A telomere binding protein in the macronuclei of the hypotrichous ciliate *Stylonychia lemnae*

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Macronuclear proteins from the hypotrichous ciliate *Stylonychia lemnae* were isolated, separated on SDS-PAGE and electroblotted onto nitrocellulose filters. Filter binding assays with ^{32}P-labeled macronuclear DNA and the synthetic telomere sequence (C_4A_4/G_4T_4) in combination with competition experiments revealed strong preference of a protein with a molecular mass of approx. 40 kDa to the terminal sequence of macronuclear gene-sized molecules.

Western blotting *DNA-binding protein* *Telomere*

1. INTRODUCTION

Macronuclear DNA of the hypotrichous ciliates exists in the form of gene-sized molecules [1–3], created by partially known events during the development of the macronucleus (for review see [4,5]). The macronuclear DNA is associated with histones typical for eukaryotes [6] and organized in nucleosomes [7]. Each DNA molecule carries the same terminal repeat sequence 5′-$C_4A_4C_4A_4C_4$ and a protruding 3′-$G_4T_4G_4T_4$ overhang [8–10]. Since this sequence is highly conserved among hypotrichous ciliates it might have an important function. In fact, it was demonstrated that this sequence, like the related sequence $(C_4A_2)_n$ in rDNA from *Tetrahymena*, functions as a telomere sequence in yeast [11–13]. Furthermore, evidence was provided that this sequence is involved in the preservation of a higher order structure in the macronucleus by nucleic acid–nucleic acid and protein–nucleic acid interactions [14]. In this report we describe a nuclear protein which has a high affinity for this terminal sequence.

2. MATERIALS AND METHODS

Stylonychia lemnae was cultured, macronuclei prepared and DNA isolated as described [15]. The synthetic 20mer $C_4A_4C_4A_4C_4/G_4T_4G_4T_4G_4$ was kindly provided by Dr H. Schott, Institute for Chemistry, University of Tübingen. After lysis of the macronuclei by the addition of 2 vols SDS sample buffer [16], vortex-mixing and heating at 100°C for 10 min, the nuclear proteins were separated on a 7.5–15% SDS-polyacrylamide gradient gel with a 4% stacking gel [16,17]. After the electrophoresis the proteins were either stained with Coomassie brilliant blue G 250 (Serva) or transferred onto nitrocellulose filters: according to Bowen et al. [18] the gels were soaked in 4 M urea, 10 mM Tris-HCl, pH 7.0, 2 mM EDTA, 0.1 mM DTT for 30 min at room temperature to remove SDS and renature the proteins. The proteins were electrophoretically transferred onto nitrocellulose filters essentially as described by Towbin et al. [19], except that the methanol in the electrophoresis buffer was omitted and a voltage gradient of 6 V/cm was applied for 4 h at 4°C.

The DNA-protein assay was carried out in principle as described by Bowen et al. [18]. DNA was labeled with ^{32}P either by nick-translation [20] or 5′-end labeling [21]. For the DNA-protein binding assay 10^5 cpm/ml of [^{32}P]DNA was used. The binding reaction was performed in 1 × protein

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binding buffer [18] for 1 h at room temperature, subsequently the filters were dried between 3MM Whatman filter paper at room temperature and subjected to autoradiography.

To remove the terminal sequences, macronuclear DNA was digested with the exonuclease *Bal*31. The reaction conditions were as follows: 600 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 0.1 U *Bal*31/μg DNA. Usually the DNA concentration was 200 μg/ml. The reaction mixture was incubated at 37°C for 30 min and stopped by the addition of EDTA to a final concentration of 50 mM followed by phenol/chloroform extraction and ethanol precipitation. To verify the removal of the terminal inverted repeat sequence, *Bal*31 digested macronuclear DNA was electrophoresed on agarose gels and the dried gel hybridized with a ^{32}P-labeled synthetic G_4A_4/G_4T_4 20mer. Hybridization conditions were 10 × Denhardt's [22], 4 × SSC, 50 μg/ml tRNA. The hybridization was performed overnight at 40°C.

3. RESULTS AND DISCUSSION

The banding pattern of Coomassie brilliant blue stained macronuclear proteins is shown in fig.1, lane a. The majority of these proteins is in the molecular mass range of 10 to 80 kDa. Following the electrophoresis proteins were transferred onto nitrocellulose filters and total ^{32}P-labeled macronuclear DNA was bound to them. As can be seen in the autoradiography (fig.1, lane b) most of the proteins bind to the DNA and the binding is proportional to the relative amount of proteins present. However, there is a slight difference in the binding effectivity. The DNA binds stronger to a protein with a molecular mass of 40 kDa than expected from its concentration seen in the Coomassie blue stained gel. This indicates that the 40 kDa protein possesses a higher affinity to the DNA than most other nuclear proteins do. Furthermore, when the filters are washed in 2 M NaCl the 40 kDa protein still binds to the DNA while none or only very little binding is observed to the other nuclear proteins (see fig.2, lane d). It was shown in an earlier study on *Oxytricha* macronuclear chromatin that a protein responsible for the preservation of a higher order structure

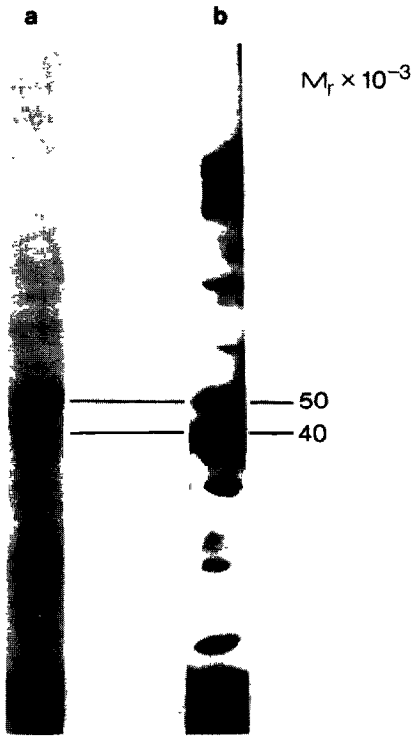

Fig.1. (a) Coomassie blue stained macronuclear proteins separated on a 7.5–15% SDS-polyacrylamide gel. (b) Proteins were transferred onto nitrocellulose filters and ^{32}P-labeled macronuclear DNA was bound to it as described in section 2.

also binds at 2 M NaCl to the termini of macronuclear DNA [14].

