

Localization of RAP1 and Topoisomerase II in Nuclei and Meiotic Chromosomes of Yeast

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Abstract. Topoisomerase II (topoII) and RAP1 (Repressor Activator Protein 1) are two abundant nuclear proteins with proposed structural roles in the higher-order organization of chromosomes. Both proteins cofractionate as components of nuclear scaffolds from vegetatively growing yeast cells, and both proteins are present as components of pachytene chromosomes, cofractionating with an insoluble subfraction of meiotic nuclei. Immunolocalization using antibodies specific for topoII shows staining of an axial core of the yeast meiotic chromosome, extending the length of the syn-

aptonemal complex. RAP1, on the other hand, is located at the ends of the paired bivalent chromosomes, consistent with its ability to bind telomeric sequences *in vitro*. In interphase nuclei, again in contrast to anti-topoII, anti-RAP1 gives a distinctly punctate staining that is located primarily at the nuclear periphery. Approximately 16 brightly staining foci can be identified in a diploid nucleus stained with anti-RAP1 antibodies, suggesting that telomeres are grouped together, perhaps through interaction with the nuclear envelope.

THE higher order structure of eukaryotic chromosomes facilitates the replication, recombination, and segregation of chromosomes, as well as maintaining the genome topologically compact and organized in metaphase (reviewed in Gasser and Laemmli, 1987). The basic unit of chromatin organization beyond the nucleosome, is thought to be torsionally constrained loops of DNA, ranging in size from <5 to >100 kb. DNA loops have been visualized by microscopy in spreads of meiotic synaptonemal complexes (Weith, 1985), amphibian lampbrush chromosomes (Callan, 1986), histone-depleted mitotic chromosomes (Paulson and Laemmli, 1977), and interphase nuclei (Cook and Brazell, 1975). Protein-protein interactions between nonhistone chromosomal proteins are thought to maintain DNA in looped domains, and a scaffold of nonhistone proteins, that putatively performs this function, can be isolated from metaphase chromosomes by various extraction procedures (Lewis and Laemmli, 1982).

On the DNA level, the best candidates to date for defining looped chromosomal domains are A + T-rich regions of DNA that show a selective affinity for nuclear and chromosomal scaffolds *in vitro* (Mirkovitch et al., