The telomerase-associated protein p43 is involved in anchoring telomerase in the nucleus

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Accepted 7 January 2003
Journal of Cell Science 116, 1757-1761 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00351

Summary

Telomere replication of eukaryotic chromosomes is achieved by a specialized enzyme, the telomerase. Although the biochemistry of end-replication is well understood, little is known about the organization of the end-replication machinery, its regulation throughout the cell cycle or the biological function of the telomerase-associated proteins. Here we investigate the function of the telomerase-associated protein p43 within the macronucleus of the ciliated protozoa *Euplotes*. It has been shown that p43 binds in vitro to the RNA subunit of telomerase and shares homology with the La autoantigen family. It therefore has been suggested that it is involved in the assembly and/or nuclear retention of telomerase. We show that the p43-telomerase complex is bound to a subnuclear structure in vivo and is resistant to electroelution. Upon inhibition of p43 or telomerase expression by RNAi, which in this study was used for the first time in spirotrichs, this complex is no longer retained in the nucleus.

Further analysis revealed that the p43-telomerase complex is bound to the nuclear matrix in vivo and that after inhibition of p43 expression, telomerase is released from this structure, strongly suggesting that p43 is involved in anchoring of telomerase in the nucleus. This is the first in vivo demonstration of the biological function of this telomerase-associated component involved in telomere replication and allows us to propose a model for the organization of the end-replication machinery in the eukaryotic cell.

Key words: Ciliates, La autoantigen, Nuclear matrix, Replication factory, Telomerase, RNAi

Introduction

Replication of chromosomal ends in eukaryotes is achieved by the enzyme telomerase, a ribonucleoprotein that adds telomeric repeats to the 3′ ends of chromosomes (Greider and Blackburn, 1996). Failure to correctly regulate telomere replication can result in premature aging or can lead to immortalization of untransformed cells (Cong et al., 2002; Shay and Wright, 2001; Vaziri and Benchimol, 1998). While the action of telomerase in vitro and its biochemistry are well understood, very little is known about its regulation in vivo, the organization of the end-replication machinery in the nucleus (Fang and Cech, 1995) and the biological function of telomerase-associated proteins. Owing to the high concentration of telomeres in the ciliate macronucleus, these cells have proven to be a model system in which to study telomere structure and their replication. Observations made in these organisms have been used to trace telomere behaviour in other species, from yeast to human. Moreover, the end-replication machinery in these cells is, in comparison with other organisms, relatively simple and their components are well characterized: telomeres in the macronucleus in spirotrichous ciliates are of homogenous length, consisting, in the case of *Euplotes*, of a 28mer 5′-C_4A_4 repeat and a 14mer 3′-$T_4G_4T_4G_2$ protruding end. These telomeres are capped by a heterodimeric telomere-binding protein (TeBP) in the case of *Oxytricha* and *Stylonychia* and a monomeric TeBP of *Euplotes* (Jonsson and Lipps, 2002). The telomerase consists of a 123 kDa catalytic subunit and a 238 bp RNA subunit (Lingner et al., 1994; Lingner et al., 1997).

We have recently shown that telomeric sequences in the spirotrichous ciliate *Stylonychia lemnae* adopt the anti-parallel G-quartet structure in vivo (Schaffitzel et al., 2001) and are bound in this structure to the nuclear matrix by a specific interaction of the TeBP with components of this subnuclear structure. In the course of replication both the G-quartet structure as well as the interaction of the telomere-binding protein with the nuclear matrix is resolved, making telomeres accessible to telomerase action (Jonsson et al., 2002; Postberg et al., 2001). In the related species *Euplotes aediculatus*, telomerase is associated with the protein p43 (Aigner et al., 2000). This protein copurifies with active telomerase and appears to be stoichiometric with both the RNA and the catalytic subunit of the telomerase complex. Recombinant p43 binds telomerase RNA in vitro. It shares homology with the La autoantigen family (Aigner et al., 2000), which has been shown to be involved in sequestering RNA in the nucleus (Maraia, 2001) and to bind to internal sequences and/or structures (Chang et al., 1994; Grimm et al., 1997) in vitro. It therefore has been suggested that p43 functions in the assembly and/or nuclear retention of telomerase (Aigner et al., 2000).

