Identification of a Novel “Chromosome Scaffold” Protein That Associates with Tec Elements Undergoing En Masse Elimination in *Euplotes crassus*

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During macronuclear development in the ciliate *Euplotes crassus*, the highly repetitive, transposon-like Tec elements possess an unusual chromatin structure. We observed that the Tec element chromatin is highly resistant to salt extraction and behaves like a nuclear matrix/chromosome scaffold-associated structure. Standard matrix/scaffold extraction procedures identified two major proteins: 1) an ~140-kDa protein that seems to be topoisomerase II based on its reactivity with anti-topoisomerase II antibodies, and 2) an 85-kDa protein that we further purified by acid extraction and have shown to be a novel protein by sequence analysis of its gene. The 85-kDa protein (p85) is a developmental stage-specific protein and is located exclusively in the developing macronucleus. Immunolocalization studies of p85 show that it colocalizes with topoisomerase II in chromatin. In addition, in situ hybridization combined with immunofluorescence localization of the proteins indicates that 100% of the Tec elements colocalize with 70% of the p85, whereas no significant colocalization with a total macronuclear sequence-specific probe is observed. p85 is the first developmental stage-specific protein identified as being specifically associated with sequences undergoing elimination in *E. crassus*.

INTRODUCTION

During the sexual phase of the life cycle of the ciliated protozoan *Euplotes crassus*, a macronucleus is formed from a copy of a micronucleus (reviewed by Jahn and Klobutcher, 2002). As in other ciliated protozoa, the macronucleus is transcriptionally active during vegetative life, whereas the micronucleus is not. The formation of a macronucleus involves polytenization of the micronuclear chromosomes, highly specific DNA-processing events, and massive chromatin elimination, such that only the genes, with very little extra noncoding DNA, remain. The macronuclear genome comprises only a small percentage of the micronuclear genome (5–10%); thus, the chromatin elimination involves as much as 95% of the DNA. Two types of DNA processing form the macronuclear chromosomes: 1) precise deletion of DNA sequences that are internal to the sequences that are retained as the macronuclear genomic sequences and hence, called internal eliminated sequences (IESs); and 2) precise fragmentation of chromosomes 5' and 3' of genes coupled with the de novo addition of telomeres, which results in the

“gene-sized” linear DNA molecules characteristic of *E. crassus* and other “hypotrichous” ciliates (Figure 1A).

In *E. crassus*, the IESs defined to date are either repeated sequences corresponding to two closely related families of transposable elements called Tec elements (referred to as Tec-IES) or they are small, unique sequences (referred to as SU-IES) (Jahn and Klobutcher, 2002). Both types of IES are eliminated by a common mechanism that generates extra-chromosomal circular forms of the IES (Jahn *et al.*, 1989; Tausta and Klobutcher, 1989, 1990; Jaraczewski and Jahn, 1993). The IES deletion occurs during the last part of each of two discrete S phases that give rise to the polytene chromosomes (Frels and Jahn, 1995) (developmental timing is shown in Figure 1B). Tec-IES can be deleted in either S phase, whereas most SU-IES are deleted in the second S phase (Frels and Jahn, 1995; Frels *et al.*, 1996).

Our laboratory has demonstrated that during the replications that form the polytene chromosomes, the Tec elements become organized into an unusual chromatin structure that differs from the chromatin structure of the macronuclear-destined sequences (Jahn, 1999). This is the first evidence that chromatin structure could play a role in defining which DNA sequences are to be eliminated in *E. crassus*. A role for chromatin structure in DNA elimination was previously demonstrated in the distantly related ciliate, *Tetrahymena thermophila*, where three proteins, Pdd1p, Pdd2p, and

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Figure 1. Types of DNA processing and their developmental timing in *E. crassus*. (A) Micronuclear precursor to a macronuclear gene is diagrammed to show the two types of DNA processing: IES excision, which involves precise deletion, and chromosome fragmentation and telomere addition, which results in a macronuclear mini-chromosome bearing a gene (open box, macronuclear-destined sequence or gene; black box, IES; black lines, eliminated surrounding DNA; gray arrowheads, telomeres). (B) Developmental timing of events in the sexual phase of the *E. crassus* life cycle, where time of mixing of two different mating types is 0 h and other events are referred to as time postmixing. A developing macronucleus (referred to as anlagen) is first apparent around 17 h and the events designated by the arrows from ~20 h on (polytene S phases, excision, etc.) occur in the anlagen.

Pdd3p, have been shown to be associated with the eliminated DNA in heterochromatic “vesicle” structures that associate with the nuclear periphery (Madireddi *et al.*, 1996; Smothers *et al.*, 1997b; Nikiforov *et al.*, 2000). Pdd1p and Pdd3p are chromodomain proteins (Madireddi *et al.*, 1996; Nikiforov *et al.*, 2000) and thus share the characteristic heterochromatic localization of this class of proteins (Koonin *et al.*, 1995). The Pdd proteins are referred to as “DNA degradation”-specific proteins because they are also associated with “old” macronuclei that are undergoing elimination

while the new macronucleus develops. Recent work using reverse genetic approaches to knock out the Pdd1 and Pdd2 genes in the parental macronucleus, which alters the timing of appearance and amounts of these proteins during development, has demonstrated that these proteins are critical for the DNA-processing events that eliminate DNA in the *Tetrahymena*-developing macronucleus (Coyne *et al.*, 1999; Nikiforov *et al.*, 1999). Nevertheless, antibodies to the Pdd1p and Pdd2p proteins and gene probes have not identified potential homologues of these proteins or genes in *E. crassus*. Thus, the goal of the experiments described herein was to use the properties of the unusual chromatin structure of the Tec elements to identify proteins that might be associated with the elements during their elimination and which might play an analogous role to the Pdd proteins.

