Site of tubulin synthesis in *Tetrahymena pyriformis*

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Free and membrane-bound polysomes were isolated from the protozoa *Tetrahymena pyriformis*, and the contribution of these two types of polysomes to tubulin synthesis were studied using immunoprecipitation of the ^{35}S-translational products in a rabbit reticulocyte lysate. One-dimensional electrophoretic analysis shows that tubulin is synthesized by polyadenylated RNA isolated from free and membrane-bound polysomes. Non-polyadenylated RNAs of free polysomes are also able to direct tubulin synthesis. Two-dimensional electrophoretic analysis using O'Farrell's system confirms these results and also reveals the existence of the α- and β-tubulin subunits.

Membrane-bound and free polysomes *In vitro protein synthesis* *Immunoprecipitation* *Tubulin*

1. INTRODUCTION

Alpha(α)- and beta(β)-tubulins, the major components of microtubules, are found in all eukaryotic cells, and play important structural and functional roles in mitosis, cell shaping, secretion and motility [1–3]. In brain, for instance, tubulins represent roughly 1/4 of the soluble proteins [4,5]. Microtubules differ in their stability under physiological conditions and in their response to physical or chemical treatment [6] but the mechanisms which underlie these differences are unknown. They may be due either to the existence of different tubulins or interaction with different components of essentially the same tubulin molecules [7]. The heterogeneity of α- and β-tubulins [8–10] may be due to the existence of several tubulin genes [11,12] or to post-translational modification processes [13]. We have previously reported that free and membrane-bound polyribosomes from *Tetrahymena pyriformis* contain different populations of poly(A)-containing RNA as determined by their expression in a rabbit reticulocyte lysate cell-free system [14]. Here we are concerned with identifying which of the two populations of polyribosomes synthesizes tubulin, or whether both do. We present evidence that poly(A)-rich RNA directing the synthesis of tubulin is present in both free and membrane-bound polysomes. In addition we show that non-polyadenylated RNA isolated from free, but not from membrane-bound polysomes contains tubulin mRNAs.

2. MATERIALS AND METHODS

2.1. *Cell culture conditions*

T. pyriformis strain CGL (amicronucleate) was grown axenically at 28°C as in [15]. For RNA extraction, cells were collected in the exponential phase at 0.5×10^5 cells/ml. Cells used for tubulin preparation were grown in PPY medium [16] and were harvested in the stationary phase at a density of 1.5×10^6 cells/ml.

2.2. *Cell fractionation and RNA preparation*

Free and membrane-bound polyribosomes were isolated as in [14]. Total RNA was extracted from these populations of polysomes by the dodecylsulphate/phenol procedure at pH 9.0 [14]. Poly(A)$^+$ RNA was separated from total RNA by affinity chromatography on oligo(dT)-cellulose Type T_3 columns and according to the procedure

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outlined by the manufacturers (Collaborative Research Inc.).

2.3. *Preparation of tubulins*

Cells were deciliated by the ethanol/calcium procedure in [17] and tubulin was isolated from an acetone powder of cilia as in [18]. ^{35}S-Labelled tubulin was prepared by the same method as for *Tetrahymena* grown in PPY medium supplemented with 0.5 μCi/ml of L-[^{35}S]methionine (Amersham).

2.4. *Preparation of antitubulin antiserum*

Tubulin prepared as described above and tested for purity by two-dimensional electrophoresis was used for the preparation of specific antibodies.

Rabbits (2.5 kg) were injected intramuscularly with 0.2 mg of tubulin in Freund's incomplete adjuvant administered at intervals of 2 weeks for a period of 3 months. Using this procedure tubulin did not cause any encephalitogenic response in the rabbits. The antibody titre of sera obtained seven days after the third immunization was determined by immunodiffusion. The rabbits were given 3 further injections and sera were recovered one week after the last immunization. The immunodiffusion test at that time gave a titre of 1:64.

IgG was purified by precipitation with 40% sat. $(NH_4)_2SO_4$. The mixture was allowed to stand overnight at 4°C. IgG were pelleted, washed twice with 2.1 M $(NH_4)_2SO_4$, dissolved in PBSA (3.35 mM KCl, 1.83 mM PO_4KH_2, 17 mM NaCl, 4 mM PO_4HNa_2) and dialysed overnight against the same buffer at 4°C. Using this procedure we obtained a partially purified anti-tubulin IgG which was stored at −20°C. The specificity of the antiserum was tested as in [19].

2.5. *Translation in vitro of poly(A)-rich RNA*

The rabbit reticulocyte lysate in vitro translation system was used as indicated by the manufacturers (Amersham) in the presence of 50 μg/ml of free and membrane-bound poly(A)-rich RNA. When immunoprecipitation was used, 100 μl of NET buffer (10 mM Tris–HCl (pH 7.5), 0.1 mM NaCl, 1 mM EDTA) and 5 μl of anti-tubulin IgG was added to 10 μl of incubation mixture. The material was left at room temperature for 2 h and at 4°C overnight; 15 μl of protein A–Sepharose (Pharmacia) were then added and the samples vigorously shaken at room temperature for 2 h. The complex formed between protein A–Sepharose and the antigen–antibody complex was pelleted by centrifugation and washed 4-times with 1 ml of NET buffer and once again with 100 μl of the same buffer. The pellet was then resuspended in SDS sample buffer. After heating at 90°C for 2 min, protein A–Sepharose was centrifuged down and the supernatant containing the antigen used for gel electrophoresis.

2.6. *SDS gel electrophoresis and fluorography*

Total translation products and immunoprecipitates were analysed by electrophoresis in one and two dimensions polyacrylamide gels containing SDS followed by fluorography as in [14].

3. RESULTS AND DISCUSSION

Antiserum against *Tetrahymena pyriformis* tubulins isolated from cilia was produced in rabbits as described in section 2. The specificity of the antibody prepared against tubulins was first confirmed using an agar double-immunodiffusion test as shown in fig.1. Only one precipitation line was

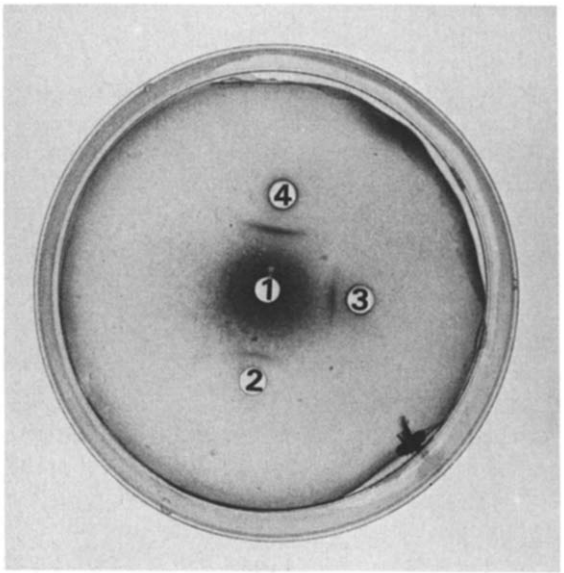

Fig.1. Double-immunodiffusion test of tubulin: 1% agar gel in 1 mM Tris–HCl (pH 7.7) and 0.02% NaN_3. Well 1: partially purified antiserum prepared as in section 2. Wells 2,3 and 4: 5, 10 and 20 μg tubulin, respectively. Immunodiffusion was performed at 4°C for 3 days.

formed against purified *T. pyriformis* tubulins. However, as the work described below involved specific tubulin immunoprecipitation, it was important to check the true specificity of the antiserum with respect to tubulins. The partially purified antibody was, therefore, tested according to the nitrocellulose transfer method in [19]. Fig.2 shows that the serum reacts strongly only with the tubulins from axoneme (lane a) and cortices (lane b) of *Paramecium*. The reaction is comparable to the one obtained when antiserum against *Paramecium* ciliary tubulin (lanes c and d) is used. Tubulin from *Tetrahymena* cilia reacted specifically with the anti-*Tetrahymena* tubulin IgG and also with antiserum against *Paramecia* ciliary tubulins (not shown). It should, however, be stressed that, in the conditions used, the α- and β-subunits migrate together and consequently the antiserum contains both antibodies (note that in fig.2 the band is very intense and large).

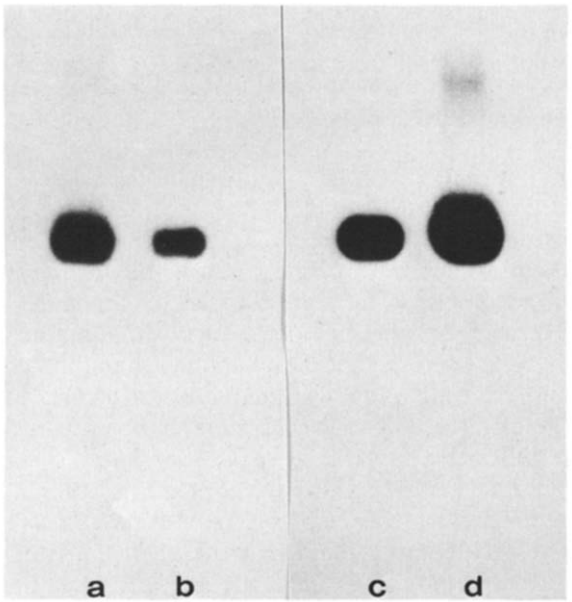

Fig.2. Specificity of anti-tubulin serum tested against *Paramecium tetraurelia* proteins. *P. tetraurelia* proteins separated by 1-D electrophoresis were transferred to nitrocellulose filters and reacted with serum against *Tetrahymena pyriformis* tubulins (1:100) followed by [^{125}I]protein A (10^5 cpm/ml). Lane a, axoneme proteins; lane b, cortical proteins; lanes c and d the same as a and b but treated with *Paramecium* antitubulin serum (1:200) as a control.

In order to find out where tubulin is synthesized in the cytoplasm, it was necessary to separate free and membrane-bound polysomes from which polyadenylated and non-polyadenylated RNAs were extracted [14]. These RNAs were then translated in a rabbit reticulocyte lysate and the [^{35}S]methionine-labelled translation products were either analysed directly or after immunoprecipitation as described in section 2. The fluorograms are shown in fig.3 where 1 intense band can be detected in all lanes, migrating like [^{35}S]tubulin used as marker (lane a). After immunoprecipitation (lanes f–j) this band disappears in the absence of