In this report we analyze the organization of the p43-telomerase complex in the macronucleus of *Euplotes* and show that both components are bound in vivo to a subnuclear

structure, the nuclear matrix or chromosome scaffold. But in contrast to telomeric sequences this complex is not released from this structure during replication. Using the powerful tool of RNAi technology we now can show that one of the major functions of the telomerase-associated protein p43 is to contribute to nuclear retention of telomerase and anchorage of this enzyme complex in the replication factory of the ciliate macronucleus. Furthermore, we made an attempt to analyze the molecular interaction involved in this retention mechanism.

Materials and Methods

Euplotes

Euplotes aediculatus cells were cultured in Pringsheim medium (0.11 mM Na_2HPO_4, 0.08 mM $MgSO_4$, 0.85 mM $Ca(NO_3)_2$, 0.35 mM KCl, pH 7.0) using either the algae *Chlorogonium elongatum* (Ammermann et al., 1974) or *E. coli* [adopted after (Timmons and Fire, 1998)] as a food source.

Isolation of nuclei

For isolation of macronuclei, cells were lysed with 2× lysis buffer (1 mg/ml spermidine phosphate, 0.1% Triton X-100) and centrifuged at 500 ***g*** for 7 minutes.

Halo preparation

Macronucleus halos were isolated using the lithium diiodosalicylate (LIS) technique described for mammalian cells (Dijkwel and Hamlin, 1988), although the light microscopical appearance is very different from mammalian nucleus halo preparations owing to the small size of macronuclear DNA (Jonsson et al., 2002). Furthermore because of mechanical forces during preparation the elongated macronuclei become fragmented in most of the cases.

Inhibition of gene expression by RNAi

Constructs used for the inhibition of p43 and telomerase were obtained by polymerase chain reaction (PCR) using the following primers (Fig. 2a and Fig. 3a):

p43-5: 5′-CAGCCAAGCTTATCGACCTTCATATATCCAATAC-GATGA-3′

p43-6: 5′-CGTTCCTCGAGTTATTTCTTTAATGATCTTCTGT-GC-3′

EuCP5′: 5′-CAGCCAAGCTTCAACTACTATTTAACAAAATC-TTGTC-3′

EuCP3′: 5′-CGTTCCTCGAGCAAATATTCTTTCTGTAGTAA-TAGGT-3′

Inhibition of gene expression was achieved by feeding *Euplotes* with *E. coli* cells HT115 (*F–, mcrA, mcrB, IN(rrnD-rrnE)1, lambda–, rnc14::Tn10(DE3 lysogen:lacUV5 promoter-T7 polymerase*) expressing the corresponding dsRNAs using vectors as described previously (Timmons and Fire, 1998). Usually a first effect could be observed after about three days of feeding. As controls, *Euplotes* were fed only with bacteria or bacteria containing the vector without insert.

In situ localization, electroelution and western blot analysis

In situ localization of telomeres, p43 and the telomerase, electroelution experiments and western blot analyses were done as previously described for *Stylonychia* (Jonsson et al., 2002; Postberg et al., 2001). The probe used for FISH analysis of the RNA telomerase subunit was the digoxigenin-labeled oligonucleotide 5′-(DIG-C12) r_m(GCUUGACAGAUUCUACA)dG-3′.

Results and Discussion

The localization of telomeric sequences, p43 and telomerase in the macronucleus of *Euplotes* was analyzed by FISH analysis and in situ antibody staining. As shown in Fig. 1a, similar to telomeres, p43 and telomerase appear in clusters distributed equally over the whole macronucleus and, as expected, show a significant concentration in the replication band, the huge replication factory of the ciliate macronucleus (Prescott, 1994). To determine whether these components are bound to a subnuclear structure in vivo we used the previously described electroelution technique (Jackson et al., 1988; Postberg et al., 2001). Living cells are embedded into agarose, permeabilized and the DNA is digested with restriction endonucleases. In a subsequent electroelution step all DNA and proteins not bound to a subnuclear structure are removed from the nucleus, although some disturbance of total nuclear architecture can take place during this procedure (Jackson et al., 1988). Similar to the observation made in *Stylonychia* (Postberg et al., 2001), telomeric repeats of *Euplotes* can not be electroeluted from the nucleus. However, they become electroeluted from the replication band, demonstrating that the interaction of telomeric repeats with this subnuclear structure is resolved during replication. In contrast telomerase and p43 cannot be electroeluted, neither from the nucleus nor from the replication