MATERIALS AND METHODS

Cell Culture and Preparation of Nuclei, Scaffolds, and Chromatin Spreads

E. crassus strains X1 and X2 were cultured, mated, and harvested as described previously (Roth *et al.*, 1985; Krikau and Jahn, 1991). Nuclei were prepared by lysis in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8) with 0.5% Triton X-100 and sonication, and MNase digested as described previously (Jahn, 1999). Protease inhibitors were used throughout all preparations and included the following: *N*-tosyl-L-phenylalanine chloromethyl ketone (100 μ g/ml), *N*-tosyl-L-lysine chloromethyl ketone (50 μ g/ml), leupeptin (2 μ g/ml), and aprotinin (1 μ g/ml) (all from Sigma-Aldrich, St. Louis, MO). Chromosome scaffolds/nuclear matrices were isolated by suspending the purified nuclei in either NaCl extraction buffer (2 M NaCl, 0.25 M sucrose, 50 mM Tris-HCl pH 7.5, 3 mM MgCl₂, 0.5 mM CaCl₂) or in LIS extraction buffer (10 mM lithium iodosalicylate, 20 mM HEPES-NaOH pH 7.5, 100 mM lithium acetate, 0.1% digitonin, 1 mM EDTA pH 8) (Mirkovitch *et al.*, 1984) for 30 min on ice, centrifuging the nuclei, and washing the nuclei once (30 min on ice) with NaCl or LIS buffer after which the nuclei were washed three times and suspended in TMS (50 mM Tris-HCl pH 7.5, 10 mM NaCl, 10 mM MgCl₂) and DNase I added at 200 μ g/ml for 30 min at room temperature. Nuclei were then washed three times with TE. (Identical results were obtained with the 2 M NaCl washes followed by three washes with 0.25 M sucrose, 50 mM Tris-HCl pH 7.5, 15 mM NaCl, 3 mM MgCl₂, 0.5 mM CaCl₂ and digestion with MNase at 5 U/ml for 30 min at 37°C.) For further extraction of the scaffolds, the nuclei (nucleoskeletons) were washed with 4 M guanidine-100 mM Tris-HCl, pH 8.0, followed by two washes with 8 M urea.

Chromatin spreads were prepared by suspending purified nuclei in 100 mM Tris-HCl pH 8, 35 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, adding trypsin at 10 μ g/ml and incubating for 10 min at room temperature. Treatment of *E. crassus* anlagen with trypsin and the effect on chromatin structure was described previously (Jahn, 1999). At this concentration of trypsin, the Tec element chromatin is not disrupted and histones are not digested. Formaldehyde was added at 5% and the nuclei/chromatin were immediately spun onto polylysine-coated coverslips by diluting the chromatin either 1/10 or 1/50 in 500 μ l of phosphate-buffered saline (PBS) and centrifuging for 5 min at 300 $\times$ g. The coverslips were rinsed twice with PBS and air-dried.

Purification of p85 and Sequence Analysis

Purified nuclei were suspended in TE, H₂SO₄ added to 0.4 N, and the nuclei sonicated and left on ice for at least 30 min. In many cases, samples were frozen immediately after addition of H₂SO₄ and processed further later. After centrifugation at 12,000 $\times$ g for 10 min the supernatant was made 3.5% in perchloric acid (PCA) and allowed to

sit on ice for at least 30 min. After centrifugation as described above, the supernatant was made 20% in trichloroacetic acid (TCA), left on ice >30 min, and then centrifuged again for 10 min. The pellet was washed with acetone, 0.1% HCl, and then with acetone and air-dried. For more highly purified preparations, such as that used for affinity purification of antibodies, the above-described TCA pellet was resuspended in 5% PCA and centrifuged at $100,000 \times g$ for 1 h followed by TCA precipitation and acetone washes as described above. Protein concentration was determined using Bio-Rad (Hercules, CA) protein assay dye reagent concentrate with bovine serum albumin as a standard or by comparison with standards on Coomassie-stained gels.

The p85 purified by acid extraction was subjected to N-terminal amino acid sequence analysis by the University of Massachusetts Proteomic Mass Spectrometry Laboratory (Amherst, MA) by using their recommended methods of blotting SDS-polyacrylamide gels to nitrocellulose membranes. In addition, p85 blotted to nitrocellulose was subjected to aspartate protease digestion, which resulted in numerous peptides, two of which were purified and sequenced by The Rockefeller University Protein/DNA Technology Center (New York, NY). Oligonucleotide primers for polymerase chain reaction (PCR) of the p85 gene were designed based on the N terminal and internal peptide sequences by using the Web-based Entelechon Backtranslation program, which includes an *E. crassus* codon usage table. The peptide sequences obtained are shown in Figure 4 and oligonucleotides that resulted in a p85 gene-specific product were as follows: 5' end (amino terminus) AAGGGTAAGATAGCCAC-CAAGGTAGCTGGAAAGGGATTAAAGACTAAGGGAAAGAA-GACAAAGGCTGCAGA, and 3' end (internal peptide) CTCCTCT-TCTACCTTACCTTTTTCTTCTC. The PCR was performed using platinum *Taq* DNA polymerase, high fidelity (0.5 U/ μ l), from Invitrogen (Carlsbad, CA) by using the buffer supplied by the manufacturer, 10 ng/ μ l total *E. crassus* DNA, 2 pmol/ μ l each primer, and 200 μ M dNTPs. The PCR was performed for 30 cycles with 30 s at 94°C, 30 s at 52°C, and 2 min at 72°C. The PCR product was cloned and sequenced and shown to contain the sequence corresponding to the second internal peptide sequence obtained at The Rockefeller University. By using the PCR product as a hybridization probe, the macronuclear DNA molecule bearing the p85 gene and cDNA clones were isolated from a macronuclear genomic library and a developmental stage-specific cDNA library described previously (Harper and Jahn, 1989; Ling *et al.*, 1997). The macronuclear genomic clone and two different cDNAs were sequenced in their entirety. DNA sequencing reactions used the ABI PRISM big dye terminators cycle sequencing kit (Applied Biosystems, Foster City, CA). Gel analysis was carried out by the Northwestern University Biotechnology Laboratory (Chicago, IL). The DNA sequences were analyzed using BLAST searches (National Center for Biotechnology Information) and several programs available through the ExPASy Web site (www.expasy.ch), including Translate Tool, NetPhos, Coils, and Paircoil.

Western Blotting and Antibody Purification

Nuclear proteins were resolved on 10 or 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk in PBST (PBS + 0.05% Tween 20) for 1 h at room temperature. After washing, primary antibodies were applied at a dilution of 1:500 for anti-p85 and 1:1000 for anti-topoisomerase II in PBST + 1% bovine serum albumin for 1–2 h at room temperature or overnight at 4°C. Membranes were washed and exposed to the appropriate peroxidase-conjugated secondary antibodies (goat anti-rat, Amersham Biosciences, Piscataway, NJ; or goat anti-rabbit, Chemicon International, Temecula, CA) at the dilution of 1:1000. Staining was detected by chemiluminescence by using the Western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) and exposure to x-ray film. To affinity purify anti-p85 antibodies, purified p85 protein was electrophoresed and blotted to polyvinylidene difluoride membrane (Schleicher &

Schuell, Keene, NH) and incubated with serum. Bound antibody was then washed off the membrane and collected.

