′-gatggaagaaacgacctg-3′, corresponding to the antisense oligonucleotide of bases 755–772 (see sequences on EMBL data base, accession number: X79403 and X79404 for Tub αA and Tub αB, respectively). The pM2.1 (Tub αB) clone was isolated by screening a mycelial *N. crassa* cDNA library in the vector Lambda Zap I [9] with the *Eco*RI–*Sca*I fragment of Tub αA (bases 702–1412) as probe. After transfer of phages onto nylon membranes, the filters were processed according to the Digoxigenin user's guide system for filter hybridisation from Boehringer-Mannheim and prehybridisation and hybridisation were performed at 68°C. The inserts were excised as Bluescript SK+ plasmids according to Stratagene and the plasmid DNA was analysed further and sequenced on both strands by the dideoxy chain termination method.

2.3. Nucleic acid purification, Southern and Northern blots

The genomic DNA was extracted from 1 g of *N. crassa* mycelium cultivated for 18 h, following the procedure described by Case et al. [10]. Ten micrograms of *N. crassa* DNA was digested to completion and electrophoresed on a 0.7% agarose gel. The

DNA was transferred onto nylon membrane and hybridisations were carried out at 42°C either with a 708 bp fragment of Tub αA (bases 702–1412) or with a 965 bp fragment of Tub αB (bases 402–1367) as probes. High-stringency washes were in 0.5×SSC, 0.1% SDS at 68°C for Tub αA and 0.2×SSC, 0.1% SDS at 68°C for Tub αB and low-stringency washes were in 3×SSC, 0.1% SDS at 42°C for both. RNAs were extracted according to Chirgwin et al. [11]. Total RNA was denatured in formamide and electrophoresed through a 1.5% agarose gel containing formaldehyde. After transfer of RNA onto nylon membrane, the filter was hybridised with the Tub αB probe at 50°C using standard procedure.

2.4. Antibody production and purification

The entire coding sequence of Tub αB was subcloned into the *Nde*I/*Bam*HI sites of the pET3a expression vector, transformed into bacterial host Bl21 plys S and the expression of the native α-tubulin B protein was induced with IPTG. This protein was insoluble, extracted in Tris buffer (20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 1 mM DTT) containing 8 M urea and purified on DEAE Sepharose column with a linear gradient of 10–500 mM KCl. The fractions containing the recombinant protein were pooled and dialysed against MES buffer (50 mM MES-KOH, pH 6.4, 1 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT). The antiserum against α-tubulin B was produced in rabbits by three injections of approximately 0.1 mg of protein per injection. The serum was prepared using standard methods followed by partial purification and concentration of IgG using a protein–A–Sepharose CL-4B column (Pharmacia) and further purified by affinity with the purified protein blotted on nitrocellulose membrane.

2.5. N. crassa protein analysis and immunoblotting

The protein extraction was carried out as described by Ortega Perez et al. [12]. SDS-PAGE, 2D gels and Western-blot analysis were performed according to standard procedures. The transferred proteins were visualised by staining with 0.05% Ponçeau in 3% acetic acid. The membrane was blocked with 5% BSA in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl (TBS), and incubated with anti-α-Tub B antibodies or with rat IgG antibodies (Yol 1/34) (1:2000 and 1:200, respectively) in TBS containing 0.5% BSA for 2 h. After washing twice with TBS containing 0.5% Tween 20 (TBST), the incubation was carried out for 1 h with secondary antibodies (1:2000, donkey anti-rabbit IgG (Amersham) or goat anti-rat IgG (Biolab) horseradish peroxidase conjugate), followed by two washes with TBST and one additional wash with TBS. The peroxidase activity was visualised with 0.5 mg ml^{-1} DAB in 100 mM Tris-HCl, pH 7.5, containing 0.03% H_2O_2.

2.6. RNases protection assays

The plasmids pM2.1 and pM5.1 were digested with *Hin*dIII (base 1075) and *Xho*I (base 1356) respectively and ligated to eliminate the 3′ end noncoding sequence. The plasmids were afterward linearised with *Bgl*I and *Drd*I, respectively. A 210 (bases 865–1075) and a 168 (bases 1188–1356) nucleotide cRNA fragments for pM2.1 and pM5.1, respectively, were generated as probes. The transcription was performed using the T7 RNA polymerase and [$^{\alpha 32}$P]UTP. Fifteen micrograms of total RNA was hybridised with the probe at 42°C according to Krieg and Melton [13]. The RNA hybrids were recovered with 10 μg of tRNA and precipitated with 1 ml of ethanol at −20°C for 30 min. The pellet was resuspended in 10 μl of formamide loading buffer and electrophoresed on a 10% polyacrylamide/8 M urea gel.

