Telomerase RNA Localized in the Replication Band and Spherical Subnuclear Organelles in Hypotrichous Ciliates

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Abstract. The intranuclear distribution of telomere DNA-binding protein and telomerase RNA in hypotrichous ciliates was revealed by indirect fluorescent antibody staining and in situ hybridization. The *Oxytricha* telomere protein colocalized with DNA, both being dispersed throughout the macronucleus except for numerous spherical foci that contained neither DNA nor the protein. Surprisingly, the telomerase RNA was concentrated in these foci; therefore, much of telomerase does not colocalize with telomeres. These foci persist through the cell cycle. They may represent sites of assembly, transport or stockpiling of telomerase and other ribonucleoproteins. During S phase, the macronuclear DNA replication machinery is organized into a disc-shaped structure called the replication band. Telomerase RNA is enriched in the replication band as judged by fluorescence intensity. We conclude that the localization of a subfraction of telomerase is coordinated with semiconservative DNA replication.

TELOMERES are the DNA/protein complexes found at the ends of linear chromosomes. Telomeres cap chromosomes, protecting them from end-to-end ligation and from nucleolytic degradation (reviewed in Zakian, 1989; Blackburn, 1991). Telomeric DNA serves as a substrate for telomerase, which adds telomeric DNA sequences to chromosome ends to ensure their complete replication (reviewed in Blackburn, 1992). Specific localization of telomeres in the nucleus in a number of species suggests their involvement in chromosome positioning and nuclear architecture (Agard and Sedat, 1983; Hochstrasser et al., 1986; Klein et al., 1992), and they are involved in premeiotic chromosome movement in fission yeast (Chikashige et al., 1994). Furthermore, telomeres have been observed to affect the expression of adjacent genes, a phenomenon called telomere position effect (Gottschling et al., 1990).

Telomeric DNA usually consists of tandem repeats of a guanine-rich sequence. For example, *Tetrahymena* telomeres have a T_2G_4 repeat sequence, *Oxytricha* and *Euplotes* telomeres have T_4G_4 and human T_2AG_3 (Zakian, 1989). The guanine-rich strand is always oriented in the 5′ to 3′ direction towards the end of the chromosome. The 3′ terminus of the guanine-rich strand protrudes as a single-stranded overhang in such evolutionarily divergent species as *Oxytricha*, *Euplotes*, *Tetrahymena*, *Didymium*, and *Saccharomyces* (Klobutcher et al., 1981; Henderson and Blackburn, 1989; Wellinger et al., 1993*a*,*b*). For example, the telomeric DNA has a $T_4G_4T_4G_4$ overhang in *Oxytricha nova*.

The diverse functions of telomeres are mediated in large part through the action of protein-containing factors that associate with telomeric DNA. Two classes of factors are known to interact with the very ends of telomeric DNA: telomerase and telomere protein.

Telomerase was first identified, characterized, and partially purified in *Tetrahymena thermophila* (Greider and Blackburn, 1985, 1987). It is a ribonucleoprotein complex and contains an internal RNA molecule with a sequence complementary to the telomeric repeat sequence. This region of telomerase functions as a template that directs addition of telomeric repeats to the single-stranded telomere overhang (Greider and Blackburn, 1989; Yu et al., 1990; Yu and Blackburn, 1991). Subsequently, telomerase activity was detected in the hypotrichous ciliated protozoans *Oxytricha and Euplotes*, which are only distantly related to *Tetrahymena* (Zahler and Prescott, 1988; Shippen-Lentz and Blackburn, 1989), in a human cell line (Morin, 1989), and in several different mouse cell lines (Prowse et al., 1993). Telomerase RNA genes have been cloned from seven species of tetrahymenine ciliates, seven different hypotrichous ciliates and two species of yeast, all of which contain template sequences complementary to their telomere sequences (Shippen-Lentz and Blackburn, 1990; Romero and Blackburn, 1991; Lingner et al., 1994; Melek et al., 1994; Singer and Gottschling, 1994; McEachern and

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Blackburn, 1995). Telomerase-mediated telomere synthesis resolves the problem of incomplete DNA replication by the semiconservative DNA replication machinery (Watson, 1972). Whether telomerase is coordinated with this replication machinery is just starting to be addressed (Wellinger et al., 1993*a*,*b*).

Telomeric DNA interacts with structural proteins (reviewed in Fang and Cech, 1995). In *Oxytricha nova*, a telomere protein consisting of α (56 kD) and β (41 kD) subunits binds specifically to each single-stranded $T_4G_4T_4G_4$ overhang, and therefore protects the macronuclear chromosomal ends from nucleolytic degradation (Gottschling and Zakian, 1986; Price and Cech, 1987, 1989; Raghuraman and Cech, 1989). Genes encoding each subunit have been cloned, sequenced and overexpressed in *Escherichia coli*, and DNA–protein and subunit–subunit interactions have been studied in detail (Hicke et al., 1990; Gray et al., 1991; Fang et al., 1993; Fang and Cech, 1993*a*,*b*,*c*; Hicke et al., 1994). In *Euplotes crassus*, a 51-kD telomere protein related to the *Oxytricha* α subunit also binds to and protects the single-stranded telomeric overhang (Price, 1990; Price et al., 1992). Similarly, a *Xenopus* activity has been found to bind specifically to the vertebrate single-stranded telomere repeats (Cardenas et al., 1993). Thus, terminus-specific telomere proteins seem to be phylogenetically widespread, although we do not yet know whether they are evolutionarily related.

Hypotrichous ciliates such as *Oxytricha* and *Euplotes* provide particularly facile systems for studying telomere structure and function due to their large number of telomeres. They have two kinds of nuclei in a single cell (Prescott, 1994). The micronucleus is the germline nucleus and contains chromosomes of conventional size. The macronucleus is the equivalent of a somatic nucleus with millions of gene-sized chromosomes (~24 million with an average length of 2.2-kb pairs in *Oxytricha nova*). The macronucleus divides amitotically during vegetative growth. This nucleus is responsible for the vast majority, if not all, of RNA synthesis during vegetative growth. One special advantage of hypotrichous ciliates for cytological studies is the ease with which S phase cells can be identified morphologically in a nonsynchronous population. In *Oxytricha*, the macronuclear DNA replication machinery is organized into a disc-shaped structure. This structure, called the replication band, initiates at one tip of the macronucleus and migrates toward the other end; the front zone contains more tightly packed chromatin, while DNA synthesis occurs in the leading portion of the diffuse rear zone (reviewed in Olins and Olins, 1994; Prescott, 1994). When the two macronuclei of a cell each have a replication band, the bands initiate from opposite tips and move towards each other as revealed by DNA labeling and autoradiography (Prescott, D. M. , personal communication).

To understand the distribution of the various telomere factors in the context of the structure of the nucleus and to begin to investigate how these factors are coordinated during DNA replication, we have undertaken a cytological study. We show that the *Oxytricha* telomere protein and DNA have a dispersed but non-uniform distribution in the macronucleus as judged by indirect immunofluorescent antibody staining. In contrast, telomerase RNA is localized in numerous spherical foci throughout the macronucleus as determined by in situ hybridization (Pardue and Gall, 1970) using a biotinylated anti-telomerase RNA oligonucleotide. Surprisingly, these foci appear to contain neither macronuclear DNA nor telomere protein. Intense staining of telomerase RNA at the replication band suggests that telomeres are extended in concert with semiconservative DNA replication, and the detailed distribution of telomerase RNA in some replication bands suggests that telomerase recruitment is an early event in the replication process.