In Situ Hybridization and Immunofluorescence

Immunohistochemistry was performed on trypsinized nuclei or whole nuclear matrices as follows. Samples were fixed with 5% formaldehyde in PBS for 10 min at room temperature, washed twice in PBS, and resuspended in 1 ml of PBS. For whole cells, urea was added to a final concentration of 4 M and allowed to incubate at room temperature for 30 min (Jacobs *et al.*, 1999). Approximately 100 μ l of sample was dropped on polylysine-coated coverslips and allowed to air dry. Dried coverslips were processed in 100% ice cold

Figure 2. NaCl extraction and MNase digestion of Tec element chromatin. (A and B) Nuclei were washed with increasing concentrations of NaCl (molarity shown above each set of lanes) then digested with MNase for increasing amounts of time (0, 5, 10, or 30 min, as shown above each lane) at 0.5 U/ml. The DNA was extracted, electrophoresed on a 1.0% agarose gel, Southern blotted, and hybridized with a 32 P-labeled Tec element probe. In A, 20-h nuclei were used, whereas in B, 43-h nuclei were used; at 43 h, the excised circular forms (supercoiled and relaxed) of the Tec elements, which are especially resistant to MNase digestion, are apparent (designated by stars). (The largest hybridizing band corresponds to some multimeric form of the Tec elements as noted [Jahn *et al.*, 1989] but not fully characterized.) (C) Nuclei were digested with MNase at 0.015 M NaCl (5 U/ml for 30 min), the digestion was stopped by addition of EDTA to 10 mM, and then the nuclei were either washed extensively with 2 M NaCl (+ lane) before extraction of the DNA or immediately extracted (– lane). Samples in C were analyzed on a 1.5% agarose gel. The lanes shown are loaded on a per nucleus basis: the NaCl + lane was approximately one-fifth the amount of DNA in the – lane. Sizes in base pairs are given to the right side of each blot and were determined from a 100-base pair ladder blotted and hybridized in parallel.

Figure 3. Analysis of proteins in the nuclear matrix fraction and acid extraction of p85. (A) Nucleoskeleton preparation (labeled NS) made by LIS extraction of 45-h anlagen electrophoresed in a 12.5% SDS-polyacrylamide gel next to a sample of pellicles (PEL) to identify the nuclear specific proteins (stained with Coomassie). The 85- and ~140 kDa proteins referred to in the text are identified by stars to the left. (B) NaCl- (2 M, NaCl lane) and LIS (LIS lane)-extracted chromosome scaffold/nuclear matrix preparations were analyzed by SDS-PAGE and stained with Coomassie to show that the two different

preparations enrich for the same proteins. The 85- and ~140-kDa proteins referred to in the text are identified by stars to the right. (C) Acid extraction of p85. The starting nuclear sample (N), the H_2SO_4 extract (H), and the PCA-soluble material from the H_2SO_4 extract (P) electrophoresed and stained with Coomassie. The 85-kDa protein (star at right) is barely visible in a H_2SO_4 extract relative to the histones but is the most abundant protein obtained in the PCA-soluble fraction. (D) Antibodies were generated that specifically react to p85. A total nuclear protein sample from 45-h anlagen (45N), the same nuclei after LIS extraction (LIS), and after LIS extraction and DNase digestion (L/D) were analyzed by SDS-PAGE at equivalent numbers of nuclei per lane and then Western blotted. The left panel is a Western blot reacted with the rat antibody to purified p85, and the right panel is a blot reacted with the total nucleoskeleton antibody (star designates p85).

methanol for 6 min followed by acetone for 30 s. Coverslips were then blocked in 5% nonfat milk in PBST for 1 h at 30°C. Primary antibody was applied for 4–12 h at 4°C at 1:50 dilution in PBST + 1% bovine serum albumin for the anti-p85 and 1:100 for the anti-topoisomerase II antibodies. Secondary antibodies rhodamine-conjugated goat anti-rat (Jackson Immunoresearch Laboratories, West Grove, PA) or fluorescein-conjugated goat anti-rabbit antibodies (Invitrogen) were used at a 1:50–1:100 dilution in PBS for 1 h at room temperature in the dark. Coverslips were washed three times in PBS and 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) was added at 5 μ g/ml to the second wash. Coverslips were blotted dry, mounted using Vectashield mounting medium for fluorescence (Vector Laboratories, Burlingame, CA), and glued down. Immunofluorescence analysis of 10- μ m sections of whole nuclei was performed similarly, except that fixed whole nuclei were resuspended in Tissue-Tek OCT embedding medium (Miles, Elkhart, IN) and flash frozen in liquid nitrogen. Frozen blocks were sectioned using a cryostat at –20°C. Sections were transferred to polylysine-coated slides and processed as stated above. In situ hybridization was performed as described in Madireddi *et al.* (1996), except that 50 ng of probe was used per coverslip for hybridizations. The rDNA probe used was a plasmid clone of the full-length rDNA molecule (Erbeznik *et al.*, 1999). The macronuclear sequence probe was prepared by Bal31 treatment and size fractionation of total *E. crassus* DNA as described previously (Jahn, 1999). The Tec 1 and Tec 2 probes consisted of plasmid subclones of restriction fragments from the Tec1-1 and Tec2-1 elements (Jahn *et al.*, 1993; Jahn, 1999). The results shown in figures were obtained by mixing the Tec1 and Tec2 probes after labeling. Results obtained with the mixed probes were consistent with what was seen with individual probes.

RESULTS

Extraction Behavior of Tec Element Chromatin: Identification of Chromosome Scaffold/Nuclear Matrix-associated Proteins

To define what proteins might be determinants of the Tec element chromatin structure, we performed salt extractions of nuclei either before or after MNase digestion to see

whether protein determinants of the chromatin structure would be stripped from the chromatin at increasing ionic strength. This demonstrated that treatment of nuclei with NaCl concentrations as high as 2 M had no effect on the MNase digestion pattern of the Tec element chromatin (Figure 2, A and B), despite the fact that the majority of DNA is completely unprotected from digestion (>80% acid soluble at the most extensive digestion). The unusual pattern of Tec element chromatin digestion products described previously (Jahn, 1999), which consists of an ~200-base pair nucleosomal repeat ladder starting at ~300 base pairs instead of ~200 base pairs, is evident regardless of the salt concentration. Furthermore, the excised circular forms of the Tec elements (Figure 2B, stars), which are resistant to nuclease digestion in chromatin (Jahn, 1999), retain their resistance to MNase digestion at all NaCl concentrations examined. In samples of nuclei that are 2 M NaCl treated and digested with MNase, <10% of the DNA remains undigested and hybridization to macronuclear sequence probes is completely eliminated. Quantitation of the Tec element hybridization indicates that they comprise at least 50% of the remaining DNA; thus, these preparations are enriched for the Tec element chromatin.