2.7. Metabolic labelling and immunoprecipitation

Ten millilitres of prewarmed Vogel's medium at 37°C was inoculated with 10^9 macroconidia and immediately pulse-labelled with 500 μCi of [^{35}S]methionine–cysteine during 1 and 4 h. The chase was carried out by centrifuging the culture at 800×*g* for 5 min, the labelled medium was discarded and the macroconidia were washed twice with 30 ml Vogel's medium and resuspended in 100 ml of Vogel's medium supplemented with 5 mM each of cold methionine–cysteine. The chase was continued until 6 h of development, then the cells were harvested by filtration through a 0.45 μm Millipore filter, frozen in liquid nitrogenous and broken in a mortar in the presence of 2 ml of extraction buffer I (50 mM

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N.c. αA  MRGEVVHIHLGQAGTQLGNSAWELYLLEHGLTQDGRKDPDS-TVAGEG----------GS 60
N.c. αB  -MREIISLNVGQAGCQIANSCWELYCLEHGIQPDGYLTEER-KAADPD----------HG
A.n. α1  -MREVISLNVGQAGCQIANSCWELYCLEHGIQPDGYLTEER-KKEDPD----------HG
A.n. α2  MRGEVCHIHIGQAGTQLGNSAWELYLLEHGLGADGRLDPEKGEDINAG----------GS
S.c. α3  -MREVISINVGQAGCQIGNACWELYSLEHGIKEDGHLEDGLSKPKGGE----------EG
D.m. α1  -MREVVSIQIGQCGIQIGNACWELYLLEHGINLDGSLKTKEELTASGSSASVGHDTSAND
D.m. α4  -MRECISIHVGQAGVQIGNACWELYCLEHGIQPDGQMPSDKTVGGGDD----------SF
                  **** *  *  **** ****    *

N.c. αA  YDTFFTESSNGKYVPRSLFVDLDPSPIDEIRTGPYRQLFHPELLISGKEDAANNYARGHY 120
N.c. αB  FSTFFSETGNGNTFPRTIYADLEPNVIDEVRTGAYRGLFHPEHMISGKEDASNNYARGHY
A.n. α1  FSTFFSETGQGKYVPRTIYADLEPNVVDEVRTGTYRSLFHPENLITGKEDASNNYARGHY
A.n. α2  FETFFTETGCGKYVPRSIFVDLDPSPIDEIRTGPYRQLFHPEQLISGKEDAANNYARGHY
S.c. α3  FSTFFHETGYGKFVPRAIYVDLEPNVIDEVRTGRFKELFHPEQLINGKEDAANNYARGHY
D.m. α1  -NTFFSETGAGKHVPRAVFVDLEPTVVDEVRTGTYRQLFHPEQLITGKEDAANNYARGHY
D.m. α4  ARTFFTETGNGKQVPRSIFVDLEPTVIDDVRNGCMRELYHPEQLISGKEDAANNYARGRY
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N.c. αA  TVGKEMAENVLDRIRKITDNCHSLQGFLVFHSFGGGTGSGFGALVLERLAQDYAKKCKLE 180
N.c. αB  TVGKELIDQVLDKVRRVADNCSGLQGFLVFHSFGGGTGSGFGALLMERLSVDYGKKSKLE
A.n. α1  TVGKEMIDQVLDKVRRMADSCSGLQGFLVFHSFGGGTGSGFGALLMERLSVDYGKKSKLE
A.n. α2  TVGKELVDTVVDRVRRLSDNCSSLQGFLVFHSFGGGTGSGFGALLLERLSTEYGKKSKLE
S.c. α3  TVGREIVDEVEERIRKMADQCDGLQGFLFTHSLGGGTGSGLGSLLLENLSYEYGKKSKLE
D.m. α1  TIGKEIVDLVLDRIRKLADQCTGLQGFLIFHSFGGGTGSGFTSLLMERLSVDYGKKSKLE
D.m. α4  SIGKEVIDRVTSRLQKIAEQCDSLQGFLIFHSLGGGTGSGFTSLLVERLSTDYSKKCKLD
           ***                 *  *****  ** ********  *  * *   * ** **