Materials and Methods

Growth of Ciliates and Preparation of Macronuclei for Cytological Studies

Cultures of *Oxytricha nova* and *Euplotes aediculatus* were grown using live *Chlorogonium* as the food source (Swanton et al., 1980). 10 to 14 h before experiments, the culture was cleaned by exchanging medium and cells were fed with excess *Chlorogonium*. Immediately before collection, 2 liters of cells were filtered through cotton and then concentrated to 20 ml by filtration through a 2 μm Nitex nylon membrane (Tetko, Inc., Lancaster, NY). Cells were mixed with 20 ml of 2× PHEM (120 mM Pipes, 50 mM Hepes, 10 mM EGTA and 2 mM $MgCl_2$, pH 6.9) containing either 0.2 or 1% Triton X-100 (Schliwa and Van Blerkom, 1981; Olins et al., 1989), incubated at room temperature for 5 min and fixed with 4% (final concentration) freshly made paraformaldehyde at room temperature for 30 min. The crude nuclei were transferred to a 50 ml tube containing 5 ml of saturated sucrose-PBS solution (11.5 g sucrose/10 ml PBS, 145 mM NaCl, 100 mM sodium phosphate, pH 7.5) at the bottom of the tube, and macronuclei were purified by centrifuging at 9,000 *g* and 4°C for 10 min. The pellet was resuspended in 10 ml of PBS and centrifuged again at 700 *g* and 4°C for 5 min. The purified macronuclei were resuspended in 500 μl of 0.5% BSA in PBS and stored at 4°C. In the case of *Euplotes*, the same protocol results in macronuclei still associated with cytoskeleton.

In some experiments, cells were lysed with 20 ml of 2× PH (120 mM Pipes, and 50 mM Hepes, pH 6.9) containing 0.2% Triton X-100. The different concentration of Triton or different buffer (2× PHEM vs 2× PH) did not affect the subnuclear distribution of telomerase and telomere protein (data not shown).

Antisera and Oligonucleotides

Recombinant α and β subunits of the *Oxytricha* telomere protein were expressed in *E. coli* and purified to homogeneity as described previously (Fang et al., 1993). Rabbit polyclonal serum against either α or β was generated by Macromolecular Resources at Colorado State University (Ft. Collins, CO). The mouse monoclonal anti–DNA antibody DNA 5 was derived from mice with systemic lupus erythematosus and is a IgG2A (Marion et al., 1982); it was a gift from Dr. Tony Marion (Univ. of Tennessee, Memphis, TN) and has been used previously to stain DNA in cytological preparations (Klymkowsky and Karnovsky, 1994). Affinity-purified lissamine rhodamine–, Texas red– or fluorescein-conjugated donkey anti–rabbit antibodies with minimal cross-reactivity with mouse serum proteins were purchased from Jackson ImmunoResearch Laboratory, Inc. (West Grove, PA). Affinity-purified fluorescein-conjugated donkey anti–mouse antibody and lissamine rhodamine– or Texas red–conjugated streptavidin were purchased from the same company. Biotinylated and fluorescein-conjugated oligonucleotides (Table I) were made on a DNA synthesizer (380B; Applied Biosystems) with the use of a LC biotin-on phosphoramidite and a fluorescein-on phosphoramidite (Clontech, Palo Alto, CA), respectively. 2′-*O*-allyl modified RNA (BTELO2 and Bcon; Table I) were made from allylribonucleoside phosphoramidites (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Western Blots

O. nova and *E. aediculatus* were lysed in PH buffer with 0.1% Triton X-100, and macronuclei were purified by centrifuging through saturated sucrose solution as described above. (Since PHEM is a microtubule-stabilizing buffer [Swanton et al. 1980], PH buffer was used to lyse cells in order to increase the efficiency of subsequent nuclear protein extraction.)

After PBS wash, the macronuclear pellet was resuspended in 3 vol of TMNP buffer (50 mM Tris/Cl, pH 7.5, 10 mM $MgCl_2$, 0.35 M NaCl, and 1 mM PMSF) and proteins were extracted in a Dounce tissue grinder at 4°C. (Although efficiency of nuclear protein extraction was found to increase at higher NaCl concentrations, there was also more proteolytic degradation of the telomere protein.) The sample was spun in an Eppendorf centrifuge at full speed for 10 min at 4°C and the supernatant (nuclear extract) was saved. The nuclear proteins were separated by 4–20% gradient SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Corp., Arlington Heights, IL) in a semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Cambridge, MA). The protein blot was analyzed using the ECL Western blotting detection system (Amersham Corp.) with a primary antiserum dilution of 1:1,000 and a secondary antibody dilution of 1:10,000.

Northern Blots

O. nova and *E. aediculatus* were lysed in PHEM buffer with 0.1 or 0.5% Triton X-100 and macronuclei were purified by centrifuging through saturated sucrose solution as described above. Nuclear RNA was prepared using TriReagent (Molecular Research Center, Inc., Cinncinati, OH) according to the manufacturer's instructions. 1 μg of RNA was analyzed by 8% polyacrylamide-8 M urea gel electrophoresis or 1% agarose–0.66 M formaldehyde gel electrophoresis, and then transferred and UV–cross-linked to a Zetabind nylon membrane (Life Science Products, Inc., St. Petersburg, FL). After blocking in 5× SET (1× = 30 mM Tris·Cl, pH 7.8, 150 mM NaCl, and 2 mM EDTA), 5× Denhardt's solution, 10% dextran T500 (Pharmacia Fine Chemicals, Piscataway, NJ) and 100 mg/ml yeast total RNA (Sigma Chemical Co., St. Louis, MO) at 45°C for 4 h, the blot was incubated with 0.17 μg/ml biotinylated oligonucleotide (BTELO1 or BTELO2) in the same solution at 45°C for 16 h. The blot was washed three times with 2× SET for 5 min each and three times with 0.1× SET for 20 min each at 45°C. The biotinylated probes were detected through biotin–streptavidin interactions using the chemiluminescent Phototope Detection Kit (New England Biolabs, Beverley, MA). The use of a streptavidin-based detection system provided a close parallel to the detection method used in the fluorescent in situ hybridization experiments.

Fluorescent Antibody Staining

Because rapidly growing *Oxytricha* are filled with green algae, which autofluoresce, isolated macronuclei were used for staining experiments. Aliquots of fixed macronuclei were smeared onto saline coated microscope slides (Polysciences, Inc., Warrington, PA) and the smears were air-dried and re-hydrated with 1% BSA in PBS (1% BSA/PBS). Slides were incubated with 200 μl of the rabbit polyclonal sera against the α or β subunit of the *O. nova* telomere protein, diluted 1:50 (for α) or 1:200–1:400 (for β) with 1% BSA/PBS, in a moist chamber for 20–24 h at 37°C. (Early experiments with short incubation times [1–4 h] resulted in fluorescence mainly at the macronuclear periphery. The longer incubation times were needed to allow the antibodies to penetrate uniformly into the macronucleus.) After rinsing with 1% BSA/PBS, samples were washed four times with the same solution for 10 min each at room temperature. Slides were then incubated with 200 μl of 7.5 μg/ml fluorescent dye–conjugated donkey anti–rabbit antibody in 1% BSA/PBS in the moist chamber for 20–24 h at 37°C and then washed as described above. Anti-DNA antibody staining was done similarly except that the primary antibody was diluted 1:5–1:20 from the tissue culture supernatant containing the anti-DNA antibody. In the double staining experiments, two sets of antibodies were incubated consecutively in the order of first primary antibody, first secondary antibody, second primary antibody and second secondary antibody.

