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Microtubule cytoskeleton perturbation induced by taxol and colchicine affects chaperonin containing TCP-1 (CCT) subunit gene expression in *Tetrahymena* cells[1]

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Abstract

We report the existence of a CCTε subunit gene that encodes subunit ε of the chaperonin CCT (chaperonin containing TCP-1) in *Tetrahymena pyriformis*. This work focuses on the study of the effects of the microtubule polymerizing agent taxol and the depolymerizing agent colchicine on microtubule dynamics and their role in the regulation of tubulin and CCT subunit genes. Under taxol treatment some TpCCT and tubulin genes are distinctly expressed until 30 min of treatment. Cytoplasmic TpCCT mRNA levels slightly decrease while tubulin transcripts are increasing. In colchicine treated cells TpCCT and tubulin transcripts decrease in the initial 30 min of treatment and then start to increase. However, both antimitotic agents induce TpCCT and tubulin gene transcription. This induction does not correlate with increased steady-state levels of TpCCT proteins and seems to be necessary to replete cytoplasmic TpCCT mRNAs. Moreover, we found that TpCCTε and TpCCTα but not TpCCTη are present in the insoluble fraction after a postmitochondrial fractionation that contains components of the ciliate cortex structure, basal bodies and cilia. This suggests that some TpCCT subunits may be associated with these structures. The association of TpCCTε subunit is stimulated either by taxol or colchicine treatment. These observations support the idea that CCT subunits could have additional roles in vivo.

Keywords: Chaperonin CCT; Microtubule; Taxol; Colchicine; *Tetrahymena*

1. Introduction

Chaperonins are a class of molecular chaperones comprised of large multisubunit assemblies essential in mediating the folding of newly synthesized polypeptides in an ATP-dependent manner. Two families of chaperonins have been identified: class I comprising GroEL in eubacteria, Hsp60 in mitochondria and Rubisco binding protein in chloroplasts; class II, comprising the thermosome, TF55 in archaebacteria and the eukaryotic cytosolic chaperonin containing TCP-1 (CCT). CCT differs from GroEL both in architecture and in function. It exists as a hetero-oligomeric complex of about 850–900 kDa containing eight different, but related gene products (for review [1]). In yeast, all CCT subunit genes are essential without any redundancy among them, i.e. overexpression of one cannot rescue the lethality of the null allele of another. This suggests that each CCT subunit has a distinct function conferring a specific property to the chaperonin and/or is required to assemble the whole complex. Indeed, actin binds to CCT by specific interactions with subunits CCTδ and CCTβ or with CCTε [2]. By genetic analysis Lin and Sherman [3] have proposed that there is a unique hetero-oligomeric arrangement of the yeast CCT subunits. In metazoan, the CCTα/TCP-1 gene is highly expressed in testis [4], embryos in early stages and rapidly growing cells in tissue culture [5]. CCT mRNA expression is strongly up-regulated during cell growth especially from G_1/S transition to early S phase [6], implicating them in cell cycle regulated events probably by assisting the folding of tubulin and/or other proteins. Recently, it was shown that CCT subunit levels are up-regulated in several mammalian

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cell lines during recovery from chemical stress [7], suggesting that they respond to protein damage and play a role in recovery of cells under stress.

In vivo CCT plays an important role in the folding of newly translated tubulins (α-, β- and γ-tubulin), actin (for review see [1]) and is intimately connected with the formation of microtubules (Mts) [8–10]. In contrast with actin the productive folding of α- and β-tubulin in vivo requires, in addition to CCT, the presence of GTP and a set of protein co-factors [11,12], which act on the polypeptides after the release of tubulin from CCT. The levels of these proteins appear to affect the dynamics of Mts in vivo. It has been proposed that some of these factors play an important role during the quality control in the assembly of tubulin heterodimer by activating certain GTPases [13]. In addition to the 900 kDa cytosolic complex, free subunits of CCT or smaller oligomeric structures consisting of subsets of CCT subunits have been detected in various different cell types [14–17]. It has been suggested that these micro-complexes may be intermediates in the assembly of the intact CCT [14]. However, there is evidence that free CCT subunits can play additional roles in cells. The TCP-1/CCTα has been detected in centrosomes and incubation of these structures in vitro with an antibody to CCTα/TCP-1 prevents Mt growth, indicating that CCTα/TCP-1 may assist in Mt nucleation [18]. More recently, Roobol et al. [19] showed that CCT subunits α, γ, ζ and θ associate with assembled Mts in vitro behaving as typical Mt associated proteins. The CCTθ subunit has also been implicated with the Ras signaling pathway during yeast morphogenesis [20].

Tetrahymena is a protozoan ciliate with a variety of distinct Mt structures displaying functional diversity and dynamics. This ciliate possesses a CCT gene family homologous to the mouse CCTs. We have previously done the molecular characterization of four members of this gene family, namely TpCCTα, TpCCTγ, TpCCTη and TpCCTθ [21–24] and shown that CCT subunit genes are up-regulated during active cell division and ciliary assembly which suggests the involvement of these CCT subunits in these processes. Here we report the isolation and characterization of the TpCCTε subunit gene from *Tetrahymena*. Mts are the targets for a large number of antimitotic agents including taxol and colchicine. These agents able to suppress Mt dynamics are useful probes to investigate the role of these polymers in vivo. We investigated the possible effects of taxol and colchicine in the Mt cytoskeleton using a confocal laser microscope and a monoclonal antibody against α-tubulin. This allowed us to establish the effects of taxol and colchicine on the Mt cytoskeleton of *Tetrahymena*. Under these conditions we have observed that tubulin and CCT subunit gene expression is affected. Moreover, CCTα and CCTε subunits but not CCTη are unexpectedly associated with insoluble fractions after post-mitochondrial fractionation and the association of CCTε subunit is stimulated either by taxol or colchicine treatment. The differential association of some CCT subunits with the insoluble fractions that contain the ciliate cortex structure, basal bodies and cilia suggests that the different CCT subunits may have distinct roles in cells either when acting as free subunits and/or as part of oligomeric structures.

2. Material and methods

2.1. Cells and culture conditions

Tetrahymena pyriformis amicronucleated CGL strain was grown axenically in enriched proteose/peptone/yeast extract medium (PPY) at 28°C [25]. Cells were harvested in the stationary phase at a density of 1.5×10^6 cells/ml for DNA extraction. For RNA and protein extraction, cells were collected in the exponential phase at 2×10^5 cells/ml. Taxol (50 μm, paclitaxel Sigma) [26] or colchicine (10 mM, Sigma) [27] were added in exponential phase. In the presence of the antimitotic agents the cells were swimming and alive for more than 6 h as revealed by optical microscopic observations; however, the shape of the cell was altered since 30 min of treatment.

2.2. Cloning of TpCCTε gene

Degenerated primers (primer 1 5′-GCCTCTAGAAAY-GAYGGTGCYACYATY-3′ and primer 2 5′-TTCGA-ACCRCCRAAGCCR-3′) were used to amplify *T. pyriformis* genomic DNA using PCR cycling conditions as previously described [22]. The PCR product (∼1.0 kb fragment for *T. pyriformis*) was cloned into pUC19 plasmid and sequenced. One of the clones, named PCRε24, contains the *T. pyriformis* 1.0 kb amplified fragment, which encodes a predicted amino acid sequence related to the mouse CCTε [28]. This fragment was used to screen a *Sau*3AI genomic library [21]. Filters were prehybridized and hybridized using the conditions described in Cyrne et al. [22], except that the hybridization and washing temperature was 42°C. One of the positive clones, named TpCCTε6.1.1, was analyzed by restriction mapping. Restriction fragments were subcloned in pUC19 plasmid. Sequencing and sequence analysis were performed as referred in [22].

2.3. Northern blot hybridization

Total cytoplasmic RNAs from *T. pyriformis* exponentially growing cells and cells treated with taxol or colchicine at different times were extracted and analyzed by Northern blot as described by Sambrook et al. [29]. When hybridizations were performed using βTT1 as probe [30], the conditions were the same as described by Soares et al. [26]. Hybridizations carried out using probes from *T. pyriformis* TpCCT subunit genes were essentially per-

formed as described by Soares et al. [26]. When hsp70 [26] and actin oligonucleotide (5′-CTCTGGGAGCATCGTCACCAGC-3′) [31] probes were used, hybridization conditions were those described in [32]. Hybridizations using TU20 gene as probe were done essentially as described by Neves et al. [33].