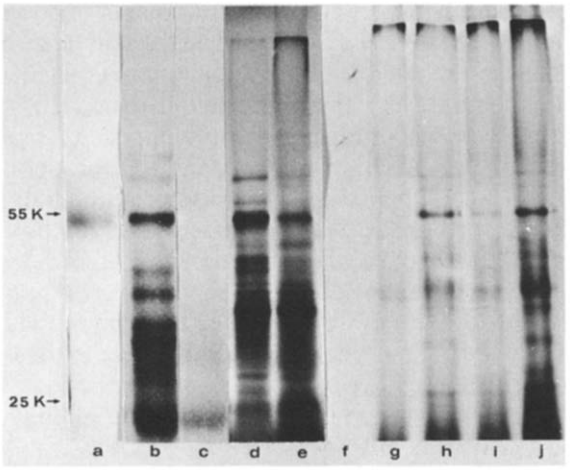

Fig.3. Fluorogram of [^{35}S]methionine-labelled polypeptides resulting from the translation in the rabbit reticulocyte system RNA from membrane-bound and free polysomes of *Tetrahymena pyriformis* (lanes a–c) and their immunoprecipitated products with antitubulin antibodies (lanes f–j): (a) [^{35}S]tubulin isolated as described in section 2 and used as a marker; (b) 10 μg of poly(A)$^-$ RNA isolated from free polysomes (200000 cpm); (c) the same amount as in lane b, isolated from membrane-bound polysomes (200000 cpm); (d) 0.5 μg of poly(A)$^+$ RNA from free polysomes (300000 cpm); (e) as in lane d, but isolated from membrane-bound polysomes; (f) absence of RNA; (g) translation products directed by poly(A)$^-$ RNA from membrane-bound polysomes (see lane c for comparison); (h) as in lane g, but using poly(A)$^-$ RNA from free polysomes (see lane b); (i) in the presence of poly(A)$^+$ RNA from membrane-bound polysomes (see lane e for comparison); (j) poly(A)$^+$ RNA from free polysomes (see lane d for comparison). Arrows indicate the migration of proteins at M_r 55000 (tubulin) and M_r 25000 (globin) used as markers.

RNA (lane f). The translational products directed by non-polyadenylated RNA from membrane-bound polysomes (lane g) and from free polysomes (lane h) are immunoprecipitated only in the latter case, forming a band which migrates like [^{35}S]tubulin marker (lane a). However, when polyadenylated RNAs from both populations of polysomes are used (lanes i,j), the in vitro synthesized tubulin is immunoprecipitated, although in the case of polyadenylated RNA from membrane-bound polysomes the band is slightly less intense. In the lower part of the gel, some (non-individualised) blurred bands can be seen which may correspond either to unfinished tubulin chains or to non-specific precipitation not removed during washing after reaction with protein A–Sepharose. However, the band corresponding to tubulin is very sharp and well separated from the lower-M_r products. For comparison, fig.3 (lanes b–e) shows the analysis of non-immunoprecipitated translational products using the same RNA samples. The differences found between lanes b and c could be interpreted as meaning that poly(A)-free RNA isolated from membrane-bound polysomes codes for small polypeptides which are not detected in the electrophoresis conditions we used. Thus, as we have already suggested before [14], it seems that poly(A) is involved in the attachment of mRNA to the membranes.

These results show that, like actin [20], tubulin is coded by non-polyadenylated as well as polyadenylated RNA, but in this case only by non-polyadenylated RNA isolated from free polysomes.

Two-dimensional electrophoretic analysis by the system in [21] of the immunoprecipitated tubulins was used for two reasons: to confirm the previous results, and because one-dimensional electrophoresis does not allow the separation of α- and β-tubulin. In this system tubulins used as markers are separated in α- and β-subunits (not shown). The fluorograms in fig.4 show the immunoprecipitation of ^{35}S-labelled translational products directed by polyadenylated RNA isolated from free polysomes (A) and from membrane-bound polysomes (B).

In all fluorograms, we have always detected two spots, one more intense which migrates like α-tubulin and the other less intense corresponding to β-tubulin. These results confirm the ones obtained

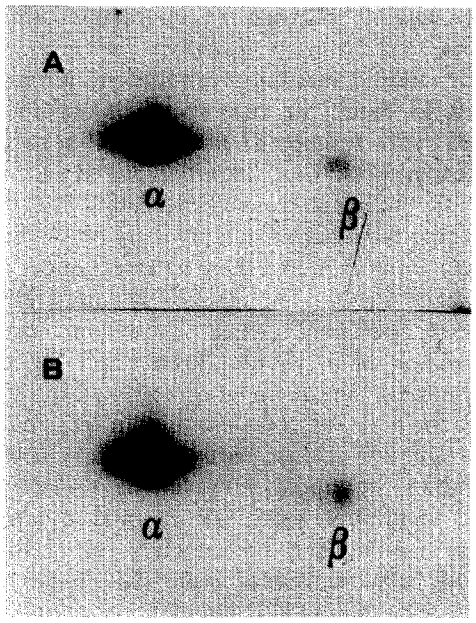

Fig.4. Fluorogram of the two-dimensional electrophoretic analysis of immunoprecipitated tubulin synthesized in a rabbit reticulocyte system: (A) directed by poly(A)$^+$ RNA from free polysomes; (B) directed by poly(A)$^+$ RNA from membrane-bound polysomes. The blanks done without RNA are not included because no spots were detected.

by one-dimensional electrophoresis (see fig.3). It is worth noting that in fig.4A and B the unspecific products seen in fig.3 are completely eliminated.

