Protein–DNA interactions in soluble telosomes from *Saccharomyces cerevisiae*

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ABSTRACT

Telomeric DNA in *Saccharomyces* is organized into a non-nucleosomal chromatin structure called the telosome that can be released from chromosome ends in soluble form by nuclease digestion (Wright, J. H., Gottschling, D. E. and Zakian, V. A. (1992) *Genes Dev.* 6, 197–210). The protein–DNA interactions of soluble telosomes were investigated by monitoring isolated telomeric DNA fragments for the retention of bound protein using both gel mobility shift and nitrocellulose filter-binding assays. Telosomal proteins remained associated with telomeric DNA at concentrations of ethidium bromide that dissociated nucleosomes. The protein–DNA interactions in the yeast telosome were also disrupted by much lower salt concentrations than those known to disrupt either the interactions of ciliate terminus-binding proteins with telomeric DNA or the interactions of histones with DNA in nucleosomes. Taken together, these data corroborate previously published nuclease mapping data indicating that telosomes are distinct in structure from conventional nucleosomes. These data also indicate that yeast do not possess telomere binding proteins similar to those detected in ciliates that remain tightly bound to telomeric DNA even in high salt. In addition, the characteristic gel mobility shift of soluble telosomes could be mimicked by complexes formed *in vitro* with yeast telomeric DNA and recombinant Rap1p suggesting that Rap1p, a known component of soluble yeast telosomes (Wright, J. H., Gottschling, D. E. and Zakian, V. A. (1992) *Genes Dev.* 6, 197–210; Conrad, M. N., Wright, J. H., Wolf, A. J. and Zakian, V. A. (1990) *Cell* 63, 739–750), is likely to be the major structural protein bound directly to yeast telomeric DNA.

INTRODUCTION

Telomeres, the ends of eukaryotic chromosomes, are essential for the stable maintenance of *Saccharomyces* chromosomes at least in part because they protect chromosome ends from degradation (3). Telomeres are also thought to be required for replication of the very end of the chromosome (4,5) and to help organize the

nucleus by associating with one another and with the nuclear periphery (reviewed in 6). In most organisms, including yeast, the ends of chromosomal DNA molecules are comprised of a variable amount of simple-sequence DNA, with the strand of telomeric DNA that runs 5' → 3' towards the chromosomal terminus rich in G residues. In ciliated protozoa such as *Oxytricha*, the G-rich strand is extended to form a short, single-stranded tail (7). Chromosomes in *Saccharomyces* end with ~350 ± 75 bp of the heterogeneous sequence, C_{2–3}A(CA)_{1–6}(TG)_{1–6}TG_{2–3}, abbreviated C_{1–3}A/TG_{1–3} (8,9). In addition to the simple repeats at the very ends of chromosomes, many organisms have middle repetitive sequences called telomere-associated repeats internal to the simple repeats. In yeast there are two types of telomere-associated elements, X and Y' (reviewed in 10).

Nuclease mapping experiments indicate that X and Y' are assembled into nucleosomes. In contrast, the C_{1–3}A/TG_{1–3} repeats on chromosome ends are protected in a structure called the telosome that encompasses the entire terminal tract of C_{1–3}A/TG_{1–3} DNA and thus contains more than twice as much DNA as a conventional nucleosome (1). The non-nucleosomal character of yeast telomeric chromatin was inferred from the fact that a larger, heterogeneous amount of DNA was protected, as well as from the presence of a DNase I hypersensitive site at the proximal boundary of the telosome. This non-nucleosomal protection pattern is conserved in other eukaryotes such as ciliates (11,12) and humans (13). In vertebrates which have long tracts of telomeric sequence that extend for several kilobases, only the most distal sequences are in telosome structures (13), with the internal runs in nucleosomes with short inter-nucleosomal linker length (14).