2.4. Radioactive probes

T. pyriformis TpCCTε gene DNA probe was prepared from pTpε6H1 plasmid DNA hydrolyzed with *SphI* to generate a ~4 kb fragment that contains the coding region of this gene. The homologous DNA probe encoding *T. pyriformis* TpCCTα subunit was prepared from pTp7 plasmid [24], TpCCTη was prepared from pTpH2.3 plasmid [22], whereas TpCCTγ subunit and βTT1 tubulin were prepared from pTpE3 and IB1 plasmid, respectively, as described in [21]. The ubiquitin probe was prepared from pTU20 plasmid [33]. Fragments were labeled using the Megaprime DNA labeling system (Amersham) and [α-^{32}P]dATP (3000 Ci/mmol) (Amersham International plc). The hsp70-specific oligomer and actin oligomer were synthesized with the gene Assembler system (Pharmacia LKB Biotechnology) and labeled using [γ-^{32}P]ATP (3000 Ci/mmol) (Amersham International plc.) as described by Soares et al. [32].

2.5. Run-on transcription assays

T. pyriformis nucleus isolation and run-on transcription assays were performed essentially as described by Soares et al. [26] using 5×10^8 nuclei. The average incorporation per assay was about 10^7 cpm. One microgram of the following purified fragments was dotted into nitrocellulose filters: α-tubulin – 8 kb *Hin*dIII fragment from IBα [30]; β-tubulin – 3 kb *Hin*dIII fragment from IB1 [30]; *TpCCTα* – ~2.9 kb *Eco*RI fragment from TpCCTα1.1 [23]; *TpCCTε* – ~4.3 kb *Sph*I fragment from TpCCTε6.1.1; *TpCCTη* – ~2.3 kb *Hin*dIII fragment from TpCCTη11.3 [22]; *TpCCTγ* – ~2.2 kb *Eco*RI fragment from pTpE3 [21]; ubiquitin – ~1.0 kb *Hin*dIII fragment from pTU20 [33].

2.6. Digitalization of autoradiograms

The autoradiograms have been digitally generated and processed after being scanned (Epson GT12000) using as software Paint Shop Pro version 4.15 and Microsoft Power Point 2000.

2.7. Production of polyclonal antibodies

A peptide from the N-terminal region of TpCCTε (see Fig. 1) was synthesized and cross-linked to PPD (purified protein derivative of *Mycobacterium tuberculosis*). This conjugated peptide mixed with equal volumes of Freund's complete or incomplete adjuvant was injected three times into rats (50 μg of TpCCTε peptide) at 2 week intervals.

2.8. Preparation of protein extracts from Tetrahymena cells

To obtain total protein extracts, *Tetrahymena* exponentially growing cells (3×10^5 cells), cells treated with taxol and colchicine for different times were pelleted at $3000\times g$ for 5 min at 4°C and washed in ice-cold buffer A (50 mM HEPES-KOH, pH 7.6, 2 mM EDTA, 100 mM NaCl and 250 mM sucrose). They were then resuspended in buffer B (50 mM Tris–HCl pH 6.8, 7.5% (v/v) 2-mercaptoethanol, 1.5% (w/v) SDS and 0.75 mM PMSF) and boiled for 3 min.

Postmitochondrial extracts of *Tetrahymena* exponentially growing cells (3×10^5 cells), cells treated with taxol or colchicine were prepared according to [23]. The soluble fraction corresponding to the postmitochondrial extract was separated from the pellet designated as insoluble fraction. The pellet was resuspended in buffer B and boiled for 3 min. For the preparation of each sample the same number of cells was used and the total amount of protein obtained was loaded in SDS–PAGE.

2.9. SDS–PAGE and Western blot analysis

SDS–PAGE was carried out according to [23]. Immunodetection was performed as described in [23]. The dilutions of the polyclonal antibodies against TpCCT subunits and of the mouse monoclonal antibody against to α-tubulin N356 purchased from Amersham are indicated in the legends of the figures.

2.10. Indirect immunofluorescence

For immunofluorescence staining *T. pyriformis* cells were grown until exponential phase. After treatment with taxol or colchicine for different times cells were washed with PHEM solution (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$; pH 6.9) followed by permeabilization with 0.15% (v/v) Triton X-100 in PHEM for 5 min at room temperature. The permeabilized cells were then fixed for 30 min with 2% (v/v) paraformaldehyde in PHEM and the monoclonal antibody anti-α-tubulin (Amersham Pharmacia, N356) was used as the primary antibody (1/100 dilution) in buffer C (0.1% (v/v) Tween 20, 2% (w/v) BSA in PBS) during 1 h. Cells were washed in buffer C and incubated with the secondary antibody (fluorescein-conjugated goat anti-mouse; Jackson ImmunoResearch Laboratories) diluted 1/200 for 1 h. After washing in buffer C, DNA was stained with DAPI (0.5 μg/ml; Sigma, D9542) and cells were mounted in a VectaShield mounting medium (Vector Laboratories). Confocal microscopy was performed according to [34].

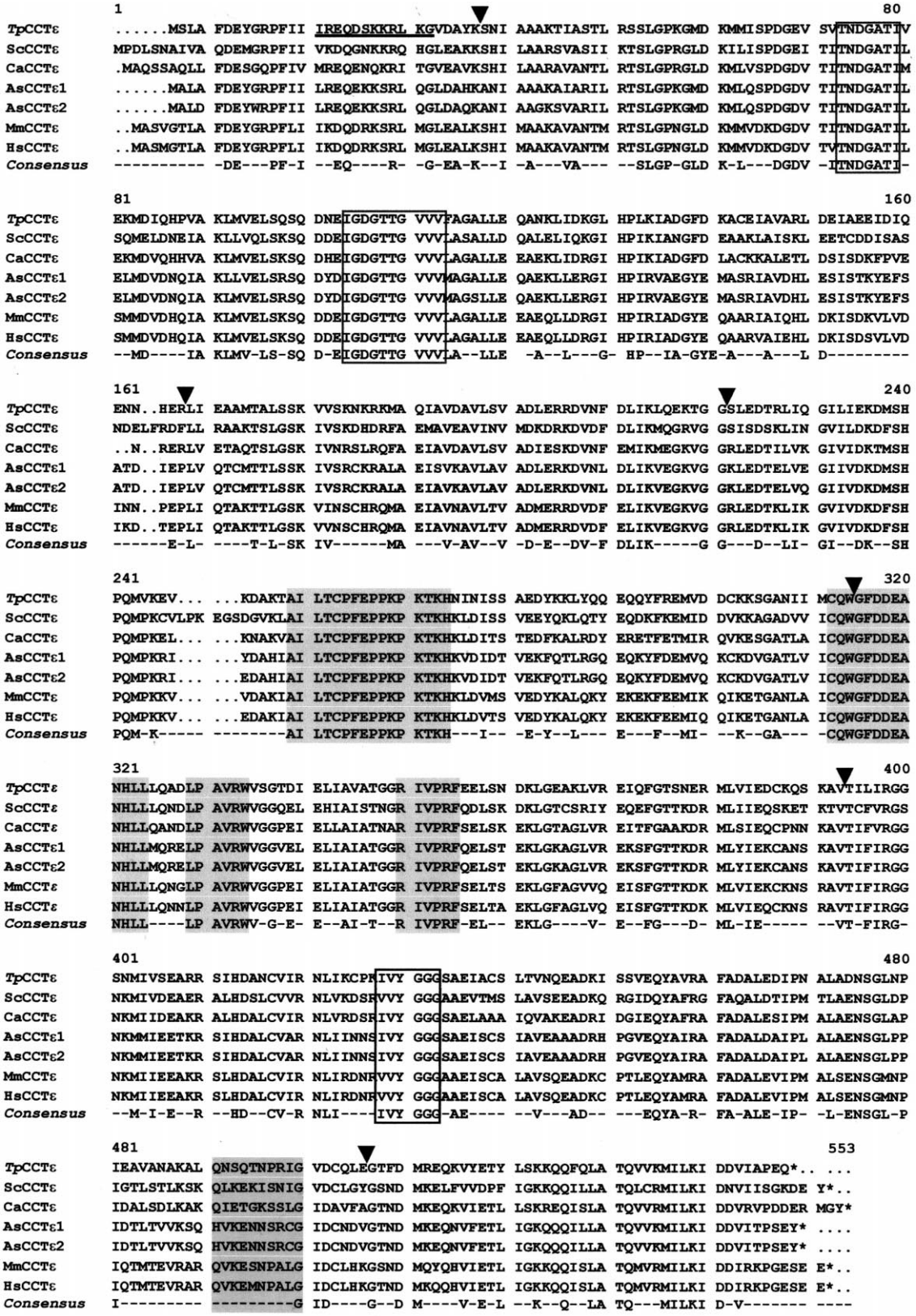
1 80
TpCCTε .....MSLA FDEYGRPFII IREQDSKKRL KGVDAYKSNI AAAKTIASTL RSSLGPKGMD KMMISPDGEV SVTNDGATIV
ScCCTε MPDLSNAIVA QDEMGRPFII VKDQGNKKRQ HGLEAKKSHI LAARSVASII KTSLGPRGLD KILISPDGEI TITNDGATIL
CaCCTε .MAQSSAQLL FDESGQPFIV MREQENQKRI TGVEAVKSHI LAARAVANTL RTSLGPRGLD KMLVSPDGDV TITNDGATIM
AsCCTε1 .....MALA FDEYGRPFII LREQEKKSRL QGLDAHKANI AAAKAIARIL RTSLGPKGMD KMLQSPDGDV TITNDGATIL
AsCCTε2 .....MALD FDEYWRPFII LREQEKKSRL QGLDAQKANI AAGKSVARIL RTSLGPKGMD KMLQSPDGDV TITNDGATIL
MmCCTε ..MASVGTLA FDEYGRPFLI IKDQDRKSRL MGLEALKSHI MAAKAVANTM RTSLGPNGLD KMMVDKDGDV TITNDGATIL
HsCCTε ..MASMGTLA FDEYGRPFLI IKDQDRKSRL MGLEALKSHI MAAKAVANTM RTSLGPNGLD KMMVDKDGDV TVTNDGATIL
Consensus ---------- -DE---PF-I --EQ----R- -G-EA-K--I -A---VA--- --SLGP-GLD K-L---DGDV -ITNDGATI-
81 160
TpCCTε EKMDIQHPVA KLMVELSQSQ DNEIGDGTTG VVVIAGALLE QANKLIDKGL HPLKIADGFD KACEIAVARL DEIAEEIDIQ
ScCCTε SQMELDNEIA KLLVQLSKSQ DDEIGDGTTG VVVLASALLD QALELIQKGI HPIKIANGFD EAAKLAISKL EETCDDISAS
CaCCTε EKMDVQHHVA KLMVELSKSQ DHEIGDGTTG VVVLAGALLE EAEKLIDRGI HPIKIADGFD LACKKALETL DSISDKFPVE
AsCCTε1 ELMDVDNQIA KLLVELSRSQ DYDIGDGTTG VVVMAGALLE QAEKLLERGI HPIRVAEGYE MASRIAVDHL ESISTKYEFS
AsCCTε2 ELMDVDNQIA KLMVELSRSQ DYDIGDGTTG VVVMAGSLLE QAEKLLERGI HPIRVAEGYE MASRIAVDHL ESISTKYEFS
MmCCTε SMMDVDHQIA KLMVELSKSQ DDEIGDGTTG VVVLAGALLE EAEQLLDRGI HPIRIADGYE QAARIAIQHL DKISDKVLVD
HsCCTε SMMDVDHQIA KLMVELSKSQ DDEIGDGTTG VVVLAGALLE EAEQLLDRGI HPIRIADGYE QAARVAIEHL DKISDSVLVD
Consensus --MD----IA KLMV-LS-SQ D-EIGDGTTG VVVLA--LLE -A--L---G- HP--IA-GYE-A---A---L D---------
161 240
TpCCTε ENN..HERLI EAAMTALSSK VVSKNKRKMA QIAVDAVLSV ADLERRDVNF DLIKLQEKTG GSLEDTRLIQ GILIEKDMSH
ScCCTε NDELFRDFLL RAAKTSLGSK IVSKDHDRFA EMAVEAVINV MDKDRKDVDF DLIKMQGRVG GSISDSKLIN GVILDKDFSH
CaCCTε ..N..RERLV ETAQTSLGSK IVNRSLRQFA EIAVDAVLSV ADIESKDVNF EMIKMEGKVG GRLEDTILVK GIVIDKTMSH
AsCCTε1 ATD..IEPLV QTCMTTLSSK IVSRCKRALA EISVKAVLAV ADLERKDVNL DLIKVEGKVG GKLEDTELVE GIIVDKDMSH
AsCCTε2 ATD..IEPLV QTCMTTLSSK IVSRCKRALA EIAVKAVLAV ADLERKDVNL DLIKVEGKVG GKLEDTELVQ GIIVDKDMSH
MmCCTε INN..PEPLI QTAKTTLGSK VINSCHRQMA EIAVNAVLTV ADMERRDVDF ELIKVEGKVG GRLEDTKLIK GVIVDKDFSH
HsCCTε IKD..TEPLI QTAKTTLGSK VVNSCHRQMA EIAVNAVLTV ADMERRDVDF ELIKVEGKVG GRLEDTKLIK GVIVDKDFSH
Consensus ------E-L- ----T-L-SK IV------MA ---V-AV--V -D-E--DV-F DLIK-----G G---D--LI- GI--DK--SH
241 320
TpCCTε PQMVKEV... ...KDAKTAI LTCPFEPPKP KTKHNINISS AEDYKKLYQQ EQQYFREMVD DCKKSGANII MCQWGFDDEA
ScCCTε PQMPKCVLPK EGSDGVKLAI LTCPFEPPKP KTKHKLDISS VEEYQKLQTY EQDKFKEMID DVKKAGADVV ICQWGFDDEA
CaCCTε PQMPKEL... ...KNAKVAI LTCPFEPPKP KTKHKLDITS TEDFKALRDY ERETFETMIR QVKESGATLA ICQWGFDDEA
AsCCTε1 PQMPKRI... ...YDAHIAI LTCPFEPPKP KTKHKVDIDT VEKFQTLRGQ EQKYFDEMVQ KCKDVGATLV ICQWGFDDEA
AsCCTε2 PQMPKRI... ...EDAHIAI LTCPFEPPKP KTKHKVDIDT VEKFQTLRGQ EQKYFDEMVQ KCKDVGATLV ICQWGFDDEA
MmCCTε PQMPKKV... ...VDAKIAI LTCPFEPPKP KTKHKLDVMS VEDYKALQKY EKEKFEEMIK QIKETGANLA ICQWGFDDEA
HsCCTε PQMPKKV... ...EDAKIAI LTCPFEPPKP KTKHKLDVTS VEDYKALQKY EKEKFEEMIQ QIKETGANLA ICQWGFDDEA
Consensus PQM-K----- --------AI LTCPFEPPKP KTKH---I-- -E-Y--L--- E---F--MI- --K--GA--- -CQWGFDDEA
321 400
TpCCTε NHLLLQADLP AVRWVSGTDI ELIAVATGGR IVPRFEELSN DKLGEAKLVR EIQFGTSNER MLVIEDCKQS KAVTILIRGG
ScCCTε NHLLLQNDLP AVRWVGGQEL EHIAISTNGR IVPRFQDLSK DKLGTCSRIY EQEFGTTKDR MLIIEQSKET KTVTCFVRGS
CaCCTε NHLLQANDLP AVRWVGGPEI ELLAIATNAR IVPRFSELSK EKLGTAGLVR EITFGAAKDR MLSIEQCPNN KAVTIFVRGG
AsCCTε1 NHLLMQRELP AVRWVGGVEL ELIAIATGGR IVPRFQELST EKLGKAGLVR EKSFGTTKDR MLYIEKCANS KAVTIFIRGG
AsCCTε2 NHLLMQRELP AVRWVGGVEL ELIAIATGGR IVPRFQELST EKLGKAGLVR EKSFGTTKDR MLYIEKCANS KAVTIFIRGG
MmCCTε NHLLLQNGLP AVRWVGGPEI ELIAIATGGR IVPRFSELTS EKLGFAGVVQ EISFGTTKDK MLVIEKCKNS RAVTIFIRGG
HsCCTε NHLLLQNNLP AVRWVGGPEI ELIAIATGGR IVPRFSELTA EKLGFAGLVQ EISFGTTKDK MLVIEQCKNS RAVTIFIRGG
Consensus NHLL----LP AVRWV-G-E- E--AI-T--R IVPRF-EL-- EKLG----V- E--FG---D- ML-IE----- --VT-FIRG-
401 480
TpCCTε SNMIVSEARR SIHDANCVIR NLIKCPKIVY GGGSAEIACS LTVNQEADKI SSVEQYAVRA FADALEDIPN ALADNSGLNP
ScCCTε NKMIVDEAER ALHDSLCVVR NLVKDSRVVY GGGAAEVTMS LAVSEEADKQ RGIDQYAFRG FAQALDTIPM TLAENSGLDP
CaCCTε NKMIIDEAKR ALHDALCVIR NLVRDSRIVY GGGSAELAAA IQVAKEADRI DGIEQYAFRA FADALESIPM ALAENSGLAP
AsCCTε1 NKMMIEETKR SIHDALCVAR NLIINNSIVY GGGSAEISCS IAVEAAADRH PGVEQYAIRA FADALDAIPL ALAENSGLPP
AsCCTε2 NKMMIEETKR SIHDALCVAR NLIINNSIVY GGGSAEISCS IAVEAAADRH PGVEQYAIRA FADALDAIPL ALAENSGLPP
MmCCTε NKMIIEEAKR SLHDALCVIR NLIRDNRVVY GGGAAEISCA LAVSQEADKC PTLEQYAMRA FADALEVIPM ALSENSGMNP
HsCCTε NKMIIEEAKR SLHDALCVIR NLIRDNRVVY GGGAAEISCA LAVSQEADKC PTLEQYAMRA FADALEVIPM ALSENSGMNP
Consensus --M-I-E--R --HD--CV-R NLI----IVY GGG-AE---- --V---AD-- ---EQYA-R- FA-ALE-IP- -L-ENSGL-P
481 553
TpCCTε IEAVANAKAL QNSQTNPRIG VDCQLEGTFD MREQKVYETY LSKKQQFQLA TQVVKMILKI DDVIAPEQ*.. ...
ScCCTε IGTLSTLKSK QLKEKISNIG VDCLGYGSND MKELFVVDPF IGKKQQILLA TQLCRMILKI DNVIISGKDE Y*..
CaCCTε IDALSDLKAK QIETGKSSLG IDAVFAGTND MKEQKVIETL LSKREQISLA TQVVRMILKI DDVRVPDDER MGY*
AsCCTε1 IDTLTVVKSQ HVKENNSRCG IDCNDVGTND MKEQNVFETL IGKQQQILLA TQVVKMILKI DDVITPSEY* ....
AsCCTε2 IDTLTVVKSQ HVKENNSRCG IDCNDVGTND MKEQNVFETL IGKQQQILLA TQVVKMILKI DDVITPSEY* ....
MmCCTε IQTMTEVRAR QVKESNPALG IDCLHKGSND MQYQHVIETL IGKKQQISLA TQMVRMILKI DDIRKPGESE E*..
HsCCTε IQTMTEVRAR QVKEMNPALG IDCLHKGTND MKQQHVIETL IGKKQQISLA TQMVRMILKI DDIRKPGESE E*..
Consensus I--------- ---------G ID----G--D M----V-E-L --K--Q--LA TQ---MILKI D-V------- ----