N.c. αA  FSVYPAPRVATAVVEPYNAVLATHSTLEHS-DVTFLVDNEAGMT-CRRNLDIPRPSYEHL 240
N.c. αB  FCVYPAPQTATSVVEPYNSILTTHTTLEQRRDCSFMVDNEAIYDICRRNLGLERPNYENL
A.n. α1  FCVYPAPQNATSVVEPYNSILTTHTTLEHS-DCSFMVDNEAIYDICRRNLGIERPSYENL
A.n. α2  FAVYPSPRVSTAVVEPYNAVLSTHSTLENS-DCTFLVDNEAVYDICRRNLDIPRPSFEHL
S.c. α3  FAVYPAPQLSTSVVEPYNTVLTTHTTLEHA-DCTFMVDNEAIYDICKRNLGISRPSFSNL
D.m. α1  FAIYPAPQVSTAVVEPYNSILTTHTTLEHS-DCAFMVDNEAIYDICRRNLDIERPTYTNL
D.m. α4  FAVYPSPKVSTAVVEPYNALLTTHSTMDHS-DCVFMVDNEAIYDICNNSLGVDRPAYRNL
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N.c. αA  NRLIAQVVSSITSSLRFDGALNVDLNEFQTNLVPFPRVHYPLISYAPVISATKSAHESFK 300
N.c. αB  NRLIAQVVSSITASLRFDGSLNVDLNEFQTNLVPYPRIHFPLVAYAPVISAAKAAHEANS
A.n. α1  NRLIAQVVSSITASLRFDGSLNVDLNEFQTNLVPYPRIHFPLVAYSPVISADKASHEANS
A.n. α2  NRLIAQVVSSITSSLRFDGALNVDLNEFQTNLVPFPRIHYPLISYAPVISSNRSLHESFK
S.c. α3  NGLIAQVISSVTASLRFDGSLNVDLNEFQTNLVPYPRIHFPLVSYAPILSKKRATHESNS
D.m. α1  NRLIGQIVSSITASLRFDGALNVDLTEFQTNLVPYPRIHFPLVTYAPVISAEKAYHEQLS
D.m. α4  NRLIAQIVSSTTASLRSFGSMNVDLNEFQTNLVPFPRIHFPLVAYAPLMSAERSAHEQHA
         * ** *  ** * ***  *  **** ******** ** * **  * *  *     **

N.c. αA  TSELTLQCFEPNNQMVVCDPRNGKYMAVALLYRGDVVPRDTSAAVAALKAKSSFNLVEWC 360
N.c. αB  VQEMTMSCFEPNNQMVKCDPRHGKYMATCLLYRGDVVPNDAHAAVATLKTKRTIQFVDWC
A.n. α1  VQDITMSCFEPNNQMVKCDPRNGKYMATCLLYRGDVVPRVDHAAVATLKTKRTIQFVDWC
A.n. α2  VQDLTLQCAEPNNQMVRCDPRNGKYMAVALLYRGDCVPRDCTQAIASLKAKASFNYVEWC
S.c. α3  VSEITNACFEPGNQMVKCDPTKGKYMANCLLYRGDVVTRDVQRAVEQVKNKKTVQMVDWC
D.m. α1  VAEITNACFEPANQMVKCDPRHGKYMACCMLYRGDVVPKDVNAAIATIKTKRTIQFVDWC
D.m. α4  ITTLTNACFESSNMMVKCDPRAGKFMACCMLYRGDVVPKDVNAAVSAIKSKRHIQFVDWC
             *  * *  * ** ***  ** **   ***** *      *    * *    * **

N.c. αA  PTGFKIGINHQKPMSVPTASPADGGLASVDRSVSMLSNTTAIAEAWSRLDHKFDLMYSKR 420
N.c. αB  PTGFKLGICYQPPHQVPNG----RSGPRLTDAVCMLSNTTAIAEAWSASFVQVDLMYSKR
A.n. α1  PTGFKIGICYQPPQQVPNG-----DLAKVNRAVCMLSNTTAISEAWSALDHKFDLMYSKR
A.n. α2  PTGFKVGINYHKPARVPGT-----ELAPVDRAVCMLSNTTAISEAWSRLDHKFDLMYSKR
S.c. α3  PTGFKIGICYEPPSVIPSS-----ELANVDRAVCMLSNTTAIADAWKRIDQKFDLMYAKR
D.m. α1  PTGFKVGINYQPPTVVPGG-----DLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKR
D.m. α4  PTGFKIGINYEKPAFVPDG-----DLAKTSRACCMLSNTTAISVAFSNLSYKFDLMFKKR
         ***** **    *   *                *********  *       ****  **