To see whether the 40 kDa protein might be specific for the telomeric sequence of *Stylonychia* macronuclear DNA the following experiments were performed. Macronuclear proteins were separated on SDS-polyacrylamide gradient gels and electrophoretically blotted onto nitrocellulose filters. To these proteins the synthetic ^{32}P-labeled 20mer C_4A_4/G_4T_4 was bound. As shown in fig.2, lane a, this sequence binds to several proteins. However, a very strong binding is observed to the 40 kDa protein while the binding to a more abundant 50 kDa protein (see fig.1, lane a) is very weak.

To obtain further evidence for the putative sequence-specific binding of the 40 kDa protein, DNA-binding competition experiments were performed. The basic idea was that competition with

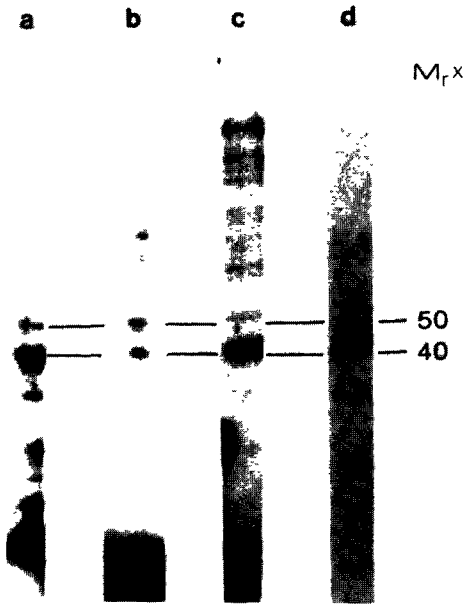

Fig.2. DNA binding competition experiments. (a) Binding of ^{32}P-labeled C_4A_4/G_4T_4 to macronuclear proteins. (b and c) Prior to the binding of ^{32}P-labeled C_4A_4/G_4T_4 to macronuclear proteins, the protein filters were preincubated with a 1000-fold excess of unlabeled total macronuclear DNA (b) and unlabeled *Bal*31 digested, i.e. telomere depicted, macronuclear DNA (c). (d) Binding of ^{32}P-labeled C_4A_4/G_4T_4 to macronuclear proteins, washed with 2 M NaCl.

a large excess of unlabeled total macronuclear DNA containing the terminal sequence followed by binding of the labeled C_4A_4/G_4T_4 should reveal no or only a weak signal with the 40 kDa protein. In contrast, competition with *Bal*31 digested, i.e. telomere depleted, macronuclear DNA should not interfere with the subsequent binding of the C_4A_4-sequence.

Protein filters were preincubated for 30 min at room temperature in 1 × protein binding buffer containing a 1000-fold excess of unlabeled macronuclear DNA. After removal of this competition solution, the final binding mixture containing ^{32}P-labeled C_4A_4/G_4T_4 was added, continuing the standard binding procedure. The results of these experiments are shown in fig.2, lanes b and c. As expected, after competition with total macronuclear DNA only a weak binding to the 40 kDa protein was detected. However, after competition with the telomere depleted macronuclear DNA a strong binding of C_4A_4/G_4T_4 sequence to the 40 kDa protein is observed, but the binding to all the other nuclear proteins is highly reduced except for histones.

To get more information about the stability of the DNA-protein complex, the ^{32}P-C_4A_4/G_4T_4 was bound to the filter immobilized proteins under standard conditions followed by extensive washes with 2 M NaCl in 1 × protein binding buffer. The autoradiography, fig.2, lane d, revealed a band corresponding to the 40 kDa protein, but no or only very weak ones with other nuclear proteins.

All the experiments described, provide strong evidence that the 40 kDa protein has a very high affinity to the terminal macronuclear sequence of *Stylonychia*. Furthermore, the binding of this protein to the C_4A_4/G_4T_4 is very strong and stable under high ionic strength (fig.2, lane d). Lipps et al. [14] proposed that a protein with a molecular mass of about 50 kDa binds to the terminal sequence of *Oxytricha* macronuclear DNA, however, no sequence-specific binding could be observed at that time. One is tempted to speculate about the possible function of this protein. The terminal sequences of macronuclear DNA are involved both in replication of the short gene-sized DNA molecules as well as in the preservation of a higher order structure. The terminal sequences form aggregates in vitro [23], such aggregates seem to be stabilized in vivo by a nuclear protein [14]. In this regard, the 40 kDa protein might have a functional similarity to the recA protein, which stabilizes multistranded DNA complexes under certain conditions [24–26]. Another possibility could be that it might be necessary for the formation of functional telomere sequences: it could either serve as a kind of recognition site for the ligation of additional telomeric sequences as it has been reported in yeast [11–13] or simply stabilize a DNA structure necessary for functional telomeres.

We are not able to distinguish between all these possibilities, however, further analysis of this DNA-protein complex might provide new insights about the exact structure of the termini of linear DNA molecules and chromosomes in eukaryotic cells.

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