Fig. 1. Alignment and comparison of *Tetrahymena* CCTε deduced amino acid sequence with those from different organisms. The deduced amino acid sequence for the *T. pyriformis* CCTε polypeptide was aligned and compared with those from other organisms (accession numbers from the EMBL data base are indicated): *Saccharomyces cerevisiae* (P40413); *C. elegans* (P47209); *A. sativum* (AS1-P40412; AS2-P54411); *Mus musculus* (P80316) and *Homo sapiens* (P48643). Gaps were inserted when required to maximize the alignment and are represented by points. Asterisks indicate the end of the open reading frames. Arrows indicate the intron positions of the *T. pyriformis* gene. The conserved sequences between almost all chaperonins, including the traditional chaperonins, are indicated by open boxes. The conserved sequences shared only by CCTε subunits are shadowed. The sequence peptide used to elicit the polyclonal antibody against TpCCTε subunit is underlined.
←

3. Results

3.1. The TpCCTε subunit gene, predicted amino acid sequence and the specific antibody against this subunit

Using degenerate primers we amplified by PCR a 1.0 kb genomic DNA fragment and determined the partial sequence of this fragment. A database search of the sequence of this fragment revealed that the amplified fragment encodes a polypeptide highly similar to the mouse *CCTε* subunit [28]. This fragment was used as a probe to screen a *T. pyriformis* genomic library constructed in λ-Dash II [21]. One of the positive clones, designated TpCCTε6.1.1, contained the complete sequence of the *TpCCTε* subunit gene as determined by sequence analysis. Analogous to other *Tetrahymena TpCCT* subunit genes the coding region of the *TpCCTε* gene is also interrupted by six introns (see Fig. 1 for the intron positions).

The protein encoded by the *TpCCTε* gene consists of 534 amino acid residues and possesses a predicted molecular mass of 58.9 kDa and a putative p*I* of 5.1. A comparative analysis of the putative p*I* of this *Tetrahymena* CCT subunit with the p*I* of the other known subunits shows that this subunit is probably the most acidic CCT subunit of *Tetrahymena*. Similarly to other TpCCT subunits, the predicted amino acid sequence of TpCCTε on comparison with other CCTε subunits from different organisms exhibits a high degree of sequence similarity, ranging from 80% with *Caenorhabditis elegans* (P47209) to 79.2% with mouse [28]. The degree of similarity decreases when this comparison is extended to the other *Tetrahymena* CCT subunits (namely 51.4% for TpCCTθ to 59.2% for TpCCTδ). The predicted amino acid sequence of *Tetrahymena* TpCCTε when aligned with the other known CCTε subunits from other organisms shown in Fig. 1 reveals that TpCCTε also contains the sequence motifs that are conserved in almost all group I chaperonins and in members of the CCT/TF55 protein family (for review see [1]). These include the motifs TNDGATIL that in TpCCTε is apparent as TNDGATIV (positions 67–73), GDGTTSV, which is conserved among all CCT but extended as –IGDGTTGVVV– (positions 98–107), and V(P/A)GGG in the form of VYGGG (positions 419–423). Interestingly, five highly conserved regions, AILTCPFEPPKPKTKH (positions 245–260), CQWGFDDEANHLL (positions 298–310), LPAVRWV (positions 315–321), RIVPRF (positions 236–241) and MILKID (positions 522–527), seem to be exclusively common to CCTε subunits.

From the predicted amino acid sequence of TpCCTε we synthesized a peptide of the N-terminal region of the protein and produced a polyclonal antibody specific to the *Tetrahymena* CCTε subunit. As can be seen from Fig. 2 the TpεNterm antibody specifically reacts with a protein of 60 kDa present in *Tetrahymena* postmitochondrial extracts, whereas no reactivity is observed with preimmune serum. The reactivity was totally eliminated when the serum was preincubated with an excess (500–700 μg) of the synthetic peptide used to produce the antibodies. These results clearly show that this antibody specifically recognizes the *Tetrahymena* CCTε subunit.

3.2. Taxol and colchicine effects on Mt arrays of Tetrahymena cells

We investigated the effects of taxol and colchicine on the morphological and subcellular status of the *Tetrahymena* Mts using a confocal laser microscope and a monoclonal antibody against α-tubulin (see Section 2). We observed in detail the intracellular Mts of exponentially

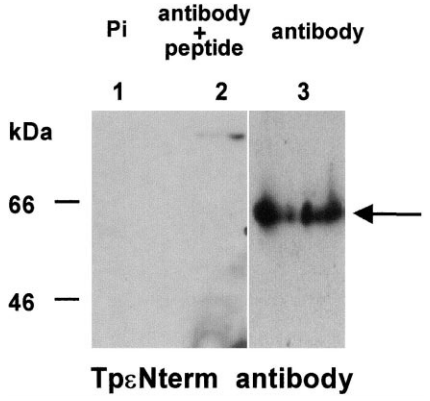

Fig. 2. Characterization of the polyclonal antibody produced against *Tetrahymena* CCTε subunit. The peptide of the N-terminal region of the predicted amino acid sequence of the TpCCTε subunit was synthesized, conjugated with the carrier protein PPD and injected into rats to produce polyclonal serum. The *Tetrahymena* preimmune and polyclonal serum was analyzed using postmitochondrial extracts (20 μg) resolved by 10% SDS–polyacrylamide gels and Western blotting. Lanes: 1, incubation with the preimmune serum; 2, incubation with the polyclonal antibody pre-absorbed with synthetic peptide (700 μg); 3, incubation with the respective polyclonal antibody. The primary antibody as well as the secondary antibody were used in all the cases in a dilution of 1/750. Molecular mass markers are indicated on the left.

