Cloning of two β-tubulin related genes in *Tetrahymena pyriformis*

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Genomic clones containing β-tubulin sequences were isolated from a pBR322 genomic library of *Tetrahymena pyriformis*. Each clone has been mapped and the identity of the tubulin-related genes has been established by cross-hybridization with a cDNA containing a complete β-tubulin coding region from *Chlamydomonas* and by the positive hybrid-selected translation assay. The respective tubulin-related genes, βTT_1 and βTT_2, differ from each other although they contain a homologous region which is also homologous with a heterologous probe. *Tetrahymena* β-tubulin-related genes are physically linked and clustered in the genome.

β-Tubulin *Gene organization* Tetrahymena pyriformis

1. INTRODUCTION

Tubulins are ubiquitous proteins found in all eukaryotic cells as constituents of microtubules particularly those of the cytoskeleton, meiotic and mitotic spindles, cilia and flagella. In the microtubules so far examined these proteins are assembled in stoichiometric amounts as heterodimers of α- and β-tubulin subunits. However, neither of these 2 types of subunits is homogenous, rather they are constituted from several isoforms as revealed by isoelectric focusing techniques [1–3]. This heterogeneity can be detected at 2 main levels. At one level different tissues or cell types may express different tubulins [4], and at another, different organelles may be assembled from different tubulins [5,6]. For instance, in the unicellular algae *Polytomella* and *Chlamydomonas* differences have been observed between the tubulins of the cytoplasmic microtubules and the flagellar microtubules, 2 organelles within the same cell type [7–9]. In the protozoan *Tetrahymena pyriformis* a microheterogeneity of α- and β-tubulins has also been detected [10] which can be due to the existence of multiple genes. In fact the presence of multiple tubulin genes has been demonstrated in *Chlamydomonas* [11], *Trypanosoma brucei* [12], *Drosophila* [13] and vertebrates [14]. The present work is specifically aimed at studying the β-tubulin-related genes in *T. pyriformis*. The high degree of conservation of these proteins in eukaryotic cells permitted us to use the cDNA of β-tubulin from *Chlamydomonas* as a probe for the isolation of 2 β-tubulin-related genes in *T. pyriformis*. We show that the 2 β-tubulin genes in this organism are organized as a cluster.

2. MATERIALS AND METHODS

2.1. *Strains and cultures*

T. pyriformis strain CGL (amicronucleate) was grown axenically at 28°C in PPY medium [15]. Cells were harvested for DNA extraction in the stationary phase at a density of 1.5×10^6 cells/ml.

For RNA extraction, cells were grown in a medium described before [16] and collected in the exponential phase at 5×10^4 cells/ml.

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Escherichia coli strains used were the highly transformable strain MC1061 and HB101 [17].

2.2. *DNA extraction and RNA preparation*

T. pyriformis cells were chilled and harvested by centrifugation, lysed and digested with proteinase K essentially as described by Karrer and Gall [18]. After 2 extractions with a mixture of phenol and chloroform (50:50) DNA was centrifuged to equilibrium in a cesium chloride density gradient, and dialysed against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. Plasmid DNA from *E. coli* was prepared by the rapid boiling method of Holmes and Quigley [19].

Total *T. pyriformis* RNA was prepared from exponentially growing cells as described [20].

2.3. *Nick-translation*

Nick-translated DNA probes (spec. act. 0.2–1 × 10^9 cpm per μg) were prepared as described by Rigby et al. [21].

2.4. *Restriction and hybridization analysis*

Enzymes were purchased from Biolabs, BRL and Boehringer and used according to the procedures recommended by the suppliers. When genomic DNA was used, incubation was done overnight at 37°C. After endonuclease digestions DNA fragments separated by agarose gel electrophoresis were isolated either by electroelution or by transfer to nitrocellulose [22] and hybridized to probes (0.5–1 × 10^6 cpm).

Prehybridizations and hybridizations were performed either at 50°C (non-stringent) in the following hybridization buffer 3 × SSC, 1 × Denhardt solution [23], 0.1% SDS, 100 μg/ml tRNA and 20 mM sodium phosphate (pH 6.5) or at 70°C (stringent) with a lower saline concentration 2 × SSC in the same buffer, for 18–48 h. After hybridization the filters were washed by several changes of 2 × SSC for 2 h at the respective hybridization temperature and finally by 4 changes of 0.2 × SSC, 0.1% SDS for 1 h at room temperature. Blots were exposed at −70°C with intensifying screens.