Fluorescent In Situ Hybridization

Fixed macronuclei were smeared onto microscope slides as described above. After prehybridizing in 100 μl of 5× SET, 5× Denhardt's solution, 10% dextran T500, 100 mg/ml yeast total RNA and 0.17 mg/ml oligonucleotide NSC (a nonspecific competitor, 5′ AAGACGACATCGCTCAGCCAGACAGACGACATCGCTCAGC 3′) in a moist chamber for 2–3 h at 45°C, samples on slides were hybridized with biotinylated or fluorescein-conjugated probes in the same solution at 45°C for 16 h at a probe concentration of 3.4 mg/ml except for BTELO2 (1.7 μg/ml). Slides were washed three times with 2× SET and once with 0.1× SET at room temperature for 5 min each, and then three times with 0.1× SET in the moist chamber at 45°C for 20 min each. Samples were incubated with 200 μl of 5 μg/ml lissamine rhodamine– or Texas red–conjugated streptavidin in the moist chamber at room temperature for 1 h and washed four times with 1% BSA/PBS at room temperature for 10 min each. (Incubation with streptavidin for 2 h did not change the pattern of fluorescence.) Double staining with antibodies was carried out in the following order: in situ hybridization, primary antibody incubation, secondary antibody incubation, and streptavidin binding.

Confocal Laser Scanning Microscopy

Cytological samples were stained as described above and mounted in Aqua-Poly/Mount solution containing 50 mg/ml 1,4-diazabicyclo(2,2,2)octane (DABCO) (Polysciences, Inc.). Fluorescence was observed under the confocal laser scanning microscope using a 60× objective lens (Multi-Probe 2001; Molecular Dynamics, Eugene, OR). The image was scanned under settings such that the signal was not over-saturated and presumably was in the linear range of the signal responsive curve. The relative intensity of fluorescence was quantitated using the ImageSpace software (Molecular Dynamics Inc.). Fluorescence in replication bands relative to adjacent regions of the nucleus was quantitated along several different lines; the separate measurements typically agreed within ±10% of the average for a given nucleus, and the agreement was ±20% for separate nuclei.

Results

Telomere Protein and Macronuclear DNA Have a Dispersed Distribution with More Staining at the DNA Replication Band

Rabbit polyclonal antisera were raised against purified α and β subunits, respectively, of the *Oxytricha* telomere protein expressed in *E. coli*. The specificity of each antiserum was assessed by Western blot analysis (Fig. 1 *A*). The α and β subunits were major protein components in an ex-

Table I. In Situ Hybridization Experiments with O. nova

Probe	Sequence*	Relationship to telomerase RNA	(nt)‡	In situ hybridization§		
				Standard conditions	RNase A_{pre}‖	RNase A_{post}¶
BTELO1	d(ABGTTTTGGGGTTTBA)	Antisense	(38–49)	+	−	Reduced signal
BETLOcon	d(ABAAACCCCAAAACCGTABA)	Sense	(38–53)	−	ND	ND
BTELO2	BB**ACGGUUUUGGGGUUUU**BBdG	Antisense	(38–52)	+	−	Reduced signal
Bcon	BB**UAAAGUCCCCAGUAC**BBdT	None		−	ND	ND
BTELO3	d(ABAGTTTACGGTTTTGBA)	Antisense	(44–57)	−	ND	ND
BTELO/E	d(ABAAT/ATGCTCTG/CAGTT/GTACBA)	Antisense	(51–67)	−	ND	ND

*B, biotinylated linkage; *d*, deoxyribonucleotides; BTELO2 and Bcon contain 2′-O-allyl-modified RNA (bold in the sequences). Degeneracy was introduced into BTELO/E so it can hybridize to both *Oxytricha* and *Euplotes* telomerase RNA; however, staining was negative in both cases.

‡Nucleotides of *Oxytricha* telomerase RNA to which the probe is complementary (or identical). Nucleotides numbered in Lingner et al. (1994); template extends from nt 37 to ≥44.

§+, strong signal intensity; −, no hybridization; ND, not determined; Reduced signal, signal intensity reduced four- to fivefold.

‖In these experiments, macronuclei were incubated with RNase A and then fixed with paraformaldehyde.

¶In these experiments, fixed macronuclei were incubated with RNase A prior to in situ hybridization.

tract of *Oxytricha* macronuclei (lane *2*), as seen previously (Gottschling and Zakian, 1986; Price and Cech, 1987). The anti-α serum reacted predominantly with a protein whose gel mobility is indistinguishable from that of α (lane *3*). This serum also reacted weakly with a band that migrates slightly faster than α. Similarly, the anti-β serum predominantly reacted with the β subunit and weakly with a band of 33 kD (lane *4*). It has been shown previously that the amino-terminal region of α (19 amino acids) and the carboxyl-terminal domain of β can be degraded under some protein extraction conditions (Gottschling and Zakian, 1986; Price and Cech, 1987; Hicke, B., J. Gray, and T. R. Cech, unpublished results), so we speculate that the minor reactive species in lanes *3* and *4* are proteolytic products of the α and β subunits.

Intranuclear distribution of the *Oxytricha* telomere protein was examined by indirect immunofluorescent antibody staining and laser scanning confocal microscopy of fixed macronuclei (Fig. 2). Both the α and β subunits have a dispersed, but non-uniform staining pattern (panels *A-D*). Numerous foci are either unstained or stained much more weakly than the bulk of the macronucleus. These foci differ in size, ranging in diameter from less than 0.5 μm (*thin arrow*, panel *B*) to greater than 3 μm (*thick arrow*). Foci of different size coexist within one macronucleus. By scanning a series of sections of a nucleus along the z axis and constructing a three-dimensional model in the computer, the number of foci in a single macronucleus was found to vary from a few to greater than one hundred. [The lack of staining of the foci by these antibodies is not due to reduced accessibility, as an anti-trimethylguanosine antibody reacts strongly with the foci (Fang, G., and T. R. Cech, manuscript in preparation).]

In the S phase macronucleus, the replication band was more intensely stained (quantitated as approximately twofold more intense) than the bulk of the nucleus (Fig. 2 *B* and *D*, *arrowheads*). Thus, at the replication band the telomere protein is present at a higher concentration, is more accessible, or exists in a conformation that reacts more strongly with the antibodies. In some S phase nuclei, telomere–protein-minus foci were found in the middle of

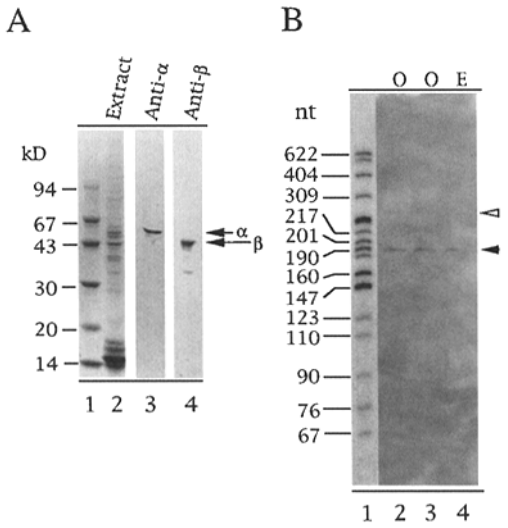

Figure 1. Specificity of the probes used in the cytological studies. (*A*) Telomere protein probes. *Oxytricha* nuclear proteins were extracted with Tris buffer containing 0.35 M NaCl, separated by SDS-PAGE and detected by staining with Coomassie blue (lane 2). Duplicate samples were transferred to nitrocellulose membranes and analyzed by Western blotting using anti-α (lane *3*) or anti-β (lane *4*) antisera. The positions where the α and β subunits migrate are marked on the right side of the figure. (*B*) Telomerase RNA probe. *Oxytricha nova* (lanes *2* and *3*) and *Euplotes aediculatus* (lane *4*) macronuclear RNA were separated by 8% polyacrylamide–8 M urea gel electrophoresis, transferred to a nylon membrane, and hybridized with biotinylated oligonucleotide BTELO1. The signal was detected using streptavidin-based chemiluminescence. RNA samples in lanes *2* and *3* were prepared from *Oxytricha* macronuclei isolated in the presence of 0.1 and 0.5% Triton X-100, respectively. The filled arrowhead points to the position of telomerase RNA and the open arrowhead points to a cross-hybridizing band. The size marker (lane *1*) was radiolabeled pBR322 cut with Msp I; DNA fragments were boiled for 5 min before loading.

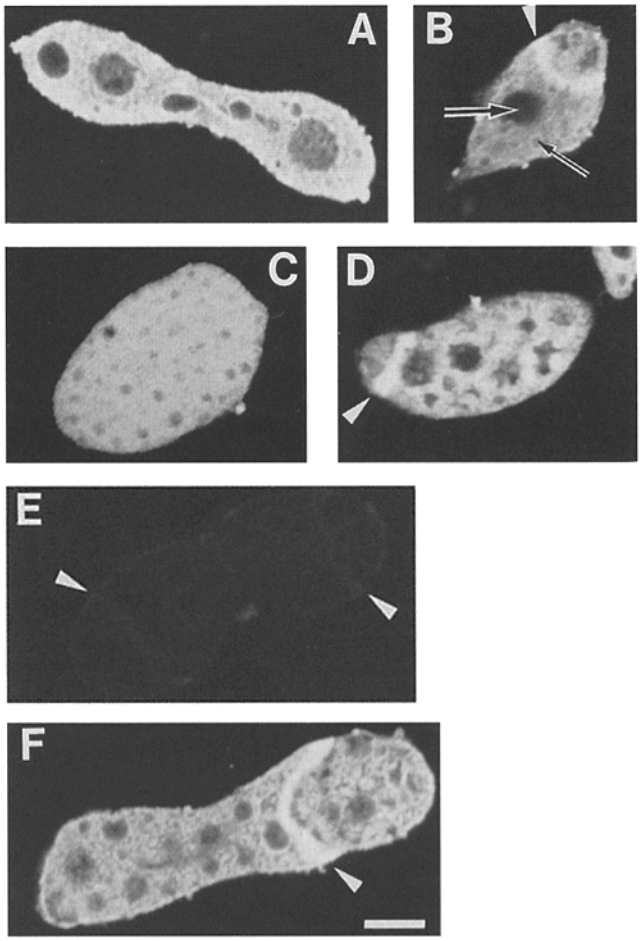

Figure 2. Dispersed but nonuniform distribution of the telomere protein and macronuclear DNA. Intranuclear distribution of the α subunit (*A* and *B*) and the β subunit (*C* and *D*) of the *Oxytricha* telomere protein was revealed by indirect fluorescent antibody staining. In a control staining (*E*), pre-immune serum (from the rabbit subsequently injected with the β subunit) was used. DNA staining (*F*) was done with a monoclonal antibody against DNA. Panels *B* and *D–F* show S phase nuclei with arrowheads pointing to replication bands. The thick arrow in *B* points to one of the larger telomere protein-minus foci and the thin arrow points to a smaller telomere protein-minus focus. All the panels have the same magnification. Bar, 5 μm.

the replication band, in which cases the band is not a continuous structure (data not shown). Series scanning and three-dimensional model building indicate that the replication band is a disc-shaped structure except at positions where such foci invade the band.

Neither the lissamine rhodamine–conjugated secondary antibody by itself nor the pre-immune sera gave rise to any staining signal (Fig. 2 *E*; data not shown). The antisera against α and β did not cross-react with any proteins in *Euplotes aediculatus* by fluorescent antibody staining of cytological preparations (data not shown), consistent with the absence of cross-reaction in Western blots (Hendrick, L. L., G. Fang, and T. R. Cech, unpublished results).

The *Oxytricha* macronuclear DNA has an intranuclear distribution similar to that of the telomere protein as revealed by fluorescent staining with a monoclonal anti-DNA antibody (Fig. 2 *F*). In the S phase macronucleus, the replication band is more intensely stained than the rest of the nucleus. The macronuclear DNA in *Euplotes aediculatus* has a similar distribution (data not shown). The DNA is dispersed throughout the *Euplotes* macronucleus with numerous foci lacking staining. Although the average size of the foci is smaller than that in *Oxytricha*, the number of foci per macronucleus is larger. As in *O. nova*, the replication band is more intensely stained than the rest of the nucleus in the S phase cell.

Telomere Protein and Macronuclear DNA Colocalize

The relative spatial distribution of the telomere protein and the macronuclear DNA was examined in pairwise antibody double staining (Fig. 3). Panel A shows the anti-α staining pattern and panel B shows the anti-DNA staining pattern. Superimposing these two patterns indicates that the α subunit and DNA colocalize in the macronucleus (Fig. 3 *C*). Similarly, the β subunit colocalizes with the DNA (Fig. 3, *D–F*). Thus the α subunit, the β subunit, and the macronuclear DNA have the same subnuclear distribution. This is not surprising since the average length of the macronuclear DNA is 2.2 kb (Klobutcher et al., 1981) and telomere protein is bound to both ends of each DNA molecule (Gottschling and Cech, 1984). Although all three molecules are more intensely stained at the replication band, whether they exactly colocalize remains to be examined.

Telomerase RNA Is Highly Localized

Surprisingly, telomerase RNA has a very different distribution from that of the telomere protein and macronuclear DNA. The intranuclear localization of telomerase RNA was revealed by in situ hybridization with either of two biotinylated oligonucleotides (BTELO1 or BTELO2) complementary to the template region. To optimize specificity the lengths of these probes were restricted to the small region of the telomerase RNA most accessible to hybridization in the ribonucleoprotein (Lingner et al., 1994). (Lengthening a probe with nucleotides that cannot hybridize because of inaccessibility can only decrease specificity.) The hybridization pattern was detected by fluorescent dye-conjugated streptavidin. In the non-replicating macronucleus the *Oxytricha* telomerase RNA was stained as numerous bright foci set in a diffuse general nuclear staining (Fig. 4 *A*). No background staining was seen with a sense oligonucleotide; we therefore believe the diffuse nuclear staining between foci is a real signal (compare panels *E* and *F*). The BTELO1 and BTELO2 antisense oligonucleotides gave the same staining pattern, as judged by double staining experiments with anti-telomere–protein antibod-