We have previously documented (Jahn, 1999) that the Tec element chromatin digestion products are tightly associated with the nuclear substructure and cannot be released by treatment of nuclei with EDTA, whereas the nucleosomal multimers of the macronuclear-destined sequences and most other sequences (based on quantitation of soluble and insoluble fractions) are solubilized by this treatment. Similarly, washing nuclei with 2 M NaCl after MNase digestion did not release the Tec element chromatin digestion products (Figure 2C). Conditions similar to those described above (MNase digestion and 2 M NaCl extraction) have previously been used to selectively purify the macronuclear telomere-binding proteins in association with *Oxytricha nova*

Figure 4. Sequence analysis of the p85 gene. The p85 gene was isolated from a macronuclear genomic library, and the macronuclear DNA molecule (1.6 kb) was sequenced in its entirety (accession no. AY155457). In addition, two cDNA clones were sequenced. The three sequences were identical except that the cDNAs were missing some of the 5' end. (A) The encoded p85 protein is shown. The three shaded sequences correspond to the peptide sequences obtained from acid extracted p85 protein (see MATERIALS AND METHODS). The two peptides with the arrows were backtranslated to generate the PCR primers used to isolate a fragment of the gene. The two regions with cccc or CCCC above them were predicted to be potential coiled coil regions. The CCCC region was predicted as a highly probable coiled coil structure by both the PAIRCOIL and COILS programs, which use the methods of Berger *et al.* (1995) and Lupas *et al.* (1991), respectively. The cccc region was only predicted as high probability by the COILS program. (B) The noncoding sequences are shown with the 5' end from the telomere to the ATG at the beginning of the p85 gene and then from the TAA stop to the 3' telomere. The sequences of two different cDNAs ended in polyA (italics) at the position shown (cDNA ends). One of the cDNAs started four base pairs downstream of the ATG and the other was missing the first 42 base pairs of the coding region.

and *E. crassus* telomeric DNA (Gottschling and Zakian, 1986; Price, 1990). Likewise, this is one method of isolating a "nuclear matrix" or "chromosome scaffold," which is operationally defined as the proteinaceous structure remaining insoluble after these treatments (Berezney and Coffey, 1977; Mirkovitch *et al.*, 1984). We therefore examined the proteins remaining in the anlagen after 2 M NaCl extraction and nuclease digestion to see whether we had selectively purified any proteins. In addition, we used a second type of nuclear matrix/chromosome scaffold preparation, involving lithium iodosalicylic acid (LIS) extraction of nuclei and DNase I digestion (Mirkovitch *et al.*, 1984), to determine whether a similar set of proteins remained postextraction.

As seen in Figure 3, A and B, one major protein species (85 kDa) is enriched in Coomassie-stained SDS-polyacrylamide gels of the nuclear matrix preparations. In addition, there is a prominent band at ~140 kDa. Comparisons were made between the NaCl- or LIS-insoluble proteins obtained from the "pellicle" or "cortical" fraction of *E. crassus*, because these would be the most abundant and most likely contaminants of the nuclear matrix preparation. Although the "140-kDa" protein in the matrix preparations comigrates with a protein seen in the pellicle fraction, we believe it is a different protein because it is always present regardless of the

amount of the other pellicle proteins that contaminate the preparation. In both types of matrix preparations, a cytologically visible "nuclear skeleton" is obtained that retains the size and shape of an intact nucleus. This structure is highly insoluble in every type of solubilizing agent we have tried (6–8 M urea, 4 M guanidine-isothiocyanate, ethylene glycol, dimethyl sulfoxide, SDS gel sample buffer supplemented with SDS at 10%, or β -mercaptoethanol at 10% and extensive boiling of the samples; high and low pH). Some portion of this nucleoskeletal material migrates into the polyacrylamide gel and remains as a smear of Coomassie-stained protein at the top (Figure 3A, NS), but the majority of the material remains particulate as spherical nucleoskeletons and is removed by centrifugation of the SDS-PAGE samples before loading of the gels. We generated rabbit polyclonal antibodies to the nucleoskeleton fraction that remained after LIS extraction, DNase digestion, and extensive washing with guanidine isothiocyanate and urea. These antibodies react to both the 85-kDa protein and the smear of protein at the top of the gel (Figure 3D, α -skeleton). We have affinity-purified antibodies to the 85-kDa protein from this sera for additional characterizations of the 85-kDa protein (described below). Due to the insolubility of most of this nucleoskeleton fraction, we chose to focus the majority of our efforts on the two

Figure 5. The ~140-kDa protein in the nucleoskeleton fraction is most likely topoisomerase II. (A) Schematic diagram of the highly conserved regions of topoisomerase II proteins (designated as GyrA and GyrB for their correspondence to the gyrase A and B subunits in *Escherichia coli*) and the locations of peptides from *Drosophila* topoisomerase II that antibodies were made to are shown across the top (antibodies kindly provided by P.A. Fisher, SUNY) (Meller *et al.*, 1995). (B) Coomassie staining of an SDS-polyacrylamide gel of a LIS-extracted nuclear matrix fraction and corresponding Western blot by using a polyclonal antibody to the whole *Drosophila* topoisomerase II protein. (C) Western blots of total nuclear proteins by using the various anti-topoisomerase II antibodies described in A. Note that in the 943 lane, a band reacts at ~50 kDa. Because of this reactivity to another protein, this antibody was not used for immunofluorescence. Reactivity to the 140-kDa protein is noted with the asterisk.

identifiable soluble proteins (85 and 140 kDa) and their potential association with the Tec element chromatin.

The Smaller Soluble Protein Is a Novel Protein with H1-Histone-like Extraction Properties

In the process of characterizing histones from *E. crassus*, we determined that the 85-kDa protein extracted from the an-

lagen with sulfuric acid (0.4 N H_2SO_4) and in addition, was the only protein that behaved like an H1 histone in that it was both H_2SO_4 and PCA soluble (addition of PCA to the H_2SO_4 -soluble fraction precipitated core histones) (Oliver *et al.*, 1972) (Figure 3C). Thus, p85 is readily purified from the anlagen based on its PCA solubility. We therefore generated rat antibodies to the PCA-extracted p85; these react with the LIS-extracted, or 2 M NaCl extracted "nucleoskeleton fraction" p85 (Figure 3D, α -p85). Likewise, antibodies to p85 that we affinity purify from the "total nucleoskeleton" (rabbit) antibody described above react to p85 from either type of preparation and show identical p85 localization as the rat antibody prepared to PCA-extracted p85 (see results below).