2.5. *Construction of a genomic library*

25 μg of supercoiled pBR322 DNA was linearised with endonuclease *Hin*dIII, phenol extracted and precipitated with ethanol. The pellet was dissolved in 10 mM Tris-HCl (pH 8.0) and 40 units of bacterial alkaline phosphatase was added. After 2 h incubation at 60°C the enzyme was removed by phenol/chloroform and the DNA was precipitated with ethanol. 1 μg *Hin*dIII digested *T. pyriformis* DNA was ligated to 5 μg pBR322 for 2 h at room temperature. The recombinant DNA was used to transform *E. coli* MC1061 using the $CaCl_2$ technique [24]. Colony hybridization was carried out by the method of Grunstein and Hogness [25] with the following modifications: prehybridization and hybridization was done at 60°C in 6 × SSC, 5 × Denhardt solution, 0.1% SDS, 50 μg/l tRNA, 50 μg/ml *E. coli* DNA and 20 mM sodium phosphate, pH 6.5. After washing, the positive clones were detected by autoradiography using Dupont cronex 4 films and intensifying screens.

2.6. *Positive hybrid selection of β -tubulin mRNA*

2 μg plasmid DNA was denatured, loaded onto nitrocellulose filters and baked for 2 h at 80°C. The filters were washed with a solution of 10 mM Tris-HCl (pH 7.5), 0.3 M NaCl, 2 mM EDTA and 0.1% SDS. Hybridization was done overnight at 37°C with 2 mg poly(A)$^+$ RNA for 10 filters in 50% formamide, 0.9 M NaCl, 0.2% SDS, 1 mM EDTA, 20 mM Pipes, pH 7.5. The filters were washed at 60°C with 5 changes of 1 × SSC, 0.1% SDS and once with 5 mM Tris-HCl (pH 7.5), 1 mM EDTA. RNA eluted from filters was translated in vitro using the RNA-dependent rabbit reticulocyte lysate system (Amersham), and [^{35}S]methionine (Amersham, 800–1200 Ci/mmol), as outlined by the manufacturers. The products were immunoprecipitated as described in [26] using 2 preparations of *T. pyriformis* anti-tubulin IgG, one prepared by us and the other kindly provided by Dr Seifert.

The proteins were analysed by SDS-urea-polyacrylamide gel [27] and fluorographed using Amplify (Amersham) according to the supplier's instructions.

3. RESULTS AND DISCUSSION

The fact that tubulins are highly conserved in all eukaryotic cells implies that the corresponding coding regions show homology between different species. We have therefore used the pcf 8-13 cDNA clone of *Chlamydomonas* [28] as a probe to search

for the tubulin genes in *T. pyriformis*. Fragments obtained by *Hin*dIII, *Eco*RI and *Bam*HI digestion of *Tetrahymena* DNA were separated electrophoretically in agarose gels, transferred to nitrocellulose and hybridized under non-stringent conditions with ^{32}P-labelled *Chlamydomonas* β-tubulin cDNA. The results thus obtained are shown in fig.1. Hybridization can be observed with 2 bands of 3 and 5 kb, in the digests obtained with *Hin*dIII and *Eco*RI. However, in a *Bam*HI-digest only 1 band of 13 kb is detected.

These results gave us the first indications that *Tetrahymena* might contain at least 2 β-tubulin-related genes which were probably located very close to each other.

To clone *Tetrahymena* β-tubulin genes, a genomic library containing *Tetrahymena Hin*dIII fragments inserted into the plasmid pBR322 was constructed and 50000 clones were screened with the *Chlamydomonas* cDNA probe. We isolated the plasmids IB_1 and IB_2, which were found to contain inserts of 3 and 5 kb, respectively. The sizes of these inserts correspond to those of the fragments detected when genomic *Tetrahymena* DNA digested with *Hin*dIII was probed with the heterologous β-tubulin cDNA (fig.1). Restriction endonuclease maps of these fragments, which we named βTT_1 and βTT_2 are shown in fig.2. As can be seen, the 2 fragments are different although the relative positions of the 2 *Eco*RI sites, the *Cla*I site to the right and the *Hha*I site between them are identical. The Northern hybridization technique has revealed that both fragments show sequence homology with mRNA of about 1800 nucleotides [29].

Restriction endonuclease mapping and blot hybridization were used to locate regions of the homology between βTT_1 and βTT_2 and also those between these 2 fragments and the pcf 8-31 tubulin cDNA of *Chlamydomonas*. When the βTT_1 is digested by the restriction enzymes shown in fig.2 and hybridized under stringent conditions with ^{32}P-labelled βTT_2, a homologous region of 1.7 kb length is found between the *Eco*RI and *Hha*I sites. Likewise, if the labelled βTT_1 is hybridized with digested βTT_2 (see fig.2), a homology region of 1.5 kb length situated to the right of the *Eco*RI site is detected.

The same kind of experiments were carried out using the pcf 8-31 cDNA from *Chlamydomonas* as a probe. In βTT_1 a homologous sequence of 1.05 kb is located between *Eco*RI and *Hae*III sites which overlaps with the sequence identified using

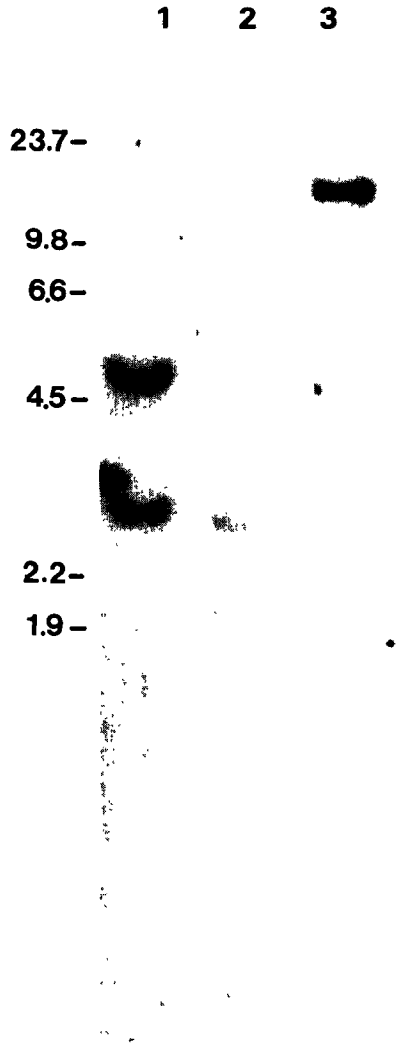

Fig.1. Southern blot hybridizations of genomic *Tetrahymena* DNA probed with a tubulin cDNA clone of *Chlamydomonas*. *T. pyriformis* DNA (10 μg) was digested by various restriction enzymes, fractionated on a 1% agarose gel and electrophoresed overnight at 50 V in 40 mM Tris-acetate buffer (pH 8.0). The DNA was denatured and blotted onto nitrocellulose. Hybridization was done at low stringent conditions (50°C and 3 × SSC) with nick-translated pcf 8-31 β-tubulin cDNA of *Chlamydomonas* [28]. Restriction enzyme digestions: lane 1, *Hin*dIII; lane 2, *Eco*RI; lane 3, *Bam*HI. The position of molecular mass markers indicated in kb in the margins was determined by running a mixture of *Hin*dIII fragments of λ DNA.