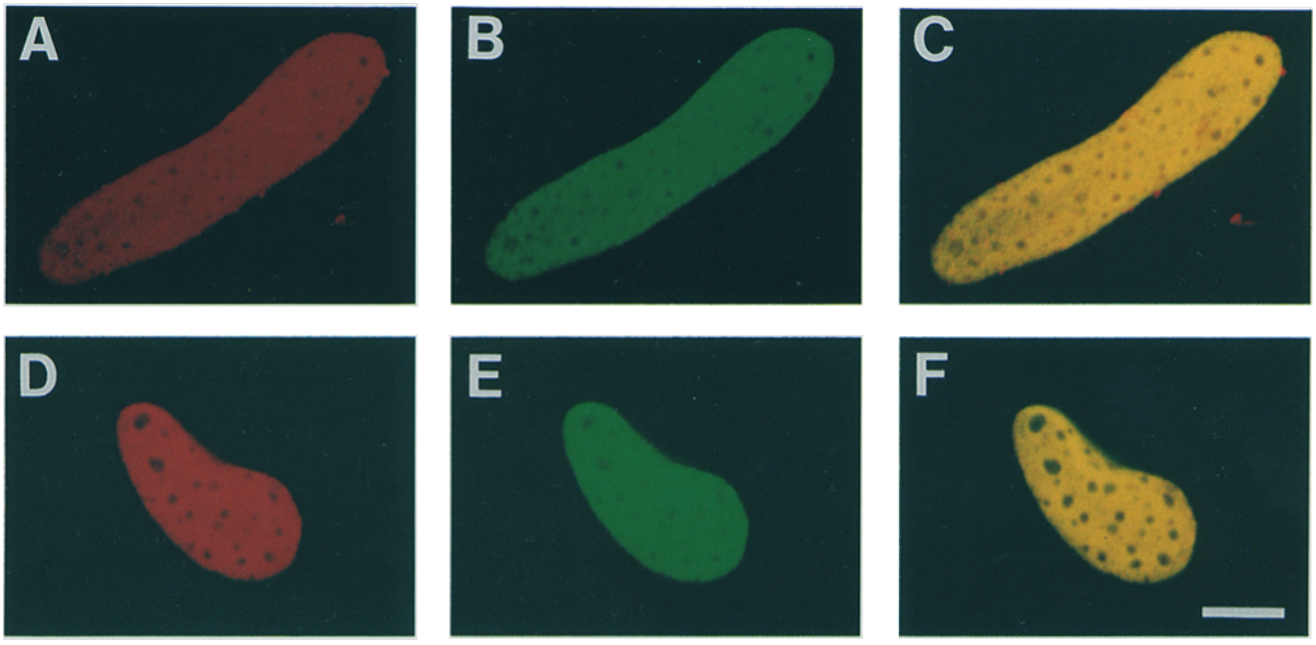

Figure 3. Colocalization of the telomere protein and macronuclear DNA. The α subunit (*A*) and the β subunit (*D*) were detected by indirect antibody staining with a Texas red–conjugated donkey anti–rabbit antibody, and the DNA (*B* and *E*) was similarly detected with a fluorescein-conjugated donkey anti–mouse antibody. Superimposing α and DNA staining of the same nucleus (*C*) and superimposing β and DNA staining (*F*) showed that the telomere protein colocalizes with the macronuclear DNA. All the panels have the same magnification. Bar, 5 μm.

ies (see Fig. 5; additional data not shown). Two other oligonucleotides complementary to different regions of the telomerase RNA gave no staining (BTELO3 and BTELO/E; Table I), consistent with the hypothesis that we are detecting telomerase RNA in its ribonucleoprotein form (see Discussion).

There are two classes of foci, one with a well-defined, brightly stained border (Fig. 4 *A*, *thick arrow*) and one with a diffuse border (*thin arrow*). Scanning serial sections revealed that the foci with the diffuse borders were not simply the edges of foci with well-defined borders. Like the telomere–protein-minus foci, the telomerase foci differ in size considerably and foci of different size coexist within one macronucleus. The number of foci also varies from nucleus to nucleus, ranging from a few to greater than one hundred (data not shown). In a fraction of the nuclei (~5–10%), we found one to four foci that stained three- to sixfold more brightly than the rest of the foci (Fig. 4 *D*).

In S phase nuclei, identified by the presence of a replication band or bands, the telomerase foci persisted (Fig. 4, *B* and *F*). Thus, the bulk of telomerase RNA has an intranuclear distribution that is constant during the cell cycle. Nor were the foci perceptibly changed upon passage of the replication band: their size, number per unit area, and staining intensity were similar in the pre-replicated and post-replicated portions of the nucleus. The replication band (Fig. 4 *B*, *arrowhead*) was approximately twofold more intensely stained than the foci and 5- ± 1-fold more intensely stained than the rest of the nucleus. (Mean ± range of values measured for eight replication bands; no background was subtracted, so these values may underestimate the enrichment of telomerase RNA in the replication band.) While more intense staining could indicate either a higher concentration of telomerase RNA or the telomerase RNA being more accessible, we consider the second possibility unlikely (see Discussion).

Euplotes aediculatus were prepared for in situ hybridization using the same protocol as for *Oxytricha*. With *Euplotes*, however, the cytoskeleton remains associated with the nucleus (Olins et al., 1989). Telomerase RNA is again distributed in numerous foci (Fig. 4 *G*). The average size of the *Euplotes* foci is smaller than that of *Oxytricha*, but the average number of foci within a single macronucleus is larger than that of *Oxytricha*. The hybridization signal in the germ-line micronucleus (Fig. 4 *G*, open arrowhead) in *E. aediculatus* is only marginally above the background signal found in the cytoplasm. We do not know whether the micronuclear signal is due to specific hybridization or not.

The in situ hybridization signal was removed by incubation of nuclei with RNase A prior to fixation (Fig. 4, compare panels *C* and *D*), and was reduced by incubation of fixed macronuclei with RNase A prior to in situ hybridization (Table I). Thus, the signal results from hybridization to RNA, not from hybridization to DNA or from binding of the probes by proteins. Fluorescent antibody staining showed that the telomere protein had a normal subnuclear distribution in these RNase-treated nuclei, suggesting that RNase A digestion did not perturb the global macronuclear structure (data not shown). The DNA and telomere protein staining patterns in the nonreplicating macronucleus do not overlap with the telomerase RNA pattern (see Fig. 5), confirming the conclusion that the in situ hy-