A non-nucleosomal protection pattern for telomeric chromatin suggests that telomeric DNA is associated with structural proteins other than, or in addition to, histones. Multiple lines of evidence suggest that the protein encoded by the essential (15) yeast gene *RAP1*, Rap1p, is a telomere-binding protein. There are ≥ 10 consensus binding sites for Rap1p at each telomere (9), and Rap1p binds to double-stranded C_{1–3}A/TG_{1–3} repeats *in vitro* (16,17). Genetic experiments indicate that Rap1p interacts with telomeres *in vivo* since mutations that alter the protein or change its expression levels affect telomere length (2,18–20). Moreover, Rap1p is a component of soluble telomeric chromatin (1,2), and immuno-localization places Rap1p at the ends of meiotic chromosomes (21). However, Rap1p also acts at sites other than

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Figure 1. Ethidium bromide stability of nucleosomes and telosomes. Aliquots of chromatin supernatants prepared from MNase treated nuclei were either loaded directly onto agarose gels (–) or treated with SDS and proteinase K prior to loading (+). Each set of samples was loaded in duplicate onto two different gels, with (panels 1 and 3) and without (panels 2 and 4) 1 μ g/ml EthBr added to the agarose before casting. Samples prepared from nuclei digested for 2 min (2') or 4 min (4') with MNase are shown. Molecular weight markers are BRL 1 kb ladder. The blots were hybridized sequentially with an X probe (panels 1 and 2) and with a TG₁₋₃ probe (panels 3 and 4).

telomeres where it can function as a transcriptional activator (for example, see ref. 22) or a transcriptional repressor (19). Genetic approaches have identified additional proteins that influence telomere length *in vivo*. It remains to be investigated whether the gene products of the TEL1 and TEL2 (23), EST1 (24), PIF1 (25) and RIF1 (26) genes are directly bound to yeast telomeric DNA *in vivo*. In addition, an *in vitro* C₁₋₃A DNA binding activity that is distinct from Rap1p has been identified, but it is not known if this represents an *in vivo* telomere binding protein (27).

In the ciliated protozoa, *Oxytricha*, *Euplotes* and *Stylonychia*, telomere specific binding proteins have been identified that bind to the very terminus of the DNA (28–30). These proteins bind very tightly, but non-covalently to the DNA end, staying bound even in 2 M salt. An activity with binding properties reminiscent of those of the *Oxytricha* proteins, but specific for vertebrate telomeric DNA, has been identified in *Xenopus* extracts (31).

In this report, we have examined the properties of soluble telosomes isolated from yeast cells as chromatin. The salt and ethidium bromide (EthBr) stabilities of soluble telosomes suggest that the telosome is not based on a conventional nucleosomal core structure. In addition, no evidence was found for salt-stable terminus binding proteins similar to those described in ciliated protozoa. A characteristic gel mobility shift of soluble telosomes could be mimicked by recombinant Rap1p–telomeric DNA complexes formed *in vitro* indicating that Rap1p is the major structural protein present in the soluble telosomes.

MATERIALS AND METHODS

Yeast strains and methods

JW1 (*MAT α lys5 ura3 Δ ::hisG leu2-3,112 trp1 Δ ::hisG ade2 ade5 can1 cyh2 prb1 Δ ::hisG*) has been described (1). In experiments where *Bam*HI was used to liberate telosomes, JW1 contained a linear plasmid called YLpFATOTEL (2). The plasmid was maintained at moderate copy number by growth on synthetic medium lacking tryptophan (YC-trp; 43). At moderate copy

number, plasmids like YLpFATOTEL have no detectable effects on telomere structure (44).