In contrast to results obtained by other authors [9] our data show that the α-tubulin subunit is synthesized in vitro in larger amounts than β-tubulin. The differences found can probably be explained by the fact that here, we have used specific immunoprecipitation and have consequently eliminated other proteins comigrating with tubulin. We must also bear in mind that there could be different amounts of α- and β-tubulin–mRNA in the cell, and that their expression in vitro could lead to the formation of quantities of α- and β-tubulins differing from those found in cilia in which they occur in equal amounts [9]; our results are indeed similar to those obtained for cultured fibroblasts [22].

The existence of different tubulin populations can result from several tubulin genes, post-transcriptional regulation or post-translational

modifications. The fact that *Tetrahymena pyriformis* tubulins are synthesized on membrane-bound polysomes may suggest that these molecules are posttranslationally modified by, for instance, glycosylation. Further experiments are aimed at clarifying this point. Molecular cloning now in progress will allow us to detect the existence of different tubulin genes.

Finally, we would like to put forward the hypothesis that the existence of distinct sites of tubulin synthesis in this protozoan may correspond to different tubulin populations in different microtubules performing specialized functions in the cell.

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REFERENCES

[1] Stephens, R.E. (1971) in: Biological Macromolecules (Timasheff, S.N. and Fasman, G.D. eds) vol.5, pp.355–391, Marcel Dekker, New York.
[2] Olmsted, I.B. and Borisy, G.G. (1973) Annu. Rev. Biochem. 42, 507–540.
[3] Korn, E.D. (1976) Trends Biochem. Sci. 1, 55–58.
[4] Olmsted, J.F. and Borisy, G.G. (1973) Biochemistry 12, 4282–4389.
[5] Luduena, R.F., Wilson, L. and Shooter, E.M. (1974) J. Cell Biol. 63, 202a.
[6] Behnke, O. and Forer, A. (1967) J. Cell Sci. 2, 169–192.
[7] Mohri, H. (1976) Biochim. Biophys. Acta 456, 85–127.
[8] Silflow, D.C. and Rosenbaum, J.L. (1981) Cell 24, 81–88.
[9] Portier, M.M., Milet, M. and Hayes, D. (1979) Eur. J. Biochem. 97, 161–168.
[10] Denoulet, P., Edde, B., Jeantet, C. and Gros, F. (1982) Biochimie 64, 165–172.
[11] Kalfayan, L. and Wensink, P.C. (1981) Cell 24, 97–106.
[12] Cleveland, D.W., Kirschner, M.W. and Cowan, N.Y. (1978) Cell 15, 1021–1031.
[13] Brunke, K.J., Collis, P.S. and Weeks, D.P. (1982) Nature 297, 516–518.
[14] Barahona, I., Galego, L. and Rodrigues-Pousada, C. (1983) Eur. J. Biochem. 131, 171–176.
[15] Rodrigues-Pousada, C. and Hayes, D. (1978) Eur. J. Biochem. 89, 407–415.
[16] Rodrigues-Pousada, C., Marcaud, L., Portier, M.M. and Hayes, D.H. (1975) Eur. J. Biochem. 56, 117–122.
[17] Watson, M.R. and Hopkins, J.M. (1962) Exptl. Cell Res. 28, 280–295.
[18] Renaud, F.L., Rowe, A.J. and Gibbons, R. (1968) J. Cell Biol. 36, 79–90.
[19] Cohen, J., Adoutte, A., Granchamp, S., Houdebine, L.M. and Beisson, J. (1982) Biol. Cell 44, 35–44.
[20] Katinakis, P.K., Slater, A. and Burdon, R.H. (1980) FEBS Lett. 116, 1–7.
[21] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[22] Ben-Ze'ev, A., Farmer, S.R. and Penman, S. (1979) Cell 17, 319–325.