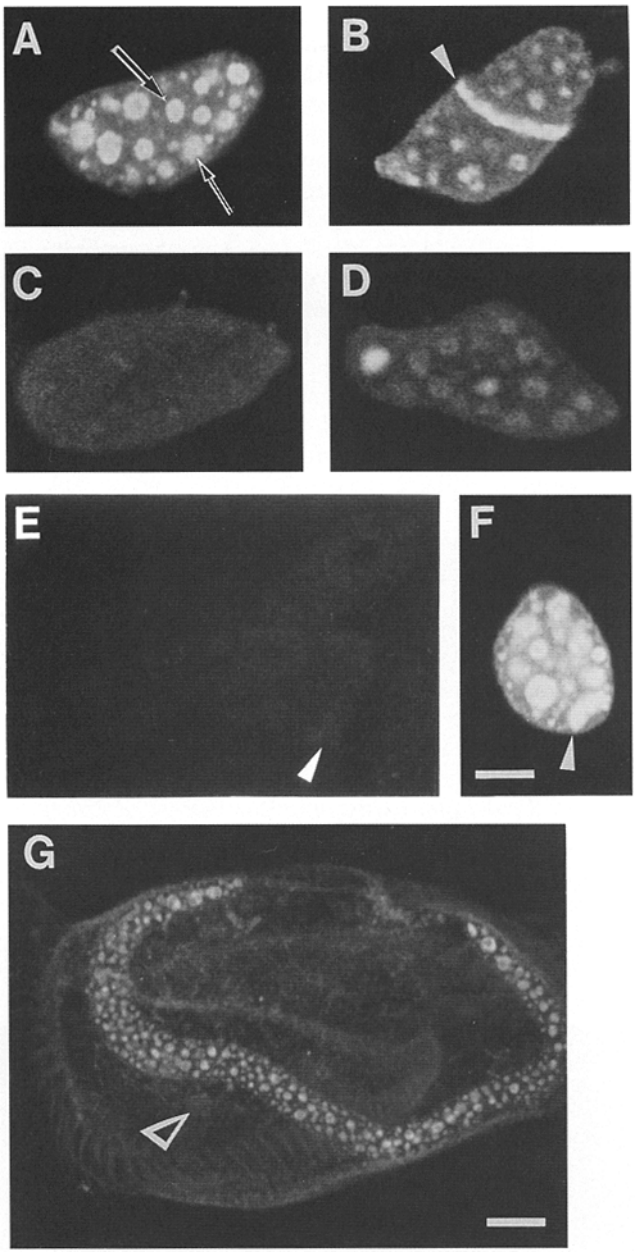

Figure 4. Telomerase RNA in replication bands and spherical subnuclear organelles. Intranuclear distribution of telomerase RNA was revealed by in situ hybridization with biotinylated oligonucleotides complementary to the template region of the telomerase RNA. Biotinylated oligonucleotides were detected by Lissamine Rhodamine-conjugated streptavidin. Panels *A–F* show *Oxytricha* macronuclei and have the same magnification. Hybridization with (*A*) BTELO1; (*B*) BTELO2; (*C*) RNase A digestion prior to fixation, then hybridization with BTELO2; (*D*) No RNase control for *C*, hybridized identically and observed under the confocal laser scanning microscope at the same settings; (*E*) BTELOcon sense-strand control; (*F*) BTELO1, prepared and scanned identically to *E*. Arrowheads in panels *B*, *E*, *F* point to the replication bands. (*G*), *Euplotes* macronucleus (the C-shaped structure), micronucleus (open arrowhead), and associated cytoskeleton. Hybridization with BTELO2. Bars: (*F*) 5 μm; (*G*) 10 μm.

bridization signal is not due to hybridization to DNA or binding to the telomere protein.

In situ hybridization with either a sense oligonucleotide or a nonspecific oligonucleotide (Table I) did not show any staining signal including the replication band (Fig. 4, compare *E* and *F*, *arrowheads* point to replication bands), indicating that in situ hybridization by BTELO1 and BTELO2 is sequence specific. Northern blot analysis with *Oxytricha* total macronuclear RNA, using the same hybridization temperature and analogous streptavidin-based detection used in the cytological hybridization, supports the conclusion that hybridization is mainly to telomerase RNA. The oligonucleotide BTELO1 only hybridized to one major band around 190 nucleotides (nt)[1] in length (Fig. 1 *B*, lanes *2* and *3*; *filled arrowhead*) and one minor band around 260 nt (*open arrowhead*). Reprobing with the entire telomerase RNA gene under stringent conditions indicated that the 190-nt band is the telomerase RNA (Hendrick, L. L., G. Fang, and T. R. Cech, data not shown).

1. *Abbreviation used in this paper*: nt, nucleotide.

Northern blot analysis of the same RNA separated on a 1% agarose–0.66 M formaldehyde gel to look for higher molecular weight cross-hybridizing species revealed no additional hybridization (data not shown). BTELO2 also hybridized to the 190-nt band, but not to the 260-nt band, although BTELO2 cross-hybridized to a set of additional bands (data not shown). The fact that both BTELO1 and BTELO2 gave the same staining pattern in fluorescent in situ hybridization indicates that these two probes recognize the same molecule in situ, i. e., telomerase RNA. For *E. aediculatus* macronuclear RNA, BTELO1 hybridized to a band of 190 nt (telomerase RNA) on a 8% polyacrylamide-urea gel (Fig. 1 *B*, lane *4*) and an additional band of 1,400 nt on a 1% agarose-formaldehyde gel (data not shown).

Telomerase RNA Colocalizes with Telomeres Mainly in the Replication Band

The relative spatial distribution of telomerase RNA, telomere protein and macronuclear DNA was examined in double staining experiments. Fig. 5 *A* shows the *Oxytricha*

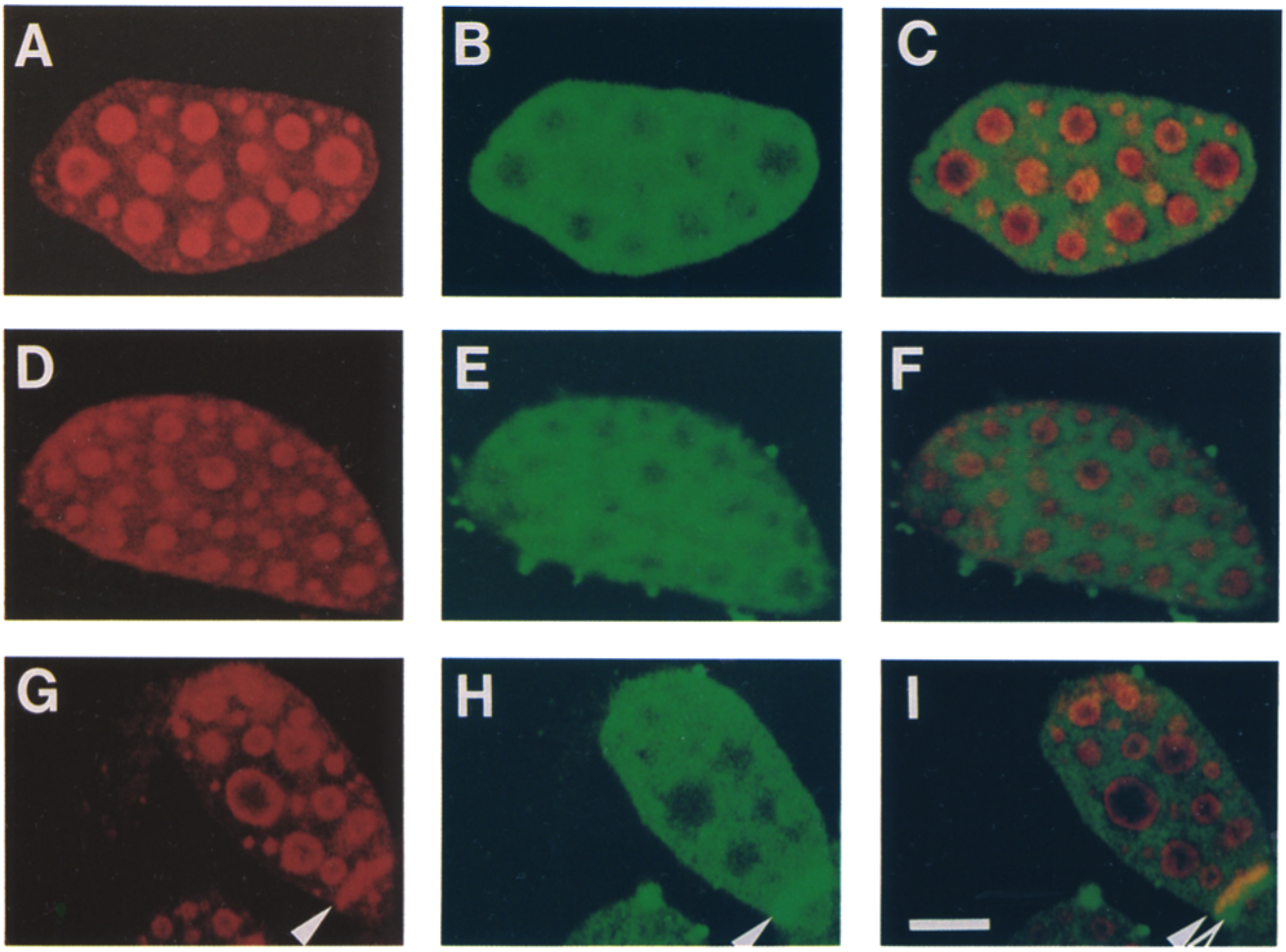

Figure 5. *Oxytricha* telomerase RNA colocalizes with telomeres mainly in the replication band. *Oxytricha* telomerase RNA was hybridized with BTELO2 (*A* and *D*) or BTELO1 (*G*) and detected by Texas red–conjugated streptavidin. Macronuclear DNA (*B*) and the α (*E*) and β (*H*) subunits of the telomere protein were stained with respective primary antibodies and fluorescein-conjugated secondary antibodies. Superimposing two staining patterns of a single macronucleus (*C*, *F*, and *I*) shows that the telomerase foci do not contain telomeres. Filled arrowheads point to the replication band and the open arrowhead points to the region of the replication band that stains for telomere protein but not telomerase RNA. All the panels have the same magnification. Bar, 5 μm.

telomerase RNA distribution and panel *B* shows the macronuclear DNA staining pattern. Superimposing the distributions of telomerase RNA and DNA indicates that they are spatially complementary to each other (panel *C*). The same distribution patterns of telomerase RNA and macronuclear DNA were also observed in *E. aediculatus* (Fig. 6). Thus, the telomerase foci do not contain detectable macronuclear DNA. Similarly, the distribution of the *Oxytricha* telomere protein complements that of the telomerase RNA (Fig. 5, *D–F*).

In the S phase nucleus, both *Oxytricha* telomerase RNA and telomere protein are more intensely stained at the replication band (*arrowheads*, Fig. 5, *G* and *H*). Superimposing the staining patterns of these two molecules showed three patterns of their relative distribution. In the first pattern, telomerase RNA overlapped only partially with the telomere protein at the replication band (panel *I*; *filled arrowhead* points to the overlapping region and *open arrowhead* points to the telomere protein that does not overlap with telomerase RNA in the replication band). Occasionally, two macronuclei were fixed in the end-to-end orientation found in the cell, so the direction of migration of their replication bands could be ascertained. In addition, a small fraction of the macronuclei contained two replication bands, one at each end of the nucleus; in this case we infer that the bands are migrating toward the middle of the nucleus by analogy to *Euplotes*, where replication bands come in pairs and are known to converge (Prescott, D. M., personal communication). In the cases where we observed two replication bands, the distribution of the telomerase RNA and telomere protein were not completely overlapping, as in Fig. 5 *I*, and the telomerase RNA was invariably found in the front edge of the replication band in the direction of band migration (data not shown). Here, "front edge" is relative to the densely stained band of telomere protein and DNA. Although front and rear zones of the replication band have been defined by ultrastructural studies (Olins and Olins, 1994), the limited resolution of light microscopy precluded assignment of the location of the telomerase RNA band and the telomere protein band to these previously defined zones.

In a second type of distribution, telomerase RNA and telomere protein were spatially separated but adjacent to each other in the replication band (data not shown). Replication bands with this kind of pattern were usually found at or near the tips of nuclei. We also found replication bands in which telomerase RNA and telomere protein colocalized, usually with replication bands located in the middle of the nucleus. Thus, we observed a correlation between the extent of distance the band migrated and the pattern of distribution of telomerase RNA and telomere protein. At the tip of the nucleus where the replication band initially forms, the telomerase RNA is separated from the telomere protein. As the band migrates, these two molecules partially overlap and eventually overlap completely. These observations suggest that the replication band is a dynamic structure. It may undergo some sort of assembly or reorganization during migration. Alternatively, because the distribution of the various macromolecules in the replication band depends on the kinetics of their migration, a change in the rate of movement of telomerase relative to other components of the replication band provides a reasonable hypothesis to explain the dynamics.

Figure 6. Most telomerase RNA does not colocalize with macronuclear DNA in *Euplotes aediculatus*. *Euplotes* telomerase RNA was hybridized with BTELO2 and detected by Texas red–conjugated streptavidin, and macronuclear DNA was stained with a monoclonal anti–DNA antibody and a fluorescein-conjugated secondary antibody. This figure shows superimposition of the telomerase staining (*red*) and DNA staining (*green*). Bar, 10 μm.

Discussion

Differential Distribution of Telomerase and Telomere Protein

The telomere protein binds to the single-stranded telomeric DNA overhangs, protecting telomeres from degradation. Telomerase elongates telomeric DNA to ensure complete replication of chromosomes. The only organisms in which both of these telomeric factors have been identified and well characterized at the biochemical level are the hypotrichous ciliates. However, due to the lack of genetic approaches for studying telomere functions in these ciliates, the functions and the dynamics of these telomere components in vivo remain largely unknown. We have begun to address these questions by cytological methods, and find that these two telomere factors have very different subnuclear distributions.

The *Oxytricha* telomere protein (both α and β subunits) and macronuclear DNA are dispersed throughout the macronucleus as revealed by indirect immunofluorescent antibody staining. This correlates with the large number of telomeres per macronucleus (48 million) and the small size of chromosomal DNA (2.2 kb on average; Klobutcher and Prescott, 1986). However, their distribution is not uniform. Numerous spherical foci (typically 0.5–3 μm in diameter) were found to lack both telomere protein and DNA. Pairwise double staining indicates that the telomere protein and macronuclear DNA colocalize. This is not surprising

since two telomeric protein/DNA complexes are formed on every 2.2-kb DNA, which as a chain of nucleosomes would have an extended length ~0.1 μm.

Our most surprising finding is that the telomerase RNA does not colocalize with telomeres, but is instead enriched in the DNA-minus foci. The specificity of the in situ hybridization by the telomerase antisense oligonucleotides (BTELO1 and BTELO2) was demonstrated in several ways: the hybridization signal was removed or reduced by RNase A digestion prior to hybridization, a sense probe and a nonspecific probe did not give any staining, and Northern blot analysis showed that the only RNA to which both probes hybridize was the telomerase RNA. There remains the concern that in the cytological preparations most of the telomerase RNA might be hidden from the hybridization probes, so that the observed signal is due to a complementary sequence in some other abundant RNA. This seems extremely unlikely because in nuclear extracts the template region of the *Oxytricha* telomerase RNA is quantitatively accessible to hybridization, unlike other regions of the ribonucleoprotein (Lingner et al., 1994).