N.c. αA  AFVHWYVGEGMEEGEFSEAREDLRSLEKDYEEVAGDYNDVDVDAEY                453
N.c. αB  AFVHWYVGEGMEEGEFSEAREDLAALERDYEEVAADSMEGEDVEAEY               451
A.n. α1  AFVHWYVGEGMEEGEFSEAREDLAALERDYEEVASDSLEEEGEEVEY               450
A.n. α2  AFVHWYVGEGMEEGEFSEAREDLAALEKDYEEVAGDSLDMEGEEAEY               451
S.c. α3  AFVHWYVGEGMEEGEFTEAREDLAALERDYIEVGADSYAEEF-----               445
D.m. α1  AFVHWYVGEGMEEGEFSEAREDLAALEKDYEEVGMDSGDGEGEGAEEY              462
D.m. α4  AFVHWYVGEGMEEGEFTEARENIAVLERDFEEVGLDNAEEGGDEDFDEF             450
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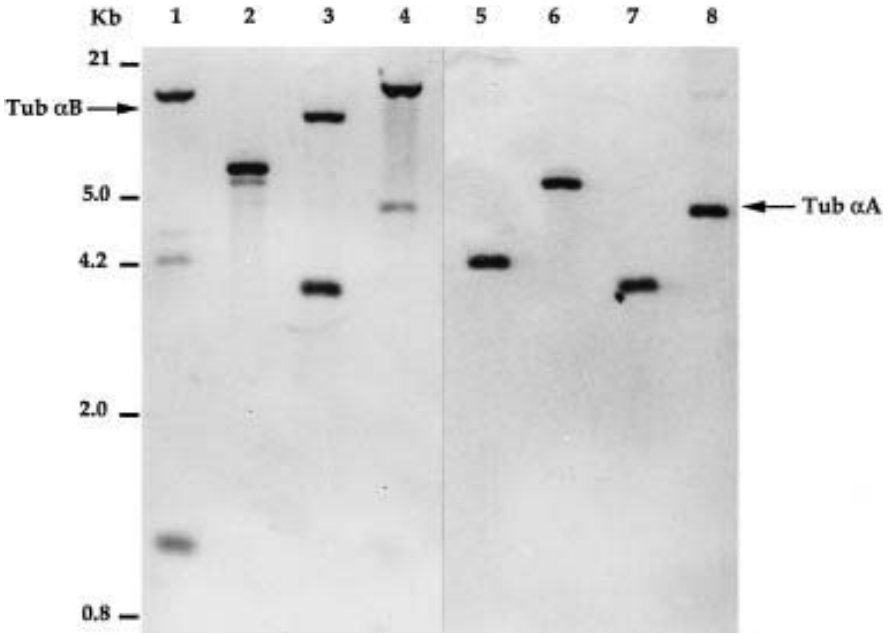

Fig. 2. Southern blot analysis of *N. crassa* genomic DNA. The DNA was digested with *Hin*dIII (lanes 1 and 5), *Pvu*II (lanes 2 and 6), *Bgl*I (lanes 3 and 7), *Sma*I (lanes 4 and 8) and hybridised with the 965 bp *Pst*I–*Sca*I fragment of *Tub αB* gene (lanes 1–4) and with the 710 bp *Eco*RI–*Sca*I fragment of *Tub αA* gene (lanes 5–8).

Tris-HCl, pH 7.6, 100 mM NaCl, 0.4% SDS, 2 mM EGTA, 10 mM benzamidine, 0.25 μg·ml^{-1} pepstatin, 20 μM aprotinin, 0.5 μg·ml^{-1} leupeptin). Triton X-100 (2%) was added and the cell lysate was centrifuged at 38 000 × *g* for 30 min at 4°C. 1/10 of the supernatant was immediately used for immunoprecipitation. Mouse IgG 1 coupled to 20 μl of agarose beads (Sigma) were incubated overnight at 4°C on a rotary shaker with 10 μl of mouse IgG antibody against chicken α-tubulin. The beads were washed twice with 0.5 ml of buffer I supplemented with 2% Triton X-100. The crude extract was added to the beads and incubation was continued for 2 h at room temperature. The pellet was washed 3 times with buffer I plus 2% Triton X-100, resuspended in sample buffer and electrophoresed on a 10% SDS-PAGE.