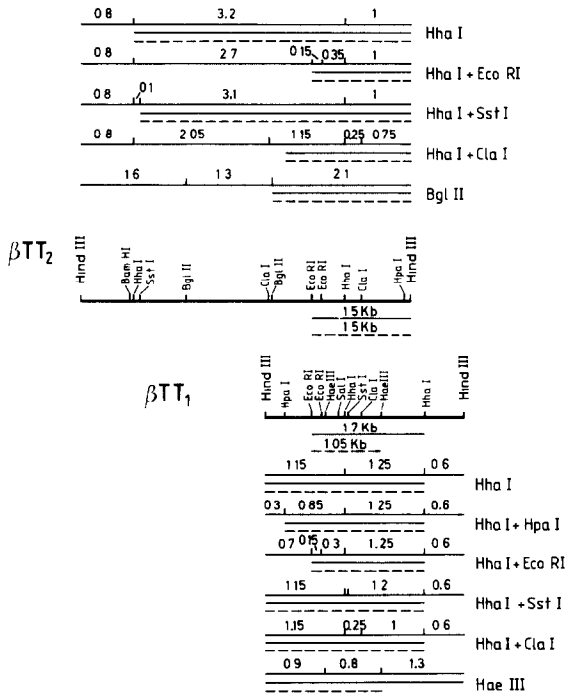

Fig.2. Restriction maps of the β-tubulin sequences βTT_1 and βTT_2. The electroeluted βTT_2 fragment of IB_2 (2 μg) and the electroeluted βTT_1 fragment of IB_1 (2 μg) were digested with several restriction enzymes, fractionated on a 2% agarose gel and transferred to nitrocellulose. The filters were hybridized at 60°C in 3 × SSC buffer with a nick-translated pcf 8-31 cDNA and at 70°C in 2 × SSC buffer with either βTT_1 fragment of IB_1 or βTT_2 fragment of IB_2. The restriction maps obtained with the different enzymes are shown. Underlining indicates which of the fragments hybridized with the labelled pcf 8-31 cDNA (– – –) and the labelled βTT_2 of IB_2 or βTT_1 of IB_1, respectively (——). A composite restriction map of each of the fragments including the regions of homology with the 2 probes is also shown.

the βTT_2 probe. The common region in βTT_2 is the same whichever probe (*β-Chlamydomonas* or *Tetrahymena*) is used (fig.2).

The presumed tubulin genes were then characterized by a positive hybrid-selected translation assay. Purified DNA from recombinant plasmid IB_1 or IB_2 was bound to nitrocellulose filters and hybridized with cytoplasmic poly(A)$^+$ RNA. The RNAs eluted from the hybrids were then translated in vitro and the products were immunoprecipitated using tubulin antibodies. After immunoprecipitation the labelled polypeptides were analysed by urea-SDS-PAGE. As fig.3 shows, only 1 band corresponding to a protein having an M_r of 52000 is seen with mRNA selected by both IB_1 and IB_2. We can therefore conclude that these 2 clones contain tubulin-related sequences.

The genomic organization of βTT_1 and βTT_2 was studied by Southern blot hybridization. When genomic DNA is digested with *Bam*HI only one band fragment of 13 kb is found to hybridize with both the βTT_1 and βTT_2 probes (fig.4a,b, lanes 1). This suggests that either the 2 fragments βTT_1 and βTT_2 are located on 2 different *Bam*HI fragments of the same size or belong to the same fragment. These 2 possibilities can be distinguished using a restriction enzyme that cuts within βTT_1 and βTT_2, for instance *Eco*RI. If βTT_1 and βTT_2 are located on 2 different fragments, at least 4 bands would have been expected. These 4 bands should be of the

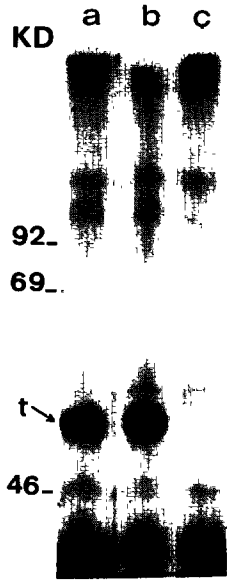

Fig.3. In vitro translation of RNA obtained by positive hybridization-selection and immunoprecipitation of translation products. 2 μg plasmids DNA (IB_1 and IB_2) were denatured, immobilized on nitrocellulose filters, and used in hybrid-selection of mRNA (see section 2). Lanes a and b show the immunoprecipitated translation products from RNA selected by hybridization to IB_1 and IB_2, respectively. Lane c represents the immunoprecipitated endogenous products of the translation system made in absence of added RNA. The molecular mass markers are on the left.

same size with each of the 2 probes since *Eco*RI cleaves within the homologous region. In contrast, if βTT_1 and βTT_2 are located on the same fragment and not separated by an *Eco*RI site only 3 bands are expected to occur. As can be seen in fig.4b (lane 3) where βTT_2 is used as a probe, 3 bands (3, 4 and 5 kb) are visible. However, in fig.4a (lane 3), where βTT_1 is used as a probe, only 2 bands are detected. These results not only demonstrate that fragments βTT_1 and βTT_2 are in the same 13 kb *Bam*HI fragment but also enable us to orientate them in this fragment (fig.4c). The fact that only 2 bands appear with the βTT_1 probe can only be explained if the βTT_2 is located on the left part of the 13 kb *Bam*HI genomic fragment. When *Bam*HI and *Eco*RI (fig.4a,b, lanes 2) are combined, the overlapping *Eco*RI fragment (5 kb) is still detected by both probes. The same is true for the 3 kb fragment which corresponds to βTT_1. On the other hand, the 4 kb fragment specific for βTT_2 is replaced by 2 fragments of 2.8 and 1.2 kb, respectively (fig.4b, lane 2). These results confirm the orientation of the βTT_1 with respect to βTT_2 on the genomic DNA. The conclusion that the 2 cloned fragments βTT_1 and βTT_2 are organized as presented in fig.4c was further substantiated by additional experiments using *Sst*I, *Hpa*I and *Hha*I cleavage (not shown). Both fragments are therefore physically linked and clustered in the genome in an arrangement similar to that found in the parasitic protozoan *Leishmania enrietti* [30].