Purified p85 was subjected to amino terminal sequence analysis as well as cleavage and internal sequence analysis, and the resulting peptide sequences were used to obtain PCR products from the p85 gene and subsequently cDNA and genomic clones of the p85 gene (see MATERIALS AND METHODS). Sequence analysis of the p85 gene (accession no. AY155457) indicates that it encodes 542 amino acids (Figure 4A), which would have a predicted size of 60 kDa. Expression of the p85 gene in bacteria results in a protein that is PCA soluble, migrates as 60 kDa in SDS-PAGE, and is recognized by anti-p85 antibodies. This size difference from what is observed for the protein extracted from *E. crassus* can be explained by the likelihood that it is a highly phosphorylated protein. The encoded p85 protein contains 62 serines and 35 threonine residues of which 57 and 13, respectively, are predicted to be phosphorylatable. These phosphorylatable residues occur within predicted sites for cAMP/cGMP-dependent protein kinase, protein kinase C, and casein kinase II. The amino acid composition of the encoded p85 protein indicates that it is highly charged (30% basic amino acids, primarily lysines, 24% acidic residues, and <1% aromatic amino acids). This composition is similar to the HMG14/17 proteins, which have similar perchloric acid extraction properties to p85 (Johns, 1982; Walker, 1982). Nevertheless, p85 does not show sequence similarity to any known HMG proteins, including the *T. thermophila* HMG proteins (Schulman *et al.*, 1991).

Protein-structure prediction programs indicate that much of the p85 protein is "low-complexity" sequence (as can be seen by the multiple lysine and glutamic acid residues in the predicted protein) and that it contains two potential regions of coiled coil structure (Figure 4). Most database matches to p85 are proteins containing a domain that is similarly low in complexity and rich in lysine, glutamic acid, and serine. High similarity was observed to the "repeat domain" (~500 aa) of the neurofilament heavy chain protein from rabbit and human, which contains a repeat of "KSP" phosphorylation sites embedded within glutamic acid residues. Likewise, the UNC-89 protein from *Caenorhabditis elegans* was similar over an ~600-aa domain that contains the KSP motif repeated in the context of multiple lysine and glutamic acid residues. The p85 protein does not contain repeats of KSP. A region of p85 from amino acids 343–465 consists of a heptamer repeat of the sequence KK(E/D)XXX(E/D), where the X denotes variable amino acids. A portion of this region is predicted to form a coiled coil structure (Figure 4A).

As is typical of many *E. crassus* genes, the macronuclear molecule carrying the p85 gene has limited amounts of noncoding DNA. As shown in Figure 4B, there are 120 base

Figure 6. Immunofluorescence localization of p85 and topoisomerase II in nuclei and chromatin spreads. (A and B) Purified 45-h anlagen (A) and LIS-extracted (without DNase digestion) anlagen (B) were formaldehyde fixed and processed for indirect immunofluorescence with antibodies to p85 (rat antibody detected with rhodamine secondary) and topoisomerase II (rabbit antibody detected with fluorescein secondary) then counterstained with DAPI to visualize the DNA. The arrowheads in A point to the spot of intense p85 and topoisomerase II staining. (C and D) Chromatin from trypsin-treated 45-h anlagen, formaldehyde fixed, and centrifuged onto coverslips. Localization of p85, topoisomerase II, and DNA was as described for A and B. The arrowheads in A point to the spot of intense p85 and topoisomerase II staining. Magnification in all cases was at 630 $\times$.

pairs between the telomere and the start of the p85 coding region (ATG). At the 3' end, there are 32 base pairs after the TAA stop codon (no internal stop codons are found). Sequence analysis of two independent cDNA clones indicates that the polyA tail is added at a residue corresponding to a position eight base pairs internal to the telomere.

The Larger Soluble Protein Is Topoisomerase II

Because topoisomerase II is associated with metaphase chromosome scaffolds (Berrios *et al.*, 1985; Earnshaw *et al.*, 1985) and is a major protein species that is resistant to the types of extractions described above, and because the larger of the two solubilized proteins was in the molecular weight range of topoisomerase II proteins from other lower eukaryotes, we tested whether this protein was topoisomerase II. To do this, we determined whether antibodies to *Drosophila* topoisomerase II (kindly provided by Paul Fisher, SUNY, Stony Brook, NY) would react with the 140-kDa band in Western blots of the polyacrylamide gels. As seen in Figure 5, four different antibodies to *Drosophila* topoisomerase II reacted with the 140-kDa band in Western blots of either total nuclear proteins or the chromosome scaffold preparations. One of these antibodies was made to the intact *Drosophila* topoisomerase II protein (Figure 5B), whereas the other three antibodies were made against large peptides corresponding to regions of *Drosophila* topoisomerase II that are related in sequence to topoisomerase II from a wide range of organisms (Figure 5, A and C) (Meller *et al.*, 1995). Given that antibodies to different regions of *Drosophila* topoisomerase II and to the intact protein react with the 140-kDa species it is highly likely that the 140-kDa protein is the *E. crassus* topoisomerase II. The antibody cross reactivity is consistent with our ability to use subfragments of the *Saccharomyces cerevisiae* topoisomerase II gene to detect a single topoisomerase II gene in *E. crassus* (Jahn, unpublished observations).

The 85-kDa Protein and Topoisomerase II Colocalize

To determine the localization of the 85-kDa protein relative to topoisomerase II in the polytene chromosome structure, we used the antibodies that we generated to PCA-extracted p85 in rats, such that double labeling with the rabbit anti-*Drosophila* topoisomerase II antibodies could be performed. We used several types of nuclear preparations to determine the subnuclear distribution of these proteins, including isolated intact nuclei, LIS-extracted nuclei, and frozen sections of nuclei. In all three cases, the localization of p85 and topoisomerase II is diffuse and little substructure is discernible (Figure 6, A and B). However, in all three types of preparations, spots of intense staining were apparent with both antibodies, and double labeling indicated that these spots are coincident for topoisomerase II and p85. Confocal microscopy indicated that these spots were internal in the nuclei and not just associated with the nuclear periphery. Because there were typically one or two spots per nucleus with about half of the nuclei showing spots, we thought that they might be related to the early differentiation of a nucleolus in the developing macronucleus. In three separate experiments combining immunofluorescence with anti-p85 or anti-topoisomerase II and in situ hybridization with an rDNA probe, colocalization of rDNA sequences with the intense spots of anti-p85 or topoisomerase II staining occurred in <20% of the nuclei. Thus, the intense spots of p85 and topoisomerase II staining do not seem to be associated with nucleoli and must be some other subnuclear region of increased concentration of these two proteins.