3. Results and discussion

3.1. Cloning and sequencing of *N. crassa* α-tubulin cDNAs

A first step in cloning of partial Tub αA and Tub αB cDNAs was the screening of two cDNA expression libraries constructed from mRNAs of mycelium cultivated for 19 and 6 h, respectively. The 5′ end sequence of Tub αA was subsequently obtained by 5′ RACE using a specific primer as described in Section 2. The two isolated cDNAs encode for α-tubulins A and B of 453 and 451 amino acids, respectively. These sequences were aligned with five α-tubulins representing a wide range of organisms (Fig. 1). The sequences of α-Tub A and α-Tub B present a divergence of 35%. This is the highest di-

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Fig. 1. Amino acid sequence alignement of *Neurospora crassa* (N.c. αA and αB) α-tubulins with *Aspergillus nidulans* (A. n. α1 and α2); *Saccharomyces cerevisiae* (S.c. α3); *Drosophila melanogaster* (D.m. α1 and α4). Bold types represent the putative sequences of GTP binding as defined by Sternlicht et al. [15].

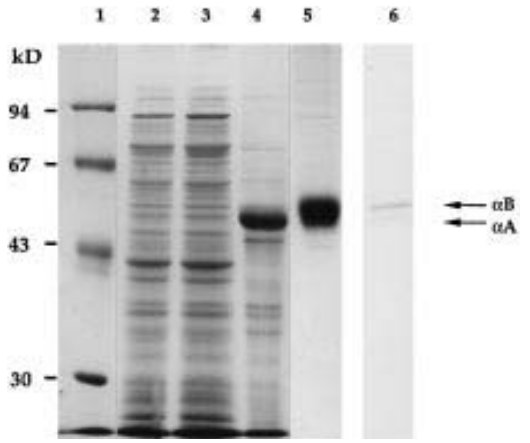

Fig. 3. Purification by DEAE-Sepharose CL-6B of *N. crassa* α-tubulin B overexpressed in *E. coli*. Molecular mass markers (lane 1). Crude protein extract (20 μg) of uninduced *E. coli* (lane 2). Soluble fraction (20 μg) of induced *E. coli* with 0.4 mM IPTG (lane 3). Inclusion bodies containing α-tubulin B fraction solubilized in 8 M urea (lane 4). Alpha-tubulin B (overloaded) purified on DEAE-Sepharose column (lane 5). Western blot of crude protein extract (20 μg) from *N. crassa* probed with the specific antibody directed against α-tubulin B (lane 6).

vergence ever observed between two tubulins in a same species. This divergence arises particularly from α-Tub A which presents a great number of substitutions and two insertions at positions 3 (G) and 368–373 (SSPADG). The only known cases of such high divergence were those observed between α1 and α4 tubulins of *D. melanogaster* (32%) and between Aα1 and Aα2 tubulins of *A. nidulans* (28%). It is particularly interesting to observe that α-Tub B from *N. crassa* is more closely related to α1 from *A. nidulans* (89% identity) than it is to α-Tub A from *N. crassa* (65% identity). This feature could be explaind if the two family members, which arose from tandem duplicated gene, were inherited from a common ancestor by the two species *A. nidulans* and *N. crassa*. Indeed, if one copy of the gene was under selection pressure to maintain function, the other diverged rapidly. One might expect the conserved copy α-Tub B in *N. crassa* to be more similar to α1 in *A. nidulans* than it would be to α-Tub A in *N. crassa*.

As previously mentioned, three α-tubulin protein isoforms have been identified by 2D gel analysis [7]. Our screening, however, revealed only two α-tubulin cDNAs. To detect a possible third gene, we performed a Southern blot analysis. In Fig. 2, genomic DNA, digested with a variety of restriction enzymes, was blotted and hybridised to the Tub αB probe (bases 402–1367, lanes 1–4) or Tub αA probe (bases 702–1412, lane 5–8). In lanes 2 and 4, the Tub αB probe hybridised strongly to a band and weakly with a second one. In the lanes 6 and 8, the Tub αA probe hybridised to identical sized bands as in lanes 2 and 4, but with opposite intensities. These results indicate that the slow migrating band contains the *Tub αB* gene, whereas the fast migrating one contains the *Tub αA* gene. The difference of intensity between the two signals is due to a low identity of the two cDNA probes (63%). To verify whether a third putative gene could be present in one of the two bands, the DNA was digested also with *Hin*dIII (lanes 1 and 5) which cleaved in the middle of the two genes and with *Bgl*I (lanes 3 and 7), which cleaved only in the middle of the *Tub αB* gene. In

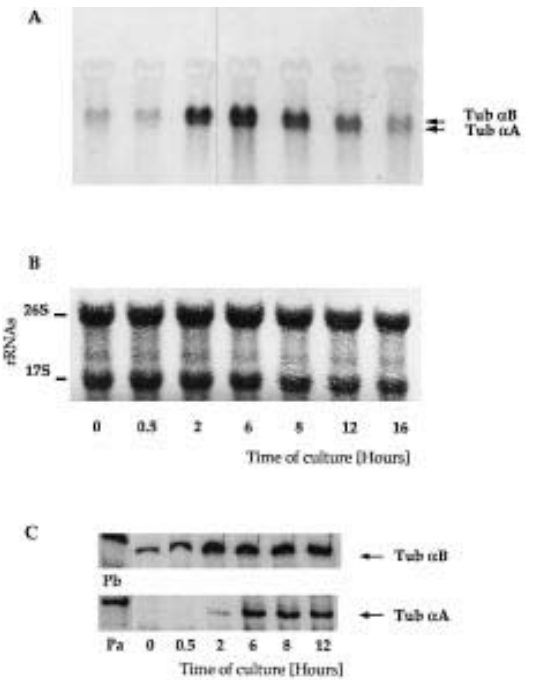

Fig. 4. Northern blot analysis of *N. crassa* α-tubulin mRNAs transcription during germination. A: Total RNA hybridised with the 965 bp *Pst*I–*Sca*I DNA fragment of *Tub αB* gene. B: Methylene blue staining of rRNAs 26S and 17S. C: RNAse protection assay. RNAs were hybridised with a 168 nucleotides cRNA probe of Tub αA (Pa) and a 210 nucleotides cRNA probe of Tub αB (Pb).

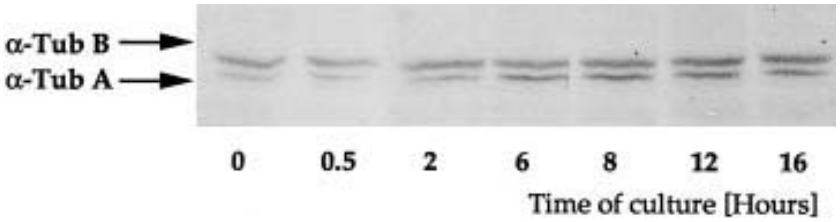

Fig. 5. Western blot analysis of *N. crassa* α-tubulin expression during germination. Twenty micrograms of total protein extract at different steps of development was separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and immunostained with the monoclonal α-tubulin antibody Yol 1/34.

lane 1, only four fragments were observed, indicating that the genes were probably not tandemly repeated. In lane 3, only two clearly visible fragments labelled with the Tub αB probe were present. The absence of a third fragment containing the *Tub αA* gene might be interpreted by its comigration with the Tub αB fragment of about 4 kbp. This interpretation was confirmed in lane 7 where the fragment containing the *Tub αA* gene was strongly visible at the expected size of 4 kbp. Under low-stringency conditions, we did not detect additional fragments (data not shown). In conclusion, these results are consistent with our previous screening and sequencing data, ruling out the presence of a putative third gene.