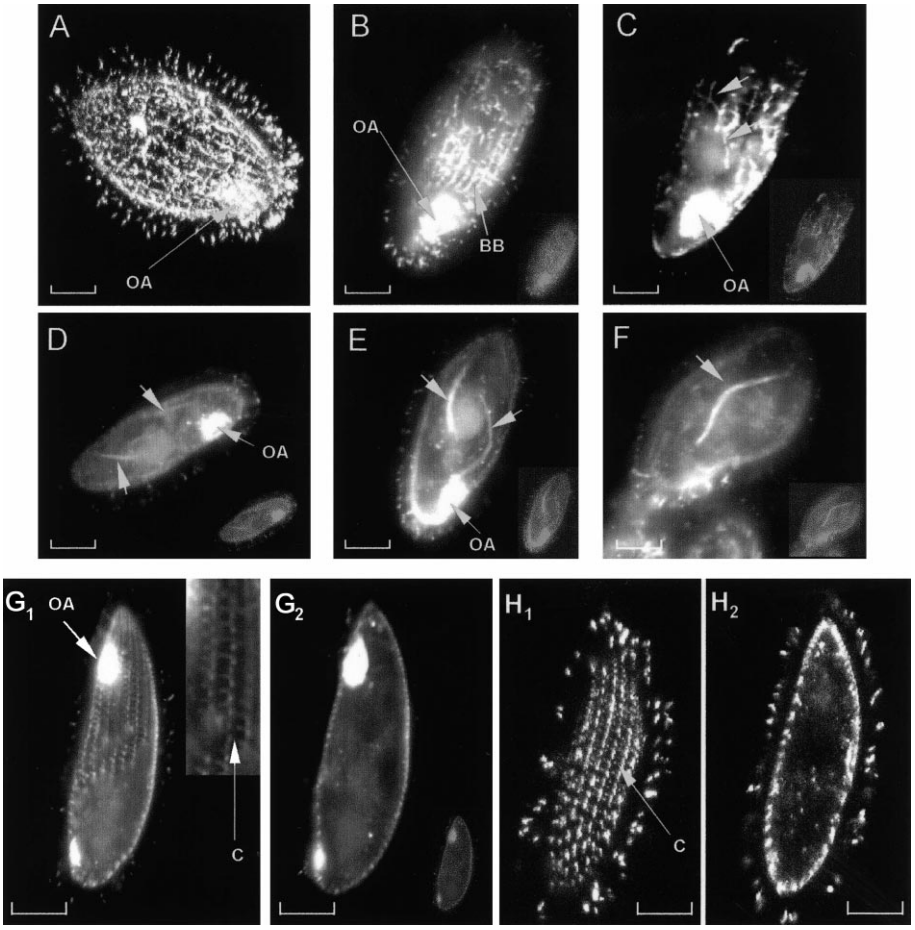

Fig. 3. Immunofluorescence analysis of *Tetrahymena* exponentially growing cells and cells treated with taxol and colchicine using a monoclonal antibody against α-tubulin. Localization of tubulin was performed in exponentially growing cells (A–C), cells treated with taxol for 90 min (D–F) and colchicine for 90 min (G_1,G_2,H_1,H_2), permeabilized with 0.15% (v/v) Triton X-100, fixed in 2% (v/v) paraformaldehyde and labeled with a monoclonal anti-α-tubulin (Amersham) (detected with fluorescein (FITC)). (A) The exponentially growing cell surface was focused on; several longitudinal rows of intact cilia are visible; the tips of cilia are preferentially labeled; the oral apparatus (OA) is also observable. (B) Using a confocal laser scanning microscope this picture was taken immediately under the surface of an exponentially growing cell; longitudinal rows of cilia are still visible; basal bodies (BB) are also decorated by the antibody (arrow); OA is strongly labeled; macronucleus stained with DAPI is starting to be visible (see small picture in the low right corner). (C) This picture was taken at the macronucleus plane as shown by DAPI staining; very thin Mts are observable surrounding the macronucleus (arrows). (D–F) Confocal optical sections at the macronucleus plane of distinct taxol treated cells for 90 min; note the visible bundles of Mts surrounding the macronucleus that were absent in exponentially growing cells (see arrows). (G_1,H_1) Confocal optical sections at the surface of two cells treated with colchicine for 90 min; note the disappearance of Mt arrays at the cortex level. In the upper right corner of H_1 a clear detail of the infraciliature is shown, suggesting that microtubular systems have been depolymerized. (G_2,H_2) Pictures at the macronucleus plane of two cells treated with colchicine for 90 min. The internal Mt networks are no longer visible. Bar = 14 μm.

growing cells and cells subjected to taxol and colchicine (Fig. 3). In control cells when the focus is on the cell surface, the α-tubulin antibody decorates the basal bodies (BB) that are arranged in longitudinal arrays with the oral apparatus (OA) and intact cilia are clearly visible; we must state that the tip of the cilia is preferentially labeled (Fig. 3A,B). Thin Mt filaments are frequently detectable under the cortex (see Fig. 3C, see arrows). At the nuclear level the images show a number of Mt surrounding the macronucleus (see Fig. 3C, see arrows). In cells treated with taxol the intracellular Mt arrays were replaced by bundles of Mts that are preferentially located around the macronucleus (see Fig. 3D–F). These bundles apparently result from the massive polymerization of Mts induced by the drug. In cells treated with colchicine, we observed the disappearance of Mt arrays at the cortex level (Fig. 3G_1,H_1). In Fig. 3G_1 a clear detail of the infraciliature is shown, suggesting that microtubular systems have been depolymerized by the action of colchicine. Additionally, the internal Mt networks are no longer visible, which indicates that they have also been affected by the colchicine treatment (see Fig. 3G_2,H_2). These data clearly show that taxol and colchicine affect the *Tetrahymena* cortex structure and Mt cytoskeleton organization.

3.3. *Expression of CCT, tubulin and actin genes in Tetrahymena cells treated with the depolymerizing and polymerizing agents colchicine and taxol*

Taxol and colchicine have been shown to regulate gene

expression in eukaryotic cells [27,35,36]. Since taxol and colchicine cause alterations in the *Tetrahymena* Mt cytoskeleton we have investigated the effects of this cytoskeleton perturbations on CCT, tubulin and actin gene expression by Northern blot analysis (Fig. 4). Using total cytoplasmic RNA prepared from exponentially growing cells and cells treated with colchicine for different times we found that the amount of the steady-state population of the *TpCCTα*, *TpCCTε*, *TpCCTγ* and *TpCCTη* subunit mRNAs and the β-tubulin mRNA (βTT1) decreases until 30 min of colchicine treatment when compared to control cells (Fig. 4). Afterwards one sees that the levels of these steady-state populations of mRNAs start to increase. At 90 min of drug treatment tubulin mRNAs already reached levels similar to those found in exponentially growing cells whereas the *TpCCT* subunit mRNAs reached levels higher than those of control cells. When cells were treated with taxol at different times, as shown in Fig. 4, we observed that in the first 30 min of treatment the *TpCCTα*, *TpCCTε*, *TpCCTγ* and *TpCCTη* subunit genes exhibit a different pattern of expression compared to that of β-tubulin genes (βTT1). Indeed, we found a decrease in TpCCT subunit mRNA levels between 15 and 30 min of taxol treatment whereas the amount of tubulin mRNAs is progressively increasing up to levels higher than that found in control cells, until 90 min of taxol treatment (Fig. 4). However, after the initial 30 min of taxol treatment the steady-state populations of TpCCT subunit mRNAs also increased to values much higher than those found in exponentially growing cells (see Fig. 4). Interestingly, the expression of actin, the other major substrate of the cytosolic chaperonin, is not significantly affected by treatment with taxol or colchicine (Fig. 4).