Yeast nuclei were prepared by growing JW1 cells to 2×10^7 to 1×10^8 cells/ml. Cells were washed once with water, resuspended in SED (1 M sorbitol, 25 mM EDTA pH 8, and 6.7 mg/ml DTT), incubated at 30°C for 10 min, spun at 3000 r.p.m. for 5 min, and resuspended in SCE-60 (1 M sorbitol, 0.1 M sodium citrate pH 5.8, 60 mM EDTA). 0.01 vol of glusulase (NEN Dupont) was added and cells were incubated until >90% of the cells could be lysed with SDS. The remaining steps of the procedure were done at 4°C. Spheroplasts were resuspended in 2 ml SPC (1 M sorbitol, 20 mM PIPES pH 6.3, 10 mM CaCl₂, 1 mM PMSF, 1 mM iodoacetic acid) and added to 40 ml FPC (9% Ficoll, 20 mM PIPES pH 6.3, 10 mM CaCl₂, 1 mM PMSF, 1 mM iodoacetic acid). The nuclei were pelleted by centrifugation at 10 000 r.p.m. for 20 min. The experiments shown in Figure 1 were done with the zymolyase spheroplasting procedure described in (1). During the course of these experiments, the glusulase procedure was adopted because it results in less proteolysis of Rap1p (A. Lustig, personal communication).

Chromatin digestions with MNase were done as described in (1). Reactions were stopped with EDTA, and diluted to 500 μ l with Binding Buffer (BB) (10 mM Tris pH 7.5, 10 mM MgCl₂, 60 mM NaCl, 0.1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 10 μ g/ml TAME). 500 μ l of glass beads (425–600 μ m diameter; Sigma) were added and tubes were shaken at 4°C for 30 min using an Eppendorf model 5432 tube mixer. The liquid was removed to new tubes, and the disrupted nuclei were centrifuged at 10 000 r.p.m. for 5 min. The supernatant contained the soluble chromatin. This method is an adaptation of procedures developed by M. Saunders and K. Bloom (personal communication). *Bam*HI (500 U; NEB) digestions were done at 4°C at the same time as the shaking with glass beads. In this case, the nuclei were resuspended directly in BB rather than PC buffer.

Gel filtration was done with A15m agarose (BioRad), which has an exclusion limit of 1.5×10^7 daltons for separation of large

macromolecules. The column had a bed volume of 80 ml (1.5 × 45 cm), was run at a flow rate of 6 ml/h for 13 h, and 80 one ml fractions were collected. All samples were run in BB + 0.002% NaN₃ with additional NaCl added for the salt stability experiments. No more than 4 ml of chromatin supernatant were loaded per run; total chromatin supernatant typically contained 150 µg/ml total protein, and thus 600 µg protein were loaded onto the A15m column.

***E. coli* methods**

For expression of Rap1p in *E. coli*, the plasmid L355, containing the RAP1 gene fused to λ *gene 10* such that the N-terminal 10 amino acids of Rap1p were replaced with 12 amino acids from *gene 10* (a generous gift of M. Roberge and S. Gasser) was introduced into the *E. coli* strain BL21(DE3) (45). BL21/DE3 contains the T7 RNA polymerase gene under *lac* control, and the *gene 10* promoter is recognized by T7 RNA polymerase. Dense cultures of BL21(DE3) cells with and without the L355 plasmid were diluted 100-fold into 50 ml LB + amp or LB. BL21(DE3) extracts lacking the L355 plasmid were used as a negative control in binding studies because the cells with L355 expressed some of the Rap1p fusion protein in the absence of IPTG induction as seen by Western blotting. Expression of the fusion protein was induced with IPTG for 2.5 h. Cells were pelleted and resuspended in 1 ml of Storage Buffer (200 mM Tris pH 8, 10 mM MgCl₂, 1 mM DTT, 10% glycerol), and frozen in 0.1 ml aliquots at -70°C. For each experiment, an aliquot of bacteria was diluted into 1 ml of Extraction Buffer (50 mM Tris pH 7.5, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, 0.1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 10 µg/ml TAME). 0.2 mg of lysozyme was added, and the mixture left on ice for 20 min. Deoxycholate was added to 0.1%, and the mixture was shaken at 37°C to lyse the cells. Extracts were then sonicated and spun at 10 000 r.p.m. for 5 min. The total amount of protein in the extracts was 1.5 mg/ml. The Rap1p fusion protein appeared to be at least 1% of the total protein by coomassie staining. For DNA binding reactions, *E. coli* extracts were diluted in Extraction buffer to 1:10 and 1:100. One, 2 or 5 µl of the undiluted extract or the dilutions were mixed with 25 µl telomeric DNA. The DNA used in the binding reaction was A15m fractions from a 370 mM NaCl column run, where the DNA retained virtually no endogenous gel mobility shift. The fraction pool was diluted 2-fold in 20 mM Tris pH 7.5, 4 mM EDTA, 1 mM DTT, 100 mM NaCl, 6% glycerol, and poly dI/dC was added to 40 ng/µl. The concentration of the telomeric DNA in the A15m fractions was <0.4 ng/µl, giving a 100 molar excess of poly dI/dC over specific DNA. Reactions were incubated on ice for 10–20 min, and analyzed by the gel mobility shift assay.