Given the abundance and dispersed localization of these proteins, we further examined their localization by spreading the chromatin, which affords higher resolution analysis of the localization. The polytene chromosomes cannot be released from the anlagen nuclear structure by any standard procedures of lysing nuclei. Thus, we resorted to mild trypsin treatment of nuclei to disrupt the nuclei and release

chromatin, which we centrifuged onto coverslips. By treating with trypsin for varying amounts of time before fixation with formaldehyde, we obtained chromatin preparations with different degrees of spreading, either with small amounts of chromosome-like structures spread away from an obvious nuclear mass to a more uniformly spread region of chromatin that was discrete but no longer spherical in structure. Analysis of the proteins from these trypsinized nuclear samples by Coomassie staining and by Western blotting with anti-p85 and anti-topoisomerase II indicated that these proteins, as well as the core histones, were not digested at the concentration of trypsin and duration of treatment used. Furthermore, MNase digestion of the nuclei after treatment with trypsin at these concentrations showed that the unusual Tec element chromatin digestion pattern was unaltered (Jahn, 1999). Disruption of the Tec element chromatin structure was only accomplished at 10^2 - to 10^3 -fold higher concentration of trypsin and digestion at 37°C instead of room temperature (Jahn, 1999).

As seen in Figure 6, C and D, the staining by anti-p85 and anti-topoisomerase II colocalizes in discrete subregions of the chromatin seen by DAPI staining of the DNA. Immunofluorescence with antibodies to core histones colocalizes with all of the DAPI staining (our unpublished data); thus, the specific subregions rich in anti-p85 and anti-topoisomerase II are not due to an unusual redistribution of chromatin proteins relative to DNA. The chromatin spreads give a better idea of the abundance of p85 and topoisomerase II. Typically, staining for these two proteins occurs within ~50% of the chromatin spread from a single nucleus. Although variations in fluorescent intensity are apparent when comparing the staining for the two proteins, the p85 and topoisomerase II staining is completely coincident (Figure 6, C and D).

Tec Elements Colocalize with p85

Although p85 and topoisomerase II are abundant in the *E. crassus* anlagen, they do not seem to associate with all of the

eliminated DNA, as observed for the *Tetrahymena* Pdd proteins, because eliminated sequences comprise 80–90% of the *E. crassus* genome. Given that the extraction of these proteins paralleled the extraction properties of the unusual Tec element chromatin, we sought to determine whether any specific association with Tec elements was apparent. We performed in situ hybridization with Tec element probes as well as a bulk “macronuclear sequence” probe (see MATERIALS AND METHODS) by using two different fluorochromes to label the two probes. We have estimated that the macronuclear sequence probe, which was Bal31 digested to remove telomeres and size fractionated to remove the abundant rDNA molecules, hybridizes to the majority of the macronuclear sequences (Southern blot hybridizations are shown in Jahn, 1999); thus, it is expected to hybridize to numerous chromosomal regions where the ~20,000 precursors to the macronuclear linear DNA molecules are clustered in the genome (Jahn *et al.*, 1988). Likewise, the Tec element probes should detect the >20,000 copies of the elements dispersed throughout the genome.

We initially performed the immunofluorescence and hybridization on intact nuclei and frozen sections of nuclei. This demonstrated that the myriad of punctate spots of anti-p85 reactivity colocalized with Tec element hybridization but not with the macronuclear sequence hybridization at least at the resolution of two-color discrimination of overlap. However, the abundance of spots made this difficult to quantitate. In addition, permeabilization for immunofluorescence relative to in situ hybridization was difficult to control in these preparations. We therefore did higher resolution localization by using the chromatin spreads described above. In hybridizations with these two probes to 40-, 42-, or 45-h anlagen chromatin spreads, we see very little overlap between the sites of hybridization of these two probes (Figure 7A). Although approximately one-third of the Tec elements would be expected to be close to macronuclear sequences because they interrupt them as IES, these time points are during or immediately after IES excision and thus the majority of the IES Tecs could be separated from the chromosomes. We quantitated the number of spots hybridizing to each probe in chromatin spreads where a substantial number (>30) of hybridizing spots were visible for each probe and where the chromatin seemed to be equivalent to one nucleus. At least 10 individual spreads from at least three different hybridizations (i.e., different coverslips) were counted. Only 0.8% of the Tec spots were seen to overlap in their hybridization with the macronuclear sequence spots.

When the in situ hybridization to Tec or macronuclear sequences is carried out with immunofluorescence to detect p85, it is apparent that the Tec elements colocalize with p85 protein and the macronuclear sequences do not (Figure 7, B–D). Quantitation of the hybridization relative to anti-p85 reactivity was carried out as described above for the hybridization probes. This demonstrated that 100% of the Tec hybridization overlapped with spots of anti-p85 staining and that 70% of the p85 staining coincided with the Tec elements. In contrast, only 5% of the p85 staining coincided with the macronuclear sequence hybridization, with 9% of the macronuclear sequence-hybridizing spots overlapping p85. Thus, the p85 protein is associated with the Tec elements as well as some other sequences, the majority of which are not macronuclear-specific sequences.

Figure 7 (facing page). Fluorescence in situ hybridization of 40- to 42-h chromatin spreads with Tec and macronuclear sequence-specific probes and immunofluorescence localization of p85. (A) Fluorescence in situ hybridization (FISH) with a bulk macronuclear sequence probe (red) and a probe that detects the Tec elements (green). DAPI staining of DNA is shown in blue. Examples of two chromatin spreads are shown. White arrows designate specific spots of hybridization. Notice that in the overlay panels most of the green and red spots do not colocalize to form yellow spots. Quantitation of spots is reported in the text. (B) FISH to chromatin spreads by using the macronuclear sequence probe in combination with immunofluorescence by using an anti-p85 antibody. Macronuclear sequence hybridization is shown in red, anti-p85 staining in green, and DAPI staining of DNA in blue. White arrows designate individual spots. Notice that in the overlay panels most of the red and green spots are distinct and do not colocalize to form yellow spots. (C) FISH with Tec element probes combined with immunofluorescence with an anti-p85 antibody for two examples of 42-h chromatin spreads. DAPI staining of DNA is shown in blue, p85 staining in red, and Tec element hybridization in green. The far right panel shows the overlay image of p85 and Tec images. White arrows indicate specific spots of p85 or Tec hybridization. Double labeling reveals that all of the Tec staining overlaps the p85 staining (seen as yellow in the overlay image). Quantitation is described in the text. (D) Same as C but with spreads from 38-h anlagen.