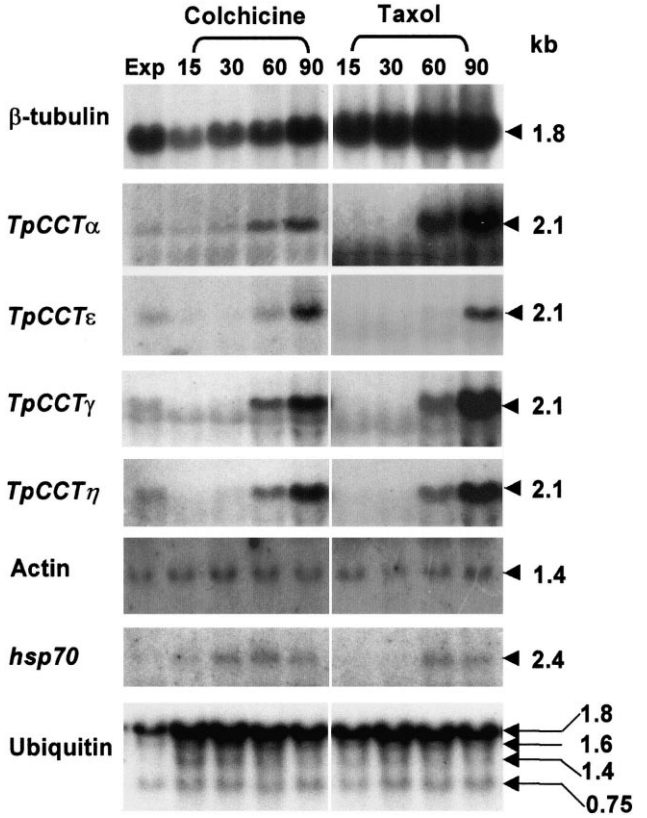

Fig. 4. β-Tubulin (βTT1), TpCCTα, TpCCTε, TpCCTγ, TpCCTη, actin, hsp70 and ubiquitin mRNA levels in *Tetrahymena* cells treated with the microtubule depolymerizing/polymerizing agents colchicine and taxol, respectively. (A) Total cytoplasmic RNA (30 μg) from exponentially growing cells, from colchicine (10 mM) treated cells for 15 min, 30 min, 60 min and 90 min and from taxol (50 μM) treated cells for 15 min, 30 min, 60 min and 90 min was analyzed in 1.5% (w/v) agarose formaldehyde gels, transferred to nitrocellulose filters, and hybridized with the following probes: β-tubulin – a 3 kb *Hin*dIII fragment from IB1 plasmid containing the β-tubulin gene (βTT1) [30]; *TpCCTα* – ~2.9 kb *Eco*RI fragment from TpCCTα1.1 [23]; *TpCCTε* – ~4.3 kb *Sph*I fragment from TpCCTε6.1.1; *TpCCTγ* – ~2.2 kb *Eco*RI fragment from pTpE3 plasmid [21]; *TpCCTη* – ~2.3 kb *Hin*dIII fragment from TpCCTη11.3 [22]; actin – a specific oligomer probe (see Section 2); hsp70 – a specific oligomer probe [26]; ubiquitin – 0.23 kb *Hin*dIII fragment from pTU20 plasmid [33]. The results shown are representative of three to five independent experiments that gave similar results. The autoradiograms have been digitally generated and processed as described in Section 2.

It is plausible to assume that dramatic modifications in Mt dynamics caused by colchicine and taxol could be a stress event for *Tetrahymena* cells. In this perspective we have studied the expression of hsp70 and ubiquitin genes in cells treated with the referred antimitotic agents. Fig. 4 shows that a transcript of about 2.4 kb, corresponding to hsp70, is expressed at barely detectable levels in exponentially growing cells, that it slightly increases after 30 min of treatment with colchicine and is maintained until 90 min of treatment. In taxol treated cells a similar type of induction of hsp70 mRNAs occurred but only after 60 min of treatment. Comparison of these results with those obtained for *Tetrahymena* cells submitted to a hyperthermic shock or recovering their cilia [26] shows that up-regulation of hsp70 by taxol and colchicine follows a different pattern. In fact, the hsp70 gene is strongly induced immediately after 15 min of heat shock or cilia recovery and rapidly decreases to the levels of control cells at 30 min. Ubiquitin genes are actively induced by heat shock [37], and we observed an increase in the 1.8 kb mRNA species after 15 min of treatment with colchicine or taxol (see Fig. 4). This induction is more visible for colchicine treated cells. A slight induction of the 1.6 kb mRNA is also detected between 15 and 60 min of colchicine treatment. Noteworthy is the appearance of a 1.4 kb mRNA species in cells treated with colchicine and taxol for 15 and 30 min. This mRNA species was never described as being specifically induced under stress conditions in *T. pyriformis* cells. On the other hand, the 0.75 kb ubiquitin fusion gene transcripts coding for 53 amino acid extension protein [33] remained almost unaltered during the treatment with the referred antimitotic agents. The effects on the induction of hsp70 and ubiquitin genes by colchicine and taxol are not comparable both with respect to time and to the extent of the expression induced by heat shock. However, our results unequivocally show that these genes are

induced, indicating that the dramatic damage in Mt arrays caused by these drugs (see Fig. 3) originates a specific stress event in *Tetrahymena* cells.

3.4. CCT, tubulin and ubiquitin gene transcription in Tetrahymena cells treated with the polymerizing and depolymerizing agents taxol and colchicine

To understand the mechanisms underlying the regulation of *TpCCTα*, *TpCCTε*, *TpCCTγ*, *TpCCTη*, tubulin and ubiquitin gene expression after treatment with the antimitotic agents we investigated the apparent rate of transcription of these genes by run-on transcription assays in nuclei isolated from cells treated with taxol and colchicine for 15 and 60 min (Fig. 5). The results show that the apparent rate of transcription of tubulin and *TpCCT* subunit genes is higher in nuclei isolated from cells exposed to taxol than in nuclei isolated from exponentially growing cells (Fig. 5A). In fact, the apparent rate of transcription of tubulin and *TpCCT* subunit genes is increased after 15 min of taxol treatment and continues to increase until 60 min.

The apparent rate of transcription of α-tubulin, *TpCCTε* and *TpCCTη* genes is maintained almost constant until 15 min of colchicine treatment, reaching values higher than those found in nuclei isolated from control cells at 60 min (Fig. 5B). In contrast, nuclei isolated from cells treated with colchicine for 15 min are already more active in producing β-tubulin, *TpCCTγ* and ubiquitin transcripts than nuclei isolated from exponentially growing cells. Nevertheless, the highest levels of the apparent rate of transcription of these genes is also observed in nuclei obtained from cells submitted to colchicine for 60 min. Interesting is the fact that *TpCCTα* presents a different pattern of induction compared to the other genes analyzed. Indeed, the transcripts of *TpCCTα* show an increase in nuclei of cells treated with colchicine for 15 min followed by a decrease to the levels found in nuclei isolated from control cells.

The taxol and colchicine results taken together show that the decrease detected in the steady-state populations of tubulin and *TpCCT* subunit mRNAs between 15 and 30 min of colchicine treatment and the slight decrease of *TpCCT* subunit mRNAs between 15 and 30 min of exposure to taxol cannot be due to down-regulation of the transcription rate of these genes. These results suggest that the decrease in the amounts of *TpCCT* subunit mRNAs might be the result of posttranscriptional events. In fact, this is consistent with our data on studies of *TpCCTη* mRNA stability that show that the half-life of this mRNA decreases in cells treated with colchicine and taxol compared to that of control cells (results not shown). Moreover, the up-regulation of the studied genes correlates with the increased levels of tubulin and *TpCCT* subunit mRNAs found in cytoplasm between 60 and 90 min of colchicine and taxol treatment. In the case of ubiquitin genes, the increased apparent rate of transcription in nuclei isolated from cells subject to colchicine supports the induction of the 1.8 kb mRNA species and the synthesis de novo of the 1.6 kb and 1.4 kb mRNA species in the cytoplasm between 15 and 90 min of exposure to the Mt depolymerizing agent. The transcription rate of the ubiquitin genes in nuclei isolated from cells treated with taxol for 15 min depicts a slight increase compared to that observed in nuclei isolated from cells treated with colchicine. This explains the fact that in taxol treated cells the in-