Gel assays and nitrocellulose binding

For gel mobility shifts, DNA was analyzed on 1% agarose gels (ultraPURE, BRL). Electrophoresis was done in TAE buffer (40 mM Tris-acetate, 1 mM Na₂EDTA, 5 mM sodium acetate pH 7.9) at 10.7 V/cm for 3 h. For protease treated samples, 4 µl of 1% SDS and 10 µg proteinase K were added to 40 µl aliquots of chromatin supernatant and incubated at 37°C for 20 min. For the EthBr stability experiments, 1 µg/ml EthBr was cast into the gel mixture. The gels were blotted to nylon membrane, and probed according to previously described methods (44). All probes were labelled using the random primer procedure (46). The X probe was a ~600 bp *EcoRI*–*EcoRV* fragment subcloned from the

plasmid pYP1-L3 (47) obtained from E. Louis. The TG₁₋₃ probe was a pair of complementary 39 base oligonucleotides containing 35 bp of TG₁₋₃ sequence whose exact sequence is based on that of a cloned yeast telomere (48).

The nitrocellulose filter binding assays were done exactly according to the previously published methods used to characterize the *Oxytricha* terminus-binding proteins (28). Chromatin supernatants were prepared from MNase digested nuclei. Half of the supernatant was treated with proteinase K, extracted with phenol and SEVAG (1 part isoamyl alcohol, 19 parts CHCl₃), precipitated with EtOH, resuspended in the same volume of isolation buffer, and used for a deproteinized DNA control. Samples were equilibrated to different concentrations of NaCl, and filtered through nitrocellulose. The DNAs from the bound and unbound fractions were analyzed by Southern hybridization.

In cases where Southern blots were quantified, autoradiograms were digitized by scanning on a Sharp JX320 flatbed scanner with a resolution of 330 d.p.i., and a transparency option. Quantification of band intensities was done using the BioScan Optimas and Microsoft Excel programs. Storage phosphorimaging (49) was used for quantification of radioactive beta particle emissions directly from the gels and blots.

RESULTS

Protein–DNA interactions in telosomes are not eliminated by ethidium bromide

Previous studies demonstrated that the DNA that links the telosome to adjacent nucleosomes is highly accessible to nucleases in chromatin (1). This feature allows the release of telosomes from the ends of chromosomes as protein–DNA complexes. Telosomes were released from the ends of yeast chromosomes by digestion with micrococcal nuclease (MNase). After nuclease digestion, the nuclei were disrupted and insoluble material was removed by centrifugation to yield a chromatin supernatant. In various experiments, the chromatin supernatant contained 60–80% of the DNA that hybridized to a TG₁₋₃ probe (data not shown). The amount of nucleosomal DNA in the chromatin supernatant, which varied from 10 to 80% of the total DNA, was roughly proportional to the extent of MNase digestion. The telomeric DNA present in these chromatin supernatants displays a protease-reversible gel mobility shift. Competition experiments indicate that the proteins responsible for the mobility shift were associated with telomeric DNA prior to the chromatin solubilization (1).

We used this gel mobility shift assay to compare the stability of soluble telosomes to that of nucleosomes. The histones in nucleosomal core particles are dissociated from DNA by small amounts of EthBr (32). To determine if telosomes are disrupted by EthBr, the gel mobility of telosomal DNA in the presence and absence of EthBr was monitored. DNA in nucleosomes was also analyzed for comparison. Aliquots of chromatin supernatants, with or without prior treatment with protease, were loaded directly onto duplicate agarose gels, one of which contained EthBr. After electrophoresis, the DNA was transferred to nylon filters and visualized by probing sequentially with radiolabeled C₁₋₃A/TG₁₋₃ DNA (to detect telosomal DNA) and X DNA (to detect nucleosomal DNA) (Fig. 1).

In gels lacking EthBr, both the nucleosomal and the telosomal DNA displayed a protease-reversible gel mobility shift [Fig. 1, panels 2 and 4; compare protease (–) and (+) lanes]. As expected,

this shift was eliminated for nucleosomal DNA when the gel was run with EthBr (Fig. 1, panel 1). In contrast, all of the telosomal DNA displayed a protein-dependent mobility shift in the gel containing EthBr (Fig. 1, panel 3). This result indicated that unlike the DNA in nucleosomes, protein remained bound to the telomeric DNA in EthBr. However, the mobility of the telosomal protein-DNA complex was increased by the EthBr, indicating that some telosomal proteins were removed by the EthBr or, alternatively, that EthBr binding to the telosomal protein-DNA complex changed its mobility.

Telosomal proteins are dissociated from telomeric DNA by moderate salt concentrations

The salt-stability of the protein-DNA interactions in telosomes was determined using a nitrocellulose filter-binding assay. This assay is based on the fact that double-stranded DNA does not bind to nitrocellulose whereas proteins bind tightly to the membrane. Consequently, duplex DNA that is protein associated is also retained on nitrocellulose, even in the presence of high salt (33). A nitrocellulose filter binding assay was used previously to demonstrate the salt-stable binding of the *Oxytricha* terminus-binding proteins to *Oxytricha* telomeres (28). These previous results illustrate the sensitivity of this assay in that even proteins present in only one molecule per telomere retain the DNA on the filter.

Chromatin supernatants were diluted into solutions containing increasing amounts of NaCl and then passed through a nitrocellulose filter. Bound DNA was extracted from the filter with detergent. The DNA in both the bound and unbound fractions was then analyzed by Southern hybridization. The deproteinized DNA, which served as a negative control, was not retained on the filter at any salt concentration (Fig. 2, naked DNA). Virtually 100% of the telomeric DNA in chromatin supernatants bound to the nitrocellulose when applied in 155 mM NaCl (Fig. 2, chromatin panel). Between 15 and 30% of the telomeric DNA was bound to nitrocellulose in NaCl concentrations of 370, 470, 570 and 670 mM. No telomeric DNA was bound when applied in 770 mM NaCl. These results indicated that most proteins in the soluble telosome were removed from the telomeric DNA by 370 mM salt, with all of them removed by 770 mM salt.

Similar results were obtained when the salt-stability of telosomes was determined by a second method. Telosomes for these experiments and for those described in the next section were prepared from a linear plasmid which had *Bam*HI sites within the nuclease hypersensitive region. Chromatin supernatants prepared with *Bam*HI from plasmid bearing JW1 cells were fractionated on an A15m agarose column that had been equilibrated to different salt concentrations. This gel filtration matrix has a high exclusion limit, allowing separation of telosomes from free protein. The fraction of telomeric DNA that was protein-bound was determined by analyzing the column fractions using the gel mobility shift assay. Telomeric DNA was recovered quantitatively in protein-DNA complexes when columns were run in ≤ 155 mM NaCl (Fig. 3). Nonetheless, the shift in the telosomal DNA mobility is lower after fractionation as compared with unfractionated chromatin supernatant, indicating that dilution into the column running buffer even in low salt may dissociate some protein (Fig. 3, compare 'Tot' lanes with numbered fractions). A small fraction of the DNA retained a mobility shift when telosomes were fractionated on columns containing 370 mM, especially in the larger sized telosomes (Fig. 3, lower panel,