Although the antisense probes only detect the RNA component of telomerase, biochemical analysis suggests that the majority of telomerase RNA is assembled into a single type of RNP complex, presumably the telomerase holoenzyme (Lingner, J., L. L. Hendrick, and T. R. Cech, unpublished results). When *O. nova* macronuclear extracts were separated by nondenaturing polyacrylamide gel electrophoresis, transferred to a nylon membrane and probed with the telomerase RNA gene, the telomerase RNA was found in a discrete band with an electrophoretic mobility slower than that of free telomerase RNA. The extent of telomerase activity in the nuclear extract correlated with the amount of the RNA in this slow migrating band, suggesting that this shifted band corresponds to active telomerase. Thus, it is likely that the antisense probes not only reveal the telomerase RNA, but also locate the telomerase holoenzyme. A critical test of this hypothesis awaits availability of antibodies against telomerase protein subunits. Antisense probes to two other regions of telomerase RNA, regions which are accessible in the purified RNA but not very accessible in the RNP (Lingner et al., 1994), gave no cytological hybridization; this is consistent with most of the cellular telomerase RNA being assembled into the RNP.

Oxytricha contain half a million telomerase RNA molecules per macronucleus (Lingner, J., L. L. Hendrick, and T. R. Cech, unpublished results), which corresponds to one telomerase every 100 telomeres on average. Based on the fluorescence intensities, we now conclude that this ratio varies widely among different parts of the nucleus. The ratio of telomerase RNA to telomeres is extremely high (perhaps infinity) in the foci. Furthermore, the ratio is considerably higher in the replication band than in nonreplicating chromatin.

Telomerase in the Replication Band

Although the mechanism of telomerase is being revealed in molecular detail (Blackburn, 1992), little is known about the coordination of telomerase action with the semiconservative replication of the bulk of the chromosome. *Saccharomyces* telomeres are extended late in S phase, and activation of the origin of replication precedes extension of the telomeric G-rich strand on a linear plasmid (Wellinger et al., 1993*a*,*b*). These experiments argue that DNA replication immediately precedes telomerase action. An attractive model for such temporal ordering postulates that the two events are linked, so that telomerase is recruited or triggered to act upon completion of DNA replication. Now, in a distant organism we provide a very different sort of evidence that DNA replication and telomerase action are coordinated: we find that telomerase RNA is concentrated near the site where DNA replication is occurring.

In hypotrichs, the macronuclear DNA replicates in a cytologically distinctive replication band (reviewed in Prescott, 1994; Olins and Olins, 1994). We found that the telomerase RNA is 5- ± 1-fold more intensely stained in the replication band than in other chromatin-containing portions of the nucleus, whereas the enhancement for telomere protein and macronuclear DNA is approximately twofold (compare Fig. 4 *B* with Fig. 2, *B*, *D*, and *F*). These measurements support the conclusion that telomerase is enriched in the replication band by an amount significantly greater than can be explained by the enrichment of chromatin in the band. Brighter telomere protein staining could result from increased accessibility of the antigenic epitopes (free α and β vs α and β in a α/β/DNA complex), a higher concentration of DNA and telomere protein in the replication band, or both. We suggest that the brighter telomerase RNA staining is probably due to a higher concentration of telomerase enzyme, since if anything active telomerase should have telomeric DNA annealed to the template region of its RNA subunit and therefore have a reduced accessibility to the antisense oligonucleotides.

By scanning serial sections of a S phase macronucleus along the z-axis and computer-aided reconstruction of the three-dimensional model, we found that the replication band is a disc-shaped structure, running through the nucleus from top to bottom. When the replication band migrates through the telomerase foci, the foci (visualized by telomerase RNA staining) simply fuse with the replication band and then pass through it. The foci seem to neither disintegrate nor change their morphology. When visualized by telomere protein staining, the foci actually disrupt the telomere protein in the replication band and cause a protein-minus hole in the staining pattern.

It seems reasonable that both telomerase and telomere protein may act in the replication band. Telomerase extends the telomeric repeats to ensure the complete replication of chromosomal ends. Telomere protein binds to the newly synthesized telomeres and protects them from nucleolytic degradation. How are these two telomere factors coordinated? Double staining of macronuclei for telomerase RNA and telomere protein indicates that these two telomere factors have a different distribution within the replication band. In a fraction of macronuclei in which the replication band has not migrated very far, the telomerase RNA band lies in front of the telomere protein band. Thus, telomerase appears to be in position prior to DNA replication, which takes place in the leading portion of the rear zone of the replication band. Its enzymatic ac-

tion presumably occurs later, after DNA replication has produced a shortened telomere.

Telomerase Foci

Double staining of telomerase RNA and macronuclear DNA, or of telomerase RNA and the telomere protein, indicates that telomerase RNA has a distribution spatially complementary to that of the telomeres except at the replication band. Thus, we conclude that the telomerase foci do not contain telomeres. It is therefore unlikely that the telomerase in these foci is actively involved in telomere elongation, although we cannot exclude the possibility that telomerase at the surface of the foci actually synthesizes telomere repeats. The foci could have any of a number of functions, ranging from assembly, maturation and transport of the telomerase RNP to storage of the enzyme. In any case, it now appears that even in the S phase nucleus only a small fraction of the telomerase could be functionally active at any given time, assuming that the in situ hybridization signal is roughly proportional to the amount of telomerase. It will be interesting to determine whether telomerase in the foci differs in protein composition, post-translational modification or RNA modification from that residing in other portions of the nucleus.

From the beginning, it seemed improbable that the foci, which occupy so much of the nuclear volume, would be dedicated to telomerase. Indeed, we have recently found 2,2,7-trimethylguanosine-capped RNAs (presumably snRNAs) in the same foci as the telomerase RNA (Fang, G., and T. R. Cech, manuscript in preparation). However, the relative concentration of these molecules varies in different foci. The staining of the foci with the anti-trimethylguanosine antibody relates the foci to "speckles" (interchromatin granules) and coiled bodies in somatic nuclei of mammalian cells and the snurposomes and sphere organelles in oocyte nuclei. All these organelles contain the splicing snRNPs (Gall, 1991; Spector, 1993; Lamond and Carmo-Fonseca, 1993), and speckles and snurposomes also contain non-snRNP proteins involved in mRNA splicing (Fu and Maniatis, 1990; Roth et al., 1990; Wu et al., 1991). Our RNP foci (0.5 to <3 μm) are similar in diameter to amphibian oocyte snurposomes, reported to range from <1 μm to ~20 μm (Gall, 1991). It has been argued that the mammalian and amphibian subnuclear organelles may function in the import, assembly, and storage of RNA processing components, but are not themselves sites of processing (Bauer et al., 1994; Zhang et al., 1994; Mattaj, 1994). This appears to be in direct analogy to the telomerase-containing foci in the hypotrichs, which may function in the assembly, storage or transport of the telomerase RNP, but are not themselves sites of telomere extension.

Future work should enable the ciliate and metozoan sub-nuclear organelles to be correlated more precisely. Additional molecular probes may allow the telomerase RNA-containing foci to be identified as ciliate versions of coiled bodies or snurposomes or both. In the other direction, it will be informative to determine whether mammalian and other non-ciliate telomerases are concentrated in specific sub-nuclear organelles. It has been difficult to explain the reorganization of speckles induced by viral infection/viral proteins in terms of requirements for the splicing snRNPs (Mattaj, 1994). Perhaps the reorganization is needed to mobilize telomerase or some other non-splicing RNP, and the snRNPs are redistributed as a secondary consequence.

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