3.2. *Recombinant α-tubulin B represents the protein with the slowest mobility*

To assign the *Tub αB* gene to its corresponding isoform, we overexpressed the recombinant α-tubulin B and we prepared an affinity purified antibody against the protein. The expressed tubulin was eluted at about 160 mM KCl and purified to homogeneity (Fig. 3). The apparent molecular mass of 55 kDa corresponds to the slowest migrating α1′ and α1″ isoforms previously described [15]. The specific polyclonal antibody, tested on a crude protein extract from *N. crassa* cultivated for 6 h, recognised specifically the slowest migrating α-tubulin isoform and had a very low affinity with the smallest subunit (Fig. 3, lane 6). These results were confirmed by a 2D gel analysis, in which our specific antibody revealed the presence of two spots of 55 kDa and of p*I* 5.77 and 5.68 and the non-specific antibody from yeast recognised additionally the third isoform of 52 kDa and p*I* 5.68 (data not shown). We therefore conclude that the α1′ and α1″ 55 kDa isoforms are encoded by the *Tub αB* gene and that the α2 isoform is encoded by the *Tub αA* gene. The third α-tubulin isoform is probably a product from a post-translational modification of α1. One of the best known modification of α-tubulins is the acetylation of a lysine. This modification occurs normally at position 40 [14]. The α1 subunit of *N. crassa* contains a lysine in position 41, which could be acetylated leading to a neutralisation of charges and therefore a lower p*I* of the protein, without modifying significantly its molecular mass. The second well-known modification is the reversible removal of the C-terminal tyrosine. However, this modification does not affect the p*I* of α-tubulin, but can occur in combination with the other modifications.

3.3. *Tub αA and Tub αB genes are developmentally regulated*

To find out whether the two genes are differentially expressed during conidial germination, a blot of electrophoretically separated total RNA from different developmental stages was probed with the Tub αB probe (bases 402–1367), which was expected to hybridise to both mRNAs. We have detected two different migrating species. The fastest should correspond to Tub αA mRNA because the isolated full-length cDNA is of 1700 nucleotides, whereas the slowest should correspond to Tub αB mRNA because its cDNA, although missing the poly A tail, is 1900 nucleotides long. The level of Tub αA and Tub αB mRNAs increased gradually, reaching a peak after 6 h of development, then decreased progressively for the next 10 h (Fig. 4A). The low resolution of an agarose gel electrophoresis led us to carry out RNase protection assays. Antisense RNA probes of Tub αA and Tub αB coding sequences of

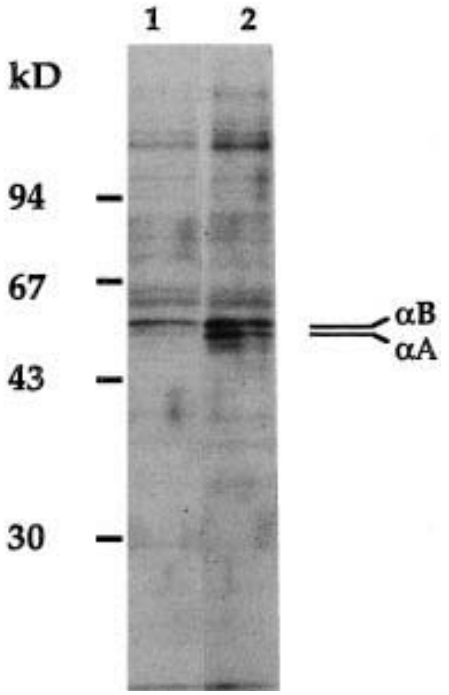

Fig. 6. Pulse-chase experiment showing the differential expression of *N. crassa* α-tubulins at the beginning of conidial germination. Cultures of macroconidia were pulse-labelled with 500 μCi of [35S]methionine–cysteine during 1 h (lane 1) and 4 h (lane 2). The chase was continued until 6 h of development. The α-tubulin subunits were immunoprecipitated with a monoclonal antibody directed against chicken α-tubulin (Amersham). After 1 h of conidial germination, only the Tub αB is expressed, whereas after 4 h both subunits are expressed.

168 bases and 210 bases, respectively, were hybridised to total RNA (Fig. 4C). The results showed that Tub αA was not transcribed during the first 30 min of germination, whereas Tub αB mRNA was already detected in freshly collected macroconidia.