Figure 2. Nitrocellulose filter-binding assays of chromatin and naked DNA at different salt concentrations. Fifty μ l aliquots of MNase prepared chromatin supernatants were diluted into 1 ml of Binding Buffer + NaCl to give final salt concentrations of: 155, 370, 470, 570, 670, 770, 870, 1070, 1270, 1570 and 2070 mM (chromatin panel). Deproteinized DNA from the same chromatin supernatant was resuspended in the same volume and included as negative controls (naked panel). The DNA in the bound (B) and unbound (U) fractions was analyzed by Southern hybridization with a 125 bp TG₁₋₃ probe.

fraction 25). No protein-dependent mobility shift was seen when fractionation was performed in 570 mM or higher concentrations of NaCl (data not shown). Thus, using this gel filtration assay, the salt stability of the majority of the telosomal proteins was between 155 and 370 mM NaCl, a result very similar to that obtained with the nitrocellulose filter-binding assay. A small fraction of telosomal DNA fragments retained a gel shift after fractionation in 370 mM salt and a small fraction was bound to nitrocellulose in 370 mM salt. These data might indicate that a small percentage of the soluble telosomes are associated with a protein that is removed only by higher salt.

Initially, we tried to assess the salt stability of telosomes by adjusting the chromatin supernatant to a given salt concentration and analyzing the DNA directly by the gel mobility shift assay. However, the proteins responsible for the gel shift were able to re-associate rapidly with telomeric DNA as the salt was diluted into the gel running buffer thereby reconstituting the gel mobility shift. An example of this phenomenon is shown in the lower panel of Figure 3. The left sets of total lanes are chromatin supernatants adjusted to 370 mM NaCl, and loaded directly onto the gel without prior fractionation on the column. When telosomes were fractionated on the column, dissociated protein was separated from telomeric DNA. However, when salt-treated telosomes were loaded directly on the gel, some of the proteins responsible for the gel shift reassociated rapidly with the DNA. These results suggest that the telomere-binding protein(s) responsible for the gel mobility shift have a high DNA association rate. This

Figure 3. Equilibrium-binding studies of telosomal proteins at different salt concentrations. Chromatin supernatants prepared with *Bam*HI from nuclei containing the linear plasmid, YLpFATOTEL, were fractionated on an A15m gel filtration column at different concentrations of NaCl. One ml fractions were collected: aliquots of fractions 25–43 are shown, along with the total chromatin supernatants both in the column buffer (left set of Tot lanes; 155 mM for upper panel, 370 mM for lower panel) and in the low salt buffer in which they were isolated (right set of Tot lanes; 70 mM NaCl). Samples were either loaded directly on the gel (–) or treated with proteinase K and SDS prior to loading (+).

hypothesis was tested directly by mixing DNA and protein fractions from an A15m column run in 2 M NaCl and incubating the mixtures on ice for various times before loading onto the gel. Surprisingly, all of the DNA molecules were completely shifted even at the 0 min time point, where the protein and DNA fractions were loaded separately into the well of the gel (data not shown).

Reconstitution of telosomes with telomeric DNA and *E. coli* made Rap1p

Previous work has shown that Rap1p is a component of the yeast telomeric chromatin (1,2). In order to assess the binding properties of Rap1p to telomeric DNA and compare them with those of isolated telosomes, Rap1p was expressed in *E. coli* as a *gene 10* fusion protein and complexed *in vitro* with telomeric DNA fragments. Telomeric *Bam*HI DNA fragments were obtained by the methods used to prepare soluble telosomes except that the DNA was deproteinized prior to use. These telomeric DNA fragments were mixed with increasing concentrations of an *E. coli* extract containing the *gene 10*–Rap1p fusion protein. These mixtures were loaded on gels with and without EthBr, along with serial dilutions of chromatin supernatants (Fig. 4). At the higher concentrations of *E. coli* extract, the protein–telomeric DNA complexes had a mobility shift very similar to that of soluble telosomes (Fig. 4, compare bracketed regions, lanes 12 and 15). A different gel mobility shift was seen with *E. coli* extracts