To establish the true identity of these fragments and also to detect regulatory sequences up- and downstream of the coding region, their sequencing is being undertaken.

Moreover, these cloned fragments should enable us to study the regulation of the β-tubulin-related genes during cell division, deciliation and reciliation. It is possible that each of these genes may be involved in controlling the assembly of the functionally specialized microtubules of either the cilia or the cytoskeleton.

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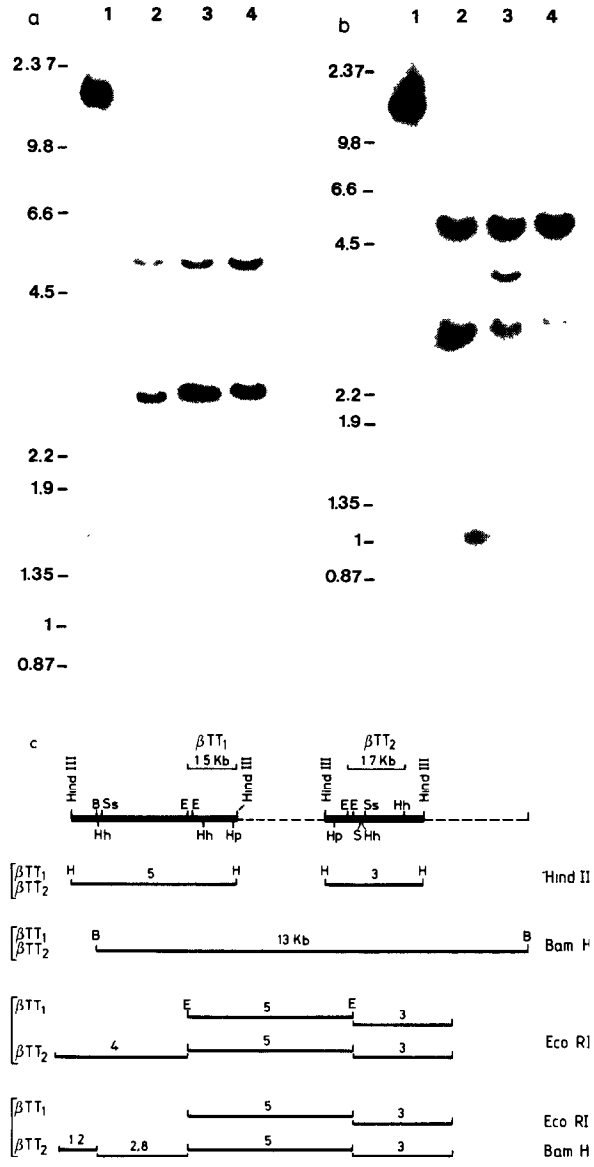

Fig.4. Southern blot hybridization of genomic *Tetrahymena* DNA probed with βTT_1 and βTT_2 fragments. Genomic DNA of *T. pyriformis* (10 μg) was digested by various restriction enzymes, fractionated on a 0.8% agarose gel and blotted onto nitrocellulose paper. Hybridization was done at highly stringent conditions (70°C and 2 × SSC with either ^{32}P-βTT_1 (A) or ^{32}P-βTT_2 fragments (B)). Restriction enzyme digestions: lanes 1, *Bam*HI; lanes 2, *Bam*HI plus *Eco*RI; lanes 3, *Eco*RI; lanes 4, *Hin*dIII. Panel C shows the genomic organization deduced from the results depicted in A and B. B, *Bam*HI; Hh, *Hha*I; Ss, *Sst*I; E, *Eco*RI; Hp, *Hpa*I.

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