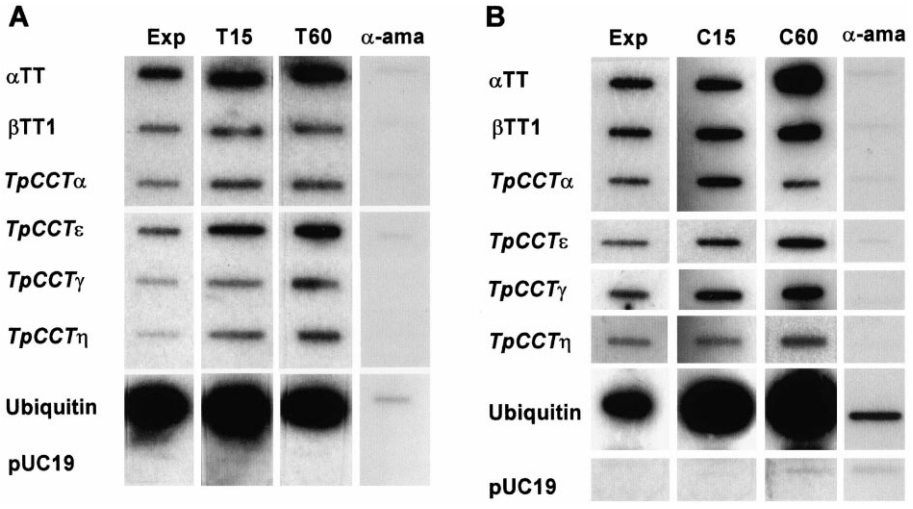

Fig. 5. Tubulin (*αTT* and *βTT1*), *TpCCTα*, *TpCCTε*, *TpCCTγ*, *TpCCTη*, and ubiquitin transcription in *Tetrahymena* cells after 15 and 60 min of treatment with taxol and colchicine. Apparent transcription rates were measured by run-on assay. For each sample an equal number of nuclei (about 5×10^8) was used. Nuclei were isolated from exponentially growing cells (Exp), exponentially growing cells incubated with α-amanitin (20 μg/ml) for 30 min (α-ama) and cells treated with taxol (A) and colchicine (B) for 15 and 60 min (T15, T60 and C15, C60, respectively). Nuclear RNA was extracted and hybridized to DNA previously bound to nitrocellulose filters. Each slot contained 1 μg of DNA fragments containing the *TpCCTα*, *TpCCTε*, *TpCCTγ*, *TpCCTη*, α- and β-tubulin (*αTT* and *βTT1*, respectively) and ubiquitin genes. The data are representative of three different experiments on separate isolations of nuclei that gave identical results. The autoradiograms have been digitally generated and processed as described in Section 2.

crease in the amount of steady-state population of ubiquitin mRNAs is smaller than in colchicine treated cells and is essential owing to the increase of the 1.8 kb mRNA species.

3.5. The steady-state protein levels of CCT subunits and tubulin in cells treated with taxol and colchicine

The effects of taxol and colchicine on transcription led us to assay the steady-state amounts of proteins of different CCT subunits and tubulin in *Tetrahymena*. We have analyzed total and postmitochondrial extracts of *Tetrahymena* exponentially growing cells and cells treated with taxol or colchicine for 15 and 90 min by 10% SDS–PAGE followed by immunoblotting with the set of antibodies produced against distinct CCT subunits and tubulin (see Section 2 and Fig. 2). Interestingly, although all CCT subunit genes studied showed similar patterns of expression in taxol and colchicine treated cells (see Fig. 4) we observed varying changes in the steady-state levels of the respective proteins in the distinct protein fractions. Analysis of total protein extracts shows that the levels of CCTα, CCTε and CCTη subunits and tubulin did not change significantly during taxol treatment (Fig. $6I_A$). In postmitochondrial extracts the amounts of CCTα and CCTη subunits are maintained almost constant in cells treated with taxol. However, in the soluble fraction of these cells there is a dramatic decrease in the levels of CCTε subunit (Fig. $6I_B$), while the levels of the CCT substrate, tubulin, almost disappear in cells treated with taxol for 15 min and subsequently increase to levels almost comparable to that of the control cells at 90 min of treatment (Fig. $6I_B$). In order to explain this apparent discrepancy between gene expression and the steady-state amounts of the CCT subunits and tubulin under taxol treatment, we decided to analyze by Western blot the corresponding pellets obtained during the preparation of the postmitochondrial extracts. Unexpectedly, we observed that CCTα as well as the CCTε subunit were detectable in the insoluble fraction of exponentially growing cells while CCTη was vestigial (Fig. $6I_C$). Moreover, the levels of CCTε were more abundant in insoluble fractions than in soluble fractions in contrast with the other CCT subunits (Fig. $6I_C$). The levels of this subunit clearly increase in the insoluble fraction in cells treated with taxol at the expense of a corresponding decrease in the soluble fraction. CCTα subunit levels in the insoluble fraction are apparently unchanged during taxol treatment. Tubulin was expected to be detected in insoluble fractions in exponentially growing cells since *Tetrahymena* is a ciliate and insoluble fractions contain all the cilia and probably part of the cortex structure. After 15 min of taxol treatment tubulin is only present in the insoluble fraction, probably due to massive polymerization, and can only be detected in the soluble fraction after 90 min of taxol treatment (see Fig. $6I_B$). This last observation can be explained by the up-regulation of tubulin genes and de novo protein synthesis as a compensation response to taxol challenge.

Fig. 6II shows the results obtained with colchicine treated cells when performing experiments similar to those above described for taxol. We observed that in total protein extracts the amounts of CCTα and CCTη subunits and tubulin slightly decrease whereas the levels of CCTε show a small increase. Interestingly, the levels of all these proteins progressively decrease until 90 min of treatment in the soluble fraction (Fig. $6II_B$). In the insoluble fraction the amounts of CCTε increase whereas the amounts CCTα remain almost constant and tubulin levels are declining (Fig. $6II_C$). As in taxol treated cells CCTη is almost undetectable in the insoluble fraction both in control cells and in cells subjected to colchicine (Fig. $6II_C$). Therefore, the depolymerization of Mt arrays by colchicine promotes the decrease of the steady-state populations of CCT subunits and tubulin in soluble fractions. In the case of CCTε subunit this decrease seems to be explained by the shift of this protein from soluble fraction to insoluble fraction. Since CCTα and CCTη subunit genes and tubulin genes are up-regulated in cells treated with colchicine, we hypothesized that the observed decrease in the soluble protein levels was due to an accelerated proteolytic degradation of these proteins. To test this hypothesis we performed experiments where colchicine was added to *Tetrahymena* cultures 15 min after the addition of cycloheximide, an elongation inhibitor of translation. These experiments showed that the half-life of these proteins was not affected by colchicine and it was longer than 90 min (results not shown). A possible explanation for these apparent incongruent observations can be the fact that the factor involved in the degradation of these proteins in cells treated with colchicine is absent due to the blockage of protein synthesis. This idea is reinforced by the fact that the decrease of tubulin in cells treated with colchicine with inhibited protein synthesis is no longer observed at 90 min (results not shown).

The presence of CCT subunits in the insoluble fraction either in exponentially growing cells or in cells treated with taxol and colchicine raises the possibility of contamination from the soluble fraction. We think that this is not the case for two reasons: (i) during preparation of insoluble fractions, pellets were extensively washed in order to avoid contamination; (ii) it is difficult to explain the presence of distinct CCT subunits in these fractions (note that CCTη is practically absent) and also their varying amounts with the physiological state of the cell. By assuming that specific CCT subunits either free or as part of small oligomeric structures can be associated with Mt arrays [18] the presence of CCT subunits in insoluble fractions treated with Mt polymerizing agent could be explained. Detection of CCTε and CCTα subunits in the insoluble fraction could be due to a preferential association of these subunits with Mts that are massively polymerized by taxol. However, this idea is not supported by the studies involving