Figure 4. Comparison of gel mobility shifts produced by recombinant Rap1p–telomeric DNA complexes with soluble telosomes. Telosomal DNA was partially purified from JW1/YLpFATOTEL chromatin supernatants and mixed with a 100-fold molar excess of poly dI/dC and *E. coli* extracts containing (lanes 3–12) or lacking (lane 2) Rap1p fusion protein. The mixtures were then loaded onto two 1% agarose gels, with and without EthBr. Lane markings refer to both gels. From left to right the lanes are as follows: lane 1, no protein; lane 2: 1.5 μ g *E. coli* protein extract from cells without Rap1p; lanes 3–12: 0.0075, 0.015, 0.03, 0.075, 0.15, 0.3, 0.75, 1.5, 3 and 7.5 μ g *E. coli* protein extract containing the Rap1p fusion protein. The amount of protein in lanes 12 and 2 is the same. Lane 13, 5 μ l chromatin supernatant treated with proteinase K/SDS; lanes 14–17, 0.5, 1, 5 and 5 μ l of soluble telosomes from chromatin supernatants for comparison with the *in vitro* Rap1p protein–telosomal DNA complexes.

prepared from cells lacking the Rap1p fusion protein, demonstrating that reconstitution of the characteristic gel mobility shift was due to the Rap1p in the *E. coli* extract (Fig. 4, lane 2). When lower concentrations of the *E. coli* extract containing Rap1p were used, the protein–DNA complexes had a greater mobility suggesting that the fully shifted *in vitro* complexes contained multiple Rap1p molecules (Fig. 4, top panel, lane 6). When the same samples were loaded on a gel containing EthBr, the mobility of the *E. coli* Rap1p–telomeric DNA complexes was altered in a similar fashion as the endogenous telosomal complexes (Fig. 4, compare top and bottom panels). Recombinant Rap1p–telomeric DNA complexes also demonstrated the rapid reassociation rate observed with the endogenous telosome structures mentioned above (data not shown). Thus, by several criteria, the DNA–protein complexes formed with *E. coli* generated Rap1p and telomeric DNA were similar to soluble telosomes, suggesting that Rap1p was the major structural protein in the soluble yeast telosomes.

DISCUSSION

As defined by nuclease mapping studies, yeast telomeric DNA is assembled into non-nucleosomal chromatin structures called

telosomes (1). Telosomes were released from chromosomes with MNase or from linear plasmids with *Bam*HI. After nuclease treatment, ~60–80% of chromosomal and plasmid telomeres were recovered as soluble protein–DNA complexes in the chromatin supernatant (Fig. 1). This high level of solubility is perhaps surprising given that vertebrate telomeric DNA (34) and yeast Rap1p (35) fractionate with the insoluble nuclear matrix and that cytological studies in many organisms suggest that telomeres are associated with the nuclear periphery (for example, 36). Perhaps only a fraction of telosomes are associated with the matrix at any one time, and that fraction is the 20% that is not solubilized in our experiments.

One model for telosome structure is that C_{1–3}A/TG_{1–3} tracts are assembled in a di-nucleosome with other proteins such as Rap1p binding to the inter-nucleosomal linker and to the additional heterogeneous length of DNA that would not be protected by a fixed-size di-nucleosome. However, several experiments herein suggest that yeast telosomes are not based on a nucleosome core. Unlike nucleosomes, the protein–DNA interactions in telosomes were not eliminated by EthBr (Fig. 1). The dissociation of nucleosomes by EthBr is thought to result from the increased stiffness of the DNA backbone upon intercalation of the dye, preventing the DNA from bending around the histone octamer (32). The stability of telosomes to EthBr may indicate that the telosome structure is not as influenced by the conformation of the DNA backbone as are nucleosomal core structures; in other words the DNA is not wrapped around a protein core. Alternatively, telosomal proteins such as Rap1p might prevent EthBr intercalation by blocking accessibility to the C_{1–3}A/TG_{1–3} repeats. However, the fact that EthBr changes the mobility of the complex indicates that intercalation of the dye is not completely blocked. A third possibility is that Rap1p or other proteins remain associated (or re-associate) with the DNA upon disassembly of the underlying nucleosomal core to give the smaller gel shift seen in the presence of EthBr. We consider this third possibility unlikely because the increase in mobility of the shifted complex with EthBr was also seen with the *in vitro* Rap1p–telomeric DNA complexes (Fig. 4, compare top and bottom panels). Hence, there is no need to invoke the loss of proteins other than Rap1p from the telosome in order to explain the altered mobility of the complex in EthBr.