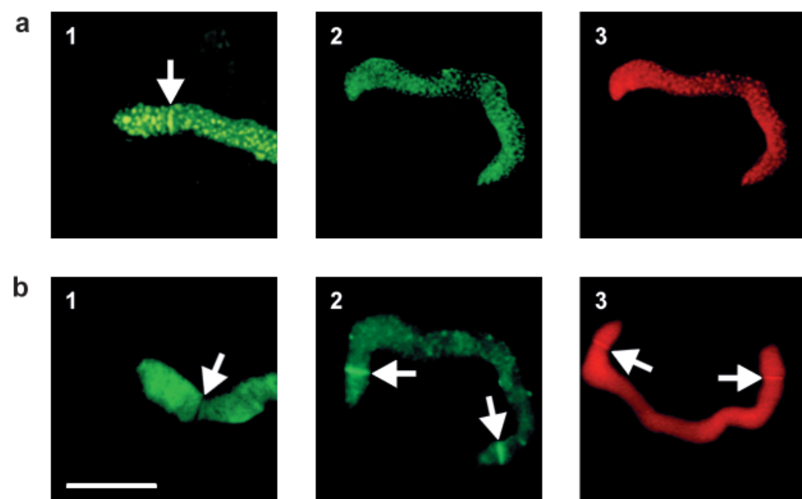

Fig. 1. Distribution of telomeric sequences, p43 and telomerase in the macronucleus of *Euplotes* before and after electroelution as described previously (Postberg et al., 2001). Telomeric sequences and telomerase were detected by FISH analysis, p43 by antibody staining. Arrows point to the replication band. (a) Distribution of telomeric sequences, p43 and telomerase in the macronucleus of *Euplotes* before electroelution. 1, telomeric sequences; 2, p43; 3, telomerase. (b) Distribution of telomeric sequences, p43 and telomerase in the macronucleus of *Euplotes* after electroelution. 1, telomeric sequences; 2, p43; 3, telomerase. Bar, 10 µm.

band (Fig. 1b), demonstrating that this complex is stably retained in the replication machinery of the ciliate macronucleus. This could be explained by either p43 and telomerase forming an insoluble complex or a complex with no net charge or by them being bound to a subnuclear structure such as the matrix or scaffold. We then inhibited the expression of p43 or the catalytic subunit of telomerase by RNAi using the technology described for *Caenorhabditis elegans* (Timmons and Fire, 1998). The effect of inhibition was analyzed after various time intervals by in situ antibody staining and western blot analysis in the case of p43 and FISH analyses in case of telomerase. As shown in Fig. 2, expression of p43 can be efficiently inhibited by this technology as demonstrated by in situ antibody staining (Fig. 2b) and western blot analysis (Fig. 2c). In a following electroelution experiment the behaviour of telomerase after inhibition of p43 expression was analyzed. In this case telomerase is no longer retained in the macronucleus and can be efficiently electroeluted from the replication band (Fig. 2d), demonstrating that p43 is required for nuclear retention of telomerase in the nucleus and for anchoring it in the replication band. This could be explained by p43 neutralizing the charge on the telomerase holoenzyme, so that in the absence of p43, telomerase can be electroeluted or that p43 anchors telomerase by a specific interaction with components of the nuclear matrix. In the course of inhibition of p43 expression by RNAi, macronuclei undergo typical morphological changes that include deformation of nuclei, loss of DNA as revealed by DAPI-staining and eventually fragmentation of macronuclei (data not shown), which finally results in the death of the cells about 2 weeks after inhibition. This strongly suggests that DNA replication is defective after alteration of telomerase location by the loss of p43.

Subsequently, expression of the catalytic subunit of telomerase (p123) (Lingner et al., 1997) was inhibited using RNAi directed against a sequence encoding 191 amino acids located at the C-terminus of telomerase (Fig. 3a), and the degree of inhibition was indirectly demonstrated by FISH analysis using a probe directed against a non-template region of the RNA subunit (Fig. 3b). Upon inhibition of telomerase, p43 distribution in the macronucleus is strongly disturbed and no longer organized in foci-like structures and can be completely electroeluted from the nucleus (Fig. 3c). In common with p43 inhibition, telomerase inhibition leads to loss of DNA from the nucleus, fragmentation of nuclei and eventually to the death of the cells. Although the overall morphology of macronuclei is still visible after inhibition of p43 or telomerase followed by electroelution, owing to the loss of a high amount of DNA no adequate DAPI staining can be obtained and, unfortunately, in these organisms no antibodies against matrix proteins are available. At this point it could be speculated that upon binding of telomerase to p43 this molecule undergoes a structural modification, and it is only after this modification that p43 can bind to the nuclear matrix. This would ensure that it is only the p43 in the p43-telomerase complex that can bind, although these binding sites cannot be occupied by free p43. This view is supported by the observation that p43 is stoichiometric with telomerase in the nucleus, that it always seems to be complexed with p43 in the macronucleus (Aigner et al., 2000) and in situ localization experiments never revealed a p43 signal without an overlapping signal for telomerase.