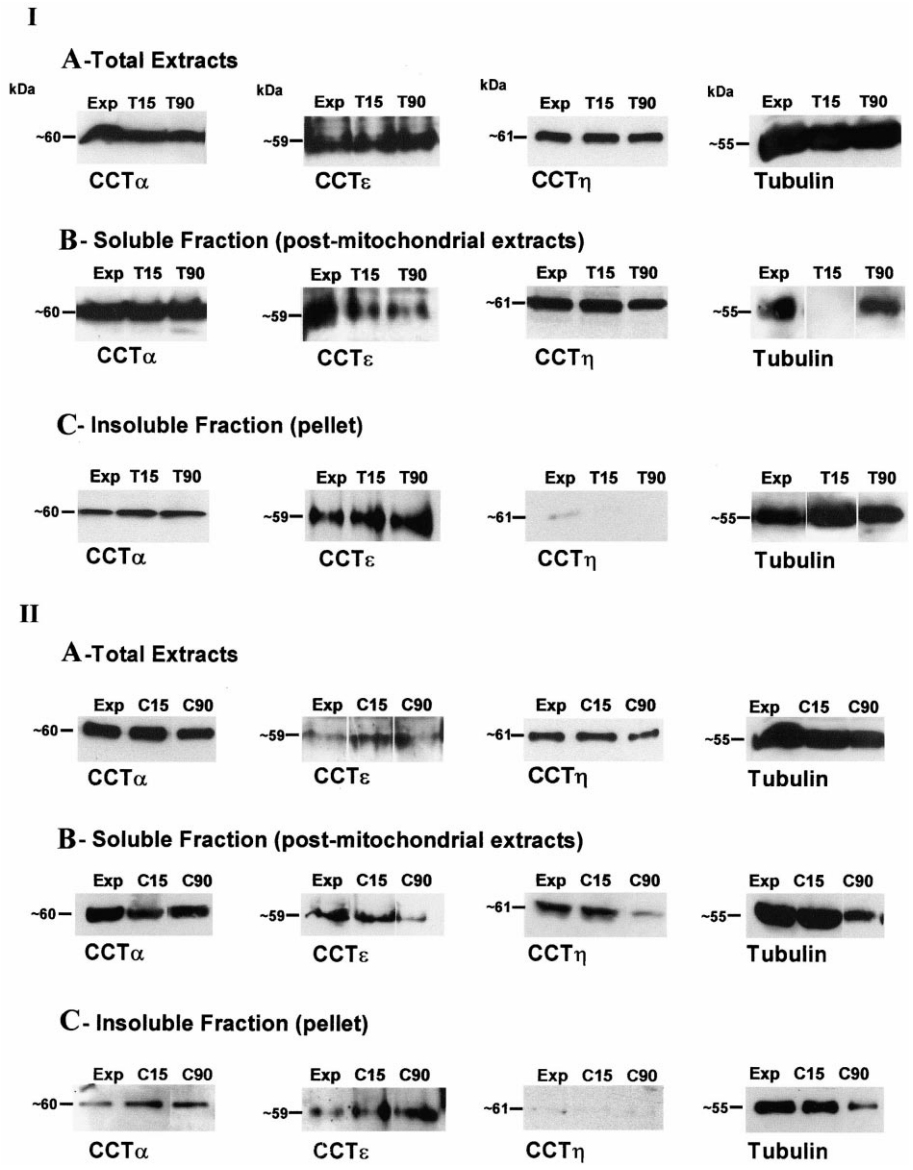

Fig. 6. Analysis of the steady-state levels of *Tetrahymena* CCT subunits and tubulin of the exponentially growing cells and cells treated with taxol and colchicine. Protein extracts obtained from exponentially growing cells (control) (Exp) and cells subjected to taxol (I) and colchicine (II) for 15 min (T15 and C15, respectively) and 90 min (T90 and C90, respectively) were analyzed by 10% (w/v) SDS–polyacrylamide gradient gels and then Western blotted. (I_A,II_A) Total protein extracts; (I_B,II_B) postmitochondrial extracts (soluble fraction); (I_C,II_C) the respective pellets (corresponding to the insoluble fraction). In all panels: CCTα, detection with TpαCterm antibody (dilution 1/1000); CCTε, detection with TpεNterm antibody (dilution 1/750); CCTη, detection with TpηCterm antibody (dilution 1/1000); tubulin, detection with monoclonal antibody against to α-tubulin N356 (see Section 2). The secondary antibody was always used at 1/1000 dilution except in the case of TpεNterm antibody where a 1/750 dilution was used. The approximate molecular mass of the identified proteins is always indicated at the left. The results shown are representative of three independent experiments that gave similar results. The autoradiograms have been digitally generated and processed as described in Section 2.

colchicine. In fact, this Mt depolymerizing agent also induces the preferentially transition of CCTε from the soluble to the insoluble fraction and CCTα subunit is still detected in the insoluble fraction in cells treated with this agent. Alternatively, the presence of CCTα and CCTε in the insoluble fractions could be due to an association of these subunits with components of cortex structure and/or with basal bodies and/or cilia. If this is true, it is possible that there is a preferential association of the CCTε subunit with these structures when dramatic alterations on Mt structures occur due to accentuated polymerization or depolymerization.

4. Discussion

We have previously shown that four members of the CCT subunit family genes of *Tetrahymena* [21–24] are

conserved from protozoan to metazoan [1]. The present work reports the identification and characterization of the fifth member of this family, TpCCTε. Comparison of the predicted amino acid sequence of TpCCTε with the other known CCTε subunits from other organisms revealed that besides the usual characteristic motifs of almost all the chaperonins of group I [1] and members of the CCT/TF55 proteins, it contains five new, highly conserved regions that seem to be exclusively shared by CCTε subunits (see Fig. 1). It is likely that these sequences could be involved in a specific function of the CCTε subunit in the chaperonin complex. In fact, it has recently been shown that the small domain of α-actin interacts with CCTδ and that the large domain interacts with CCTβ or CCTε, indicating that the binding of this substrate to CCTs is subunit-specific [2,38]. Moreover, tubulin interacts with CCT using five specific CCT subunits with the strong interactions being through either CCTε or CCTβ [39].

This study focuses on the effects of taxol and colchicine on Mt arrays and on the regulation of the expression of CCT subunit genes. Our data show that taxol induced polymerization and bundling of *Tetrahymena* Mt arrays preferentially localized around the macronucleus (Fig. 3), whereas colchicine clearly induces the depolymerization of cytoplasmic Mt networks and also affects the cortex Mts. Colchicine provokes a similar pattern of expression of tubulin and CCT subunit genes and does not affects actin expression. On the other hand, taxol induces tubulin but not actin transcripts to progressively increase to levels much higher than those found in control cells. Interestingly, at the same time the mRNA levels of the CCT subunit genes decrease in cytoplasm between 15 and 30 min and then rise to levels higher than those found in untreated cells. The apparent rate of transcription of the tubulin and CCT subunit genes is up-regulated in cells treated with colchicine and taxol. However, the pattern of induction is not exactly the same in both cases (see Fig. 5). The results taken together suggest a different type of regulation mechanism of CCT subunit gene expression between cells treated with Mt polymerizing and Mt depolymerizing agents. Besides this, it is relevant to note that CCT subunit gene expression is modified by perturbations in Mts arrays caused either by dramatic depolymerization or polymerization, suggesting a complex mechanism of regulation between these genes and Mt cytoskeleton status.

The transcription studies also show that the decrease of CCT mRNA levels in cytoplasm between 15 and 30 min in cells subjected either to taxol or colchicine, and the decrease of tubulin mRNA amounts in cells treated with colchicine cannot be transcriptional and may be a result of a posttranscriptional regulation at the level of mRNA stability. Both antimitotic drugs increase transcription of α- and β-tubulin presumably to replenish the cytoplasmic soluble pool of functional tubulin [27]. The up-regulation of CCT subunit genes has been linked to the increased synthesis of tubulin, [4,21,22,40] and is probably required to chaperone the folding of the newly synthesized tubulin pool. However, the increase of transcription of CCTs does not correlate with a corresponding increase in the steady-state levels of these proteins (see Fig. 6). Therefore, the up-regulation of CCT genes is probably required to replete the physiological levels of the CCT mRNA in cytoplasm since, as explained earlier, taxol and colchicine induce CCT mRNA degradation in cytoplasm. Although the regulatory mechanisms of CCT subunits and tubulin mRNAs stability are different and largely unknown, it is tempting to suggest that they could be related with the integrity and dynamics of Mt cytoskeleton. Several lines of evidence indicate that cytoskeleton is important for translation since translational components including ribosomes and initiation factors are physically associated with it [41,42]. In many instances translation has a direct role in determining mRNA stability as mRNA degradation may be coupled with translation [26,42].

The detection of TpCCTα, TpCCTε but not TpCCTη in the insoluble protein fraction in exponentially growing cells is a striking observation (see Fig. 6B). These results are consistent with the idea that some CCT subunits exist as free subunits or as part of oligomeric particles associated with Mt arrays and/or with MTOCs (microtubule-organizing centers). Indeed CCTα, as well as CCTζ, have been described as part of mammalian centrosomes [18,19]. Recently, Roobol et al. [19] have shown that CCTα, γ, ζ and θ but not CCTε were able to associate with Mt polymerized in vitro by taxol. CCTα subunit was detected in sea urchin embryonic and rabbit tracheal cilia [43] and progressively appeared in regenerating embryonic cilia as their growth slowed down, suggesting a regulatory role correlated with growth or turnover. In our in vivo experiments CCTα and CCTε subunits appear in the insoluble fraction that contains parts of the cortex structure, including basal bodies and cilia, indicating a putative association of these subunits with the mentioned Mt structures. Unexpectedly, the CCTε association is stimulated by either taxol or colchicine, suggesting that it is not a simple consequence of association with Mts due to a massive polymerization. Instead this association may be related with the damage of Mt organization/dynamics in the Mt containing structures present in the insoluble fraction. Our data contribute to strengthen the hypothesis that CCT subunits free or as part of oligomeric complexes could have distinct additional roles in vivo since they are differentially detected associated with structures where protein synthesis is not supposed to occur and consequently protein folding is not expected. Studies are in progress in order to identify whether TpCCTε binds to the microtubule structures or some other non-soluble organelle(s) present in the insoluble fraction.

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