Figure 8. p85 is a developmentally regulated and anlagen specific protein. (A) Western blotting of a developmental time course of whole cell extracts (SDS-PAGE) was performed using an anti-p85 antibody. The same samples run in parallel and blotted were probed with anti-tubulin as a loading control. V, vegetative. P85 shows up by 18 h, which is the beginning of the polytene stage of macronuclear development. (B) Immunofluorescence analysis on whole cells (vegetative or developing *E. crassus*) with p85 antibodies reveal that p85 is only present in the anlagen and first shows up at 15–20 h postmixing. Note that *E. crassus* cells are extremely difficult to permeabilize (see MATERIALS AND METHODS for description) and are autofluorescent, particularly their cilia and ciliary ridge, which is visible in some of

We also did the in situ hybridization of Tec elements and localization of p85 relative to topoisomerase II on chromatin spreads prepared from developing macronuclei at 38 h, which is before the second round of Tec element deletion (40–42 h of development). These spreads are similar to the 40- to 45-h chromatin spreads and demonstrate colocalization of topoisomerase II and p85 (Figure 6, C and D) and the colocalization of p85/topoisomerase II with the Tec elements (Figure 7D). In the 38-h spreads, however, the chromatin fibers seem more continuous, which may be a function of how the eliminated chromatin behaves before vs. after the second round of Tec element excision from the chromosomes (i.e., pre or post 42 h). The numerology of colocalization of the DNA sequences with p85 or topoisomerase II at 38 h was as follows: 100% of the Tec spots colocalize with the p85 spots and account for 75% of the total p85 spots. Note that these numbers are similar to what we observed with the 40- to 45-h spreads.

p85 Is a Developmental Stage and Anlagen-specific Protein

Because the above-mentioned data suggest that p85, possibly in association with topoisomerase II, could be generating the unusual chromatin structure of the Tec elements, we would expect the p85 protein to be present during the developmental time periods that the unusual Tec element chromatin structure is evident and that the protein would be specific to the anlagen, because the unusual chromatin structure is not apparent in the micronucleus. We have detected the altered chromatin structure as early as 20 h of development (Jahn, 1999) and the structure is evident as long as Tec elements are present and undergoing elimination from the anlagen (through 65 h). Thus, we examined a developmental time course of total cellular protein samples to determine when p85 is present. As seen in Figure 8, p85 is not detected in any vegetative cell sample by Western blotting. In mated cells, at 5 h the protein is not detected in total protein samples. At 18 h, p85 is detected and the amounts increase by 24 h and remain fairly constant through 65 h, the latest time point we tested. We have previously demonstrated that the excised circular forms of Tec elements are abundant as late as 65 h (Jahn *et al.*, 1989) and are just beginning to be degraded at that time. We have not detected the circular forms after 80 h.

We used immunofluorescence on fixed whole cells to determine which nuclei reacted with anti-p85. Immunofluorescence on whole cells is technically difficult with *E. cras-*

sus because the outer pellicle of the cells is difficult to permeabilize. Procedures for immunofluorescence by using urea treatment after formaldehyde fixation have allowed reliable detection of some antigens (Jacobs *et al.*, 1999). In our hands, this procedure leads to a high background fluorescence with the secondary antibodies used alone (40-h control in Figure 8). Nevertheless, we are able to demonstrate reactivity of the p85 antibodies on anlagen in whole cells at 45 h of development as being significantly greater than the background fluorescence by using this procedure; thus, we examined a time course of samples to determine whether p85 is present in any other nuclei and to determine whether the same time course of appearance is evident as that observed by Western blotting. As seen in Figure 8, p85 is not detectable above this background fluorescence in vegetative cell nuclei.

We performed immunofluorescence with antibodies to p85 on mated cells at 2, 4, 6, 10, 12, 15, 18 and 20 h postmixing. Anti-p85 reactivity is not present in any nuclei before 15 h and is faintly apparent at 15 h, which is the earliest time point where we have seen a detectable anlagen (Figure 8). After 18 h, the staining with anti-p85 becomes much more visible and clearly differs from the background staining seen with secondary antibody used alone (see 20-h example compared with the $-\alpha$ p85 control and vegetative or 15-h samples; Figure 8). In cells undergoing macronuclear development, the old macronuclei and micronuclei do not react with antibodies to p85, whereas developing macronuclei show increasing staining between 18 and 24 h with intense staining at various time points between 45 and 65 h. At 70–80 h, the staining of the anlagen decreased and we noticed vesicle-like structures external to the anlagen that reacted with both anti-p85 (70 h; Figure 8) and anti-topoisomerase II (our unpublished data) and which stained faintly with DAPI (70 h; Figure 8). These structures are similar in size to food vacuoles seen in vegetative, fed cells and we suspect that they are involved in degrading the eliminated chromatin. After 70–80 h, staining is no longer seen.

DISCUSSION

We have identified p85 as a developmental stage and anlagen-specific protein that colocalizes and coextracts with a subset of the eliminated sequences in *E. crassus*. Most notably, the highly abundant Tec elements show 100% colocalization with 70% of the p85 throughout macronuclear development while they are being eliminated. In contrast, a macronuclear sequence hybridization probe that we estimate would detect a majority of the macronuclear genomic sequences (Jahn, 1999) shows very little colocalization with p85. In addition to the localization data, the solubilization properties of the Tec element chromatin micrococcal nuclease digestion products parallel the p85 and topoisomerase II extraction properties; both the Tec element micrococcal nuclease digestion products and these two proteins become highly enriched relative to other sequences and proteins in salt extracted nuclei. In contrast, macronuclear-destined sequences (i.e., anlagen sequences hybridizing to macronuclear sequence probes) are readily digested to subnucleosomal sizes after salt extraction and are readily solubilized as nucleosome multimers and monomers at low salt concentrations. Because of the unusual solubility properties of the

Figure 8 (cont). the micrographs and which is seen even without the addition of primary antibody (shown at the top left panel as the minus anti-p85 [$-\alpha$ p85], 40-h control). Immunofluorescence analysis was performed using an anti-p85 polyclonal antibody. Left panels (black and white) show DAPI staining to indicate the nuclei; right panels (green) show anti-p85 staining. White lines indicate specific nuclei: A, developing macronucleus (anlagen); M, macronuclei (intact or degrading); and m, micronuclei. The arrow in the 70-h picture indicates a food vacuole-like structure external to the anlagen that faintly stains with DAPI and reacts with anti-p85. The exposure times used for the [$-\alpha$ p85] 40-h control, and the vegetative and 15-h samples were much longer to show that the nuclei do not stain above the background staining seen without α p85.