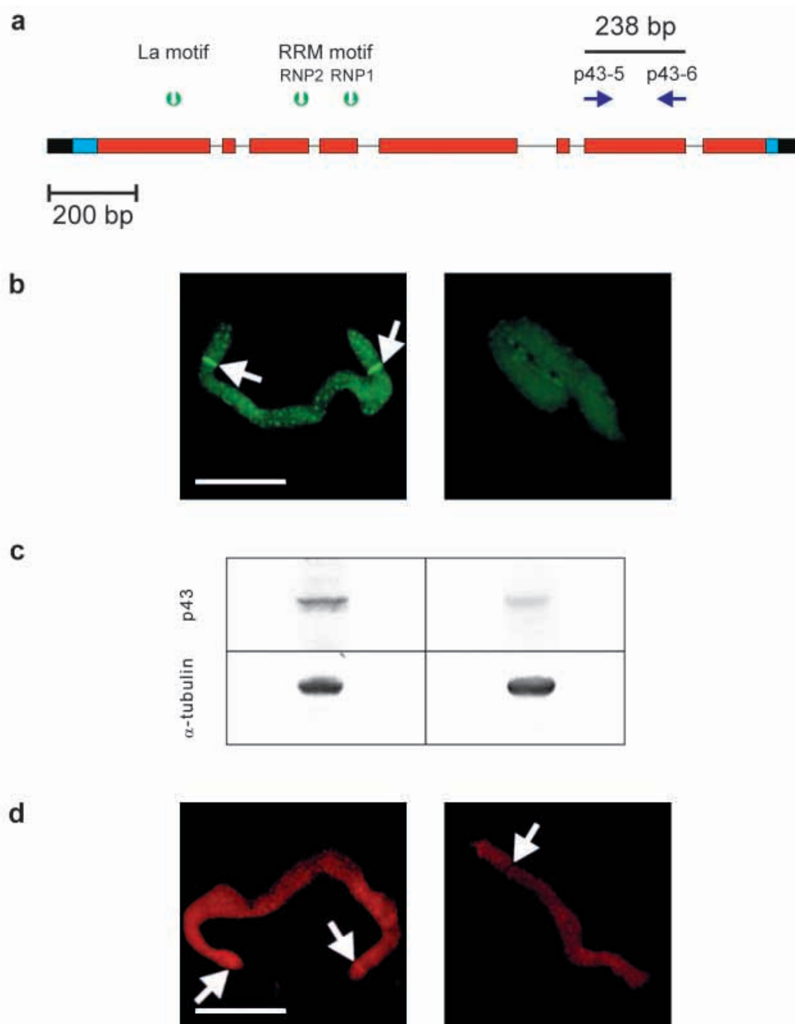

Fig. 2. Inhibition of p43 expression by RNAi. (a) Construct used for the inhibition of p43. A map of p43 shows the 238 bp construct cloned into p4440. Bacteria were transfected with this construct and the expression of dsRNA was induced as described previously (Timmons and Fire, 1998). *Euplotes* were fed daily with bacteria. The position of the La motif, the RNA-binding motif (RRM with the submotifs RNP1 and 2) and primers used for PCR amplification are indicated (Aigner et al., 2000). (b) In situ staining of macronuclei with an anti-p43 antibody before (left) and after (right) RNA inhibition. (c) Western blot analysis of cells before (left) and after inhibition (right) of p43 expression. Filters were incubated simultaneously with an anti-p43 antibody and as a control with an anti-α-tubulin antibody (Sigma). (d) FISH analysis of telomerase RNA subunit after inhibition of p43 before (left) and after (right) electroelution. Control macronuclei (left), macronuclei after inhibition of p43 expression (right). Arrows point to the replication band. Bar, 10 µm.

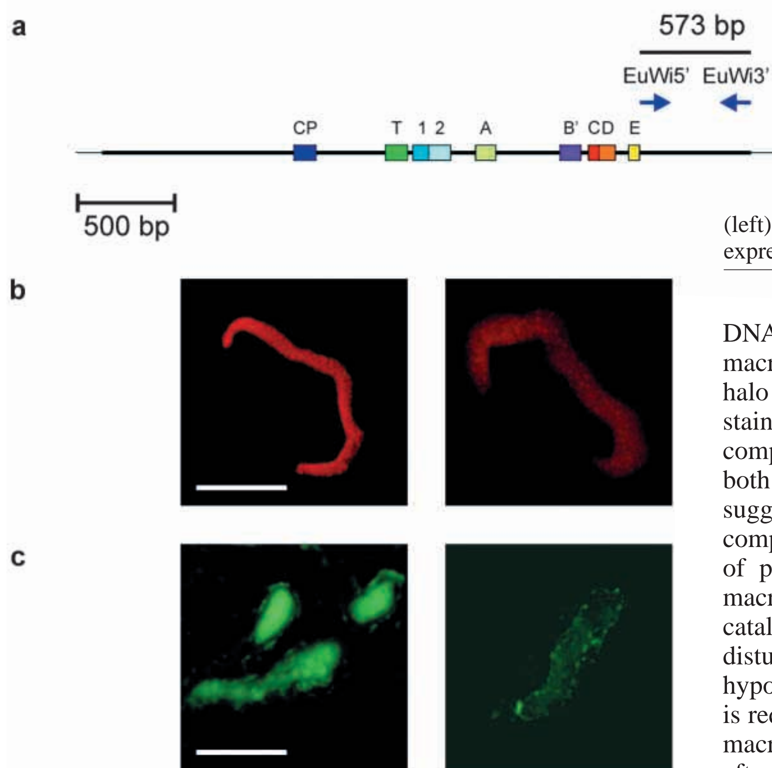

Fig. 3. Inhibition of the catalytic subunit of telomerase by RNAi. (a) Construct used for the inhibition of telomerase. A map of the gene encoding the catalytic subunit of telomerase (p123) is shown. The 573 bp construct cloned into p4440 and the primers used are indicated. Motifs CP, T, 1, 2, A, B′, C, D and E are shown (Bryan et al., 1998). (b) Before (left) and after (right) inhibition. (c) Distribution of p43 in the macronucleus after inhibition of telomerase expression (left), electroeluted macronuclei after inhibition of telomerase expression stained with an anti-p43 antibody (right). Bar, 10 μm.

To distinguish between all the possibilities discussed above and to analyze the nature of interactions involved in telomerase retention, we first prepared macronuclear halos, which are relaxed lengths of looped DNA with attachment to the nuclear matrix. However, because of the small size of macronuclear DNA the light microscopical appearance of *Euplotes* macronuclear halos is very different from mammalian nuclear halo preparations. In this structure we tested by in situ antibody staining and FISH analysis whether the p43-telomerase complex copurifies with this structure. As shown in Fig. 4a,b, both components are bound to the nuclear matrix strongly suggesting that nuclear retention is due to an interaction of this complex with components of this structure. Upon inhibition of p43 by RNAi, telomerase is no longer retained in the macronucleus. Similarly, upon inhibition of the telomerase catalytic subunit p43 distribution in the macronucleus is also disturbed and can be electroeluted (Fig. 3a,b). To test the hypothesis that binding of the telomerase RNA subunit to p43 is required for binding of p43 to the nuclear matrix we treated macronuclei with RNAse and studied the behaviour of p43 after this treatment. As shown in Fig. 4c,d under these conditions p43 is efficiently electroeluted from the nucleus. Although the overall morphology of macronuclei after RNAse treatment did not change at the light microscopical level, some as yet unknown modifications of nuclear structure cannot be excluded. However, these experiments strongly indicate that it is indeed the p43-RNA complex that binds to the nuclear matrix. To further strengthen this assumption we inhibited expression of p43 by RNAi and tested in halo preparations whether telomerase is still attached to the nuclear matrix. Fig. 4e,f shows that this is no longer the case, demonstrating that telomerase itself does not bind to the nuclear matrix, but that p43 acts as an adaptor protein, which only after binding to the RNA subunit of the telomerase, adopts a modification in which it can bind to this subnuclear structure.

Taking all these data together, it is evident that only the p43-telomerase complex binds to a subnuclear structure, and any disturbance of one of these components leads to the release of the other. Furthermore, our data allow us to propose a model for the organization of the end-replication machinery during the replication process (Fig. 5). In the course of replication, the G-quartet structure of telomeric sequences as well as the interaction of the telomere-binding protein with the nuclear matrix is resolved, releasing the DNA (Jonsson et al., 2002; Postberg et al., 2001). It might well be that the telomere-binding protein is replaced by a replication-specific binding

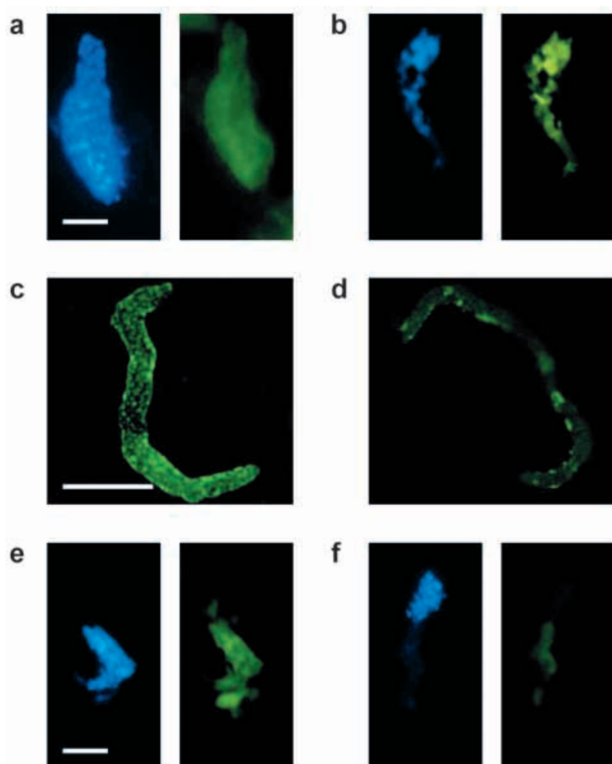

Fig. 4. Binding of the p43-telomerase complex to the nuclear matrix. (a) In situ staining of macronuclear halos with DAPI (left) and an anti-p43 antibody (right). (b) Macronuclear halos stained with DAPI (left) and FISH analysis of the telomerase RNA subunit (right). p43 staining after electroelution (c) and after RNAse treatment followed by electroelution (d). Macronuclear halos before (e) and after inhibition of p43 expression by RNAi (f) stained with DAPI (left) and FISH analysis of the telomerase RNA subunit (right). Bar 10 μm.

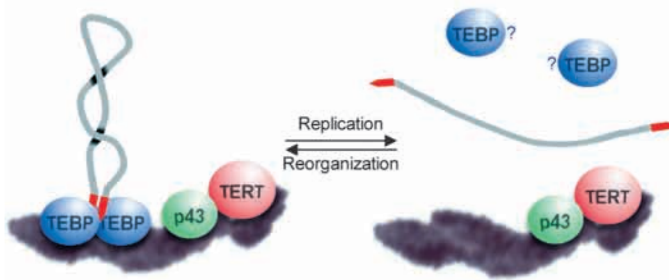

Fig. 5. Model for the organization of the end-replication machinery in the macronucleus of *Euplotes*. Telomeric sequences are bound to the nuclear matrix by an interaction of the telomere-binding protein (TEBP) with components of this structure. Similarly, the p43-telomerase complex (p43, TERT) is also bound to this structure. In the course of replication the interaction of telomeres with the nuclear matrix is resolved, and at this stage it may well be that the telomere-binding protein is replaced by a replication-specific binding protein (Carlson et al., 1997). In contrast, the enzymatic machinery stays bound to this structure during replication.

protein, as suggested earlier (Carlson et al., 1997). By contrast, in the replication band the enzymatic machinery stays bound to this subnuclear structure, a phenomenon that has been suggested to be true for other enzymes involved in replication and transcription in mammalian replication and transcription factories (Cook, 1999).

This work was supported by the Deutsche Forschungsgemeinschaft, the National Science Foundation (subcontract no. 128-6114-1) and the Alfried Krupp von Bohlen and Halbach Foundation. We thank S. Aigner and T. Cech, Boulder, for providing us with DNA probes, antibodies directed against p43 and extremely fruitful discussions.

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