Other evidence against the di-nucleosome core model includes the fact that the characteristic gel mobility shift of the soluble telosomes could be regenerated by rapid reassociation of protein and DNA fractionated in high salt (data not shown). In contrast, DNA reassociation with histones is a slow process (37,38). Finally, experiments by others show that changes in histone stoichiometry disrupt centromeric (39) but not telomeric chromatin (data cited in 40). Thus, if the telosome is based on nucleosomal cores, additional proteins must alter their EthBr stability, as well as the kinetics of nucleosome assembly.

The fact that no telomeric DNA was protein-associated in 770 mM or higher salt also indicates that soluble yeast telosomes do not contain salt-stable terminus-binding proteins similar to those detected in *Oxytricha* (28) and other ciliates. The nitrocellulose filter-binding assay used here is identical to that used to demonstrate the salt stability of the ciliate proteins. The telomeric DNA in *Oxytricha* macronuclear DNA differs from that of most other organisms in having a fixed length (reviewed in 10). In contrast, the number of base pairs of telomeric DNA at a given telomere in yeast and most other organisms is dynamic,

increasing or decreasing spontaneously (41) or in response to a variety of perturbations (for example, see 2). Thus, if terminus-binding proteins exist in organisms, like yeast, with variable length telomeres, perhaps they bind more loosely than the *Oxytricha* proteins and/or bind only during part of the cell cycle, allowing length changes to occur.

The behavior of soluble yeast telosomes *in vitro* is consistent with the *in vivo* nuclease protection data suggesting that telosomes are not based on conventional nucleosomal cores. *E.coli* produced Rap1p was able to reconstitute the native gel mobility shift of telosomes in chromatin supernatants when mixed with deproteinized telomeric DNA fractions (Fig. 4). Furthermore, at least some properties of the gel mobility shift, including the EthBr stability (Fig. 4), and the rapid DNA association rate (data not shown), were mimicked by complexes formed *in vitro* from *E.coli* produced Rap1p and telomeric DNA. These data are consistent with a model in which Rap1p is the major structural protein that binds directly to C_{1–3}A/TG_{1–3} DNA *in vivo*. Other experiments have shown that recombinant Rap1p can coat a 270 bp tract of C_{1–3}A/TG_{1–3} DNA, protecting 80% of it from DNase I digestion *in vitro* (42). These results suggest that Rap1p alone could generate the nuclease protection pattern observed in nuclei. Moreover, the presence of Rap1p at telomeres protects chromosome ends from degradation (2,18), and can mediate telomere interactions with other telomeres and with the nuclear scaffold (21,35).

While Rap1p may be the most abundant structural protein bound directly to the telomeric DNA, genetic experiments indicate that other proteins interact with telosomes *in vivo*. Perhaps other proteins that are important for telomere function are not solubilized with the nuclease-released telosome structure or are present in the ~20% of telosomes that are not solubilized using these methods. In addition, any protein that does not contribute measurably to the gel mobility shift and that is released from telomeric DNA by ≤370 mM salt would not be detected in these experiments. The biochemical analysis of telosomes described herein may be most useful for asking if a putative telomere binding protein is in telosomes using a specific antiserum as was done previously for Rap1p (2).

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