To determine whether the expression profile of α-tubulin A and B proteins parallel the ones of their mRNAs, we carried out a Western blot analysis of crude protein extracts from developmental times corresponding to the mRNA analysis, using a non-specific monoclonal antibody (Yol 1/34). The content of α-tubulin B protein increases during the development as expected from RNA analysis. In contrast, the results reveal differential expression of Tub αA mRNA and the α-tubulin A protein (Fig. 5). Although mRNA was not detected by RNAse protection assay, α-tubulin A was present in low, but significant, amounts at the beginning of conidial germination. Therefore, the α-tubulin A detected by Western blot is a product already present in freshly collected macroconidia. These results were further confirmed by pulse-chase experiments followed by immunoprecipitations. After 1 h of in vivo labelling and 5 h of chase, only α-tubulin B was detected to a significant high level (Fig. 6, lane 1), whereas both were expressed to the same level after a pulse of 4 h (lane 2). This differential expression observed at the beginning of germination could be correlated to the divergent primary structure of the proteins, particularly concerning the amino-terminus end, which was already shown to be important in the β-tubulin mRNA stability [5]. Moreover, these results suggest that the expression of the *Tub αB* gene is required throughout the vegetative life cycle of *N. crassa*, whereas the *Tub αA* gene must be necessary only in particular developmental stages. To further understand a possible specific function of each α-tubulin gene during the development, we are now looking forwards to disrupt the *Tub αA* and *Tub αB* genes and to study the effects of the knock-out during the different stages of conidial germination.

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References

[1] Raff, E.C. (1994) The role of multiple tubulin isoforms in cellular microtubule function. In: Microtubules (Hyams, J.S. and Lloyd, C.W., Eds.), pp. 85–109. Wiley-Liss, New York.
[2] Stargell, L.A., Heruth, D.P., Gaertig, J. and Gorovsky, M.A. (1992) Drugs affecting microtubules dynamics increase α-tubulin mRNA accumulation via transcription in *Tetrahymena thermophila*. Mol. Cell. Biol. 12, 1443–1450.
[3] Gu, L., Gaertig, J., Stargell, L.A. and Gorovsky, M.A. (1995) Gene-specific signal transduction between microtubules and tubulin genes in *Tetrahymena thermophila*. Mol. Cell. Biol. 15, 5173–5179.
[4] Cleveland, D.W. and Theodorakis, N.G. (1994) Regulation of tubulin synthesis. In: Microtubules (Hyams, J.S. and Lloyd, C.W., Eds.), pp. 47–58. Wiley-Liss, New York.
[5] Bachurski, C.J., Theodorakis, N.G., Coulson, R.M.R. and

Cleveland, D.W. (1994) An amino-terminal tetrapeptide specifies cotranslational degradation of β-tubulin but not α-tubulin mRNAs. Mol. Cell. Biol. 14, 4076–4086.
[6] Orbach, M.J., Porro, E.B. and Yanofsky, C. (1986) Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospoira crassa* and its use as a dominant selectable marker. Mol. Cell. Biol. 6, 2452–2461.
[7] Hoang-Van, K., Rossier, C., Barja, F. and Turian, G. (1989) Characterization of tubulin isotypes and of β-tubulin mRNA of *Neurospora crassa* and effects of benomyl on their developmental time course. Eur. J. Cell Biol. 49, 42–47.
[8] Hoang-Van, K., Rossier, C. and Turian, G. (1991) Construction of two *Neurospora crassa* Lambda Zap II-cDNA libraries. Fungal Genet. Newslett. 38, 73–74.
[9] Orbach, M.J., Sachs, M.S. and Yanofsky, C. (1990) The *Neurospora crassa arg-2* locus. J. Biol. Chem. 265, 10981–10987.
[10] Case, M.E., Schweizer, M., Kushner, S.R. and Giles, N.H. (1979) Efficient transformation of *Neurospora crassa* by utilizing hybrid plasmid DNA. Proc. Natl. Acad. Sci. USA 76, 5259–5263.
[11] Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rumer, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18, 5294–5299.
[12] Ortega-Perez, R., Irminger-Finger, I., Arrighi, J.-F., Capelli, N., van Tuinen, D. and Turian, G. (1994) Identification and partial purification of calmodulin-binding microtubule-associated proteins from *Neurospora crassa*. Eur. J. Biochem. 226, 303–310.
[13] Krieg, P.A. and Melton, D.A. (1987) In vitro RNA synthesis with SP6 RNA polymerase. Methods Enzymol. 155, 397–415.
[14] Le Dizet, M. and Piperno, G. (1987) Identification of an acetylation site of *Chlamydomonas* α-tubulin. Proc. Natl. Acad. Sci. USA 84, 5720–5724.
[15] Sternlicht, H., Yaffe Michael, B. and Farr Georges, W. (1987) A model of the nucleotide-binding site in tubulin. FEBS Lett. 214, 226–235.