Tec element chromatin and the p85 protein, it has not been possible to demonstrate their association by other techniques such as chromatin immunoprecipitation.

p85 is the first protein identified that shows any association with eliminated sequences in the *E. crassus* anlagen. The localization of the p85 protein exclusively to the anlagen differs from the *Tetrahymena* Pdd proteins in that the Pdd proteins are found in both the anlagen and the degrading old macronucleus (Madireddi *et al.*, 1996; Smothers *et al.*, 1997b; Nikiforov *et al.*, 2000). There are many features of DNA elimination in *Euplotes* that differ from elimination in *Tetrahymena* (reviewed by Coyne *et al.*, 1996), and the degree to which these processes involve conserved functions is debatable. At present, there seems to be a correlation between the formation of "heterochromatic," higher order, condensed chromatin structures and the elimination of DNA. The condensed structure of the eliminated DNA in *Tetrahymena* has been defined cytologically (Madireddi *et al.*, 1994, 1996; Smothers *et al.*, 1997a), whereas the condensed structure of the Tec elements is inferred from their inaccessibility to nucleases (Jahn, 1999) and their extraction properties (this article).

The salt extraction properties of p85, which parallel the extraction properties of the unusual Tec element chromatin structure, and its high lysine and glutamic/aspartic acid contents suggest that it could be involved in the unusual chromatin structure of the Tec elements in the anlagen. The MNase digestion properties of the Tec element chromatin (Jahn, 1999) indicate that the nucleosomal structure is modified such that the linker region between nucleosomes is completely inaccessible to digestion and only sites within the nucleosomal core become accessible. Because no histone H1 is present in the anlagen (Ray *et al.*, 1999; Jahn, unpublished observations), the high lysine content of p85 may result in binding of p85 in the linker region. In addition, the numerous patches of acidic vs. basic residues occurring throughout p85 make it possible that p85 interacts with both histones and DNA. Thus, it may span between and interact along nucleosomes, which could result in complete occlusion of the linker region. The potential coiled-coil structure within p85 indicates that it has a protein interaction domain; this may be important in associations with other proteins or in the self-association of p85 and could explain the unusual insolubility of the p85 containing chromatin. Further experiments to reconstitute chromatin with p85 and binding studies of p85 with nucleosomes and their multimers should give us a better idea of how p85 may function with respect to chromatin structure.

Our means of extracting and selectively enriching the Tec element chromatin and the p85 and topoisomerase II proteins suggests that we are looking at a chromosome scaffold structure. The best-studied example of a chromosome scaffold structure is the residual scaffold left from mammalian metaphase chromosomes after extractions similar to what we used herein. Topoisomerase II, lamins, and "XCAP" proteins, now known to be part of the condensin complex, are the major proteins identified in metaphase scaffold preparations (Berrios *et al.*, 1985; Earnshaw *et al.*, 1985). Thus, our finding of topoisomerase II as one of the major proteins in the *E. crassus* anlagen "scaffold/matrix" fraction after extraction with high salt or LIS was expected. In mammalian metaphase chromosomes, topoisomerase II colocalizes with

AT-rich DNA sequences referred to as the "AT queue," which are detected by fluorescent dyes that intercalate with AT-rich DNA (Saitoh and Laemmli, 1994). It is thought that both histone H1 and topoisomerase II preferentially interact with AT-rich MARs/SARs (matrix attachment regions or scaffold attachment regions) to facilitate chromosome condensation (Hart and Laemmli, 1998) and that topoisomerase II, as a chromosome scaffold protein, may play a major role in the looped organization of chromatin fibers. This is interesting with respect to our findings of topoisomerase II enrichment in association with the Tec elements (and p85) because it has long been recognized that the IES sequences in ciliates, or at least their boundaries, are AT rich. For instance, the Tec element inverted repeat is 96–98% AT in the first 68 base pairs (Jaraczewski and Jahn, 1993). Thus, these sequences may be equivalent to the AT-rich MARs/SARs defined in other systems and may be preferential sites of topoisomerase II interaction. In *Drosophila*, a similar sequence preference for a repetitive DNA is apparent. Localization studies with injected fluorescently tagged topoisomerase II and the mapping of in vivo topoisomerase II cleavage sites indicate that topoisomerase II is preferentially associated with the AT-rich satellite III (359-base pair repeat) DNA (Kas and Laemmli, 1992; Marshall *et al.*, 1997). The functional significance of this association is presently unknown.

The presence of p85 and topoisomerase II in discrete chromosomal subregions associated with the Tec elements and other eliminated sequences suggests that there may be a role for topoisomerase II in DNA-processing events. Given the known properties of topoisomerase II (Nitiss, 1998), it is likely to be involved in decatenation of DNA during the polytene replications. In mitotic cells, topoisomerase II is required for sister chromatid separation postreplication and loss of topoisomerase II function can result in a late S-phase block (DiNardo *et al.*, 1984; Holm *et al.*, 1985). We are intrigued by the fact that Tec element excision is a post replication or late replication event in that it occurs at the end of each of the two polytene S phases, and we wonder whether topoisomerase II could be directly involved in the excision. If the polytene chromatin strands in the *E. crassus* anlagen remain entangled with each other postreplication and undergo a controlled separation, the inverted repeat structure of the Tec elements could allow these entanglements to become trapped via recombination junctions at the ends of the elements, with the elements held as catenated loops. Resolution of such a structure, potentially by topoisomerase II, might result in excision of the Tec element loops. Alternatively, topoisomerase II may control the chromatin condensation state of eliminated vs. retained DNA. As an alternative or adjunct to direct binding of p85 to nucleosomes to generate a higher order structure, the unusual Tec element MNase digestion properties could reflect a particular coiling state of the chromatin that is maintained by p85 and topoisomerase II. The Tec elements may be a discrete loop controlled by topoisomerase II, and the combination of p85 with topoisomerase II anchoring may create a higher order structure with altered MNase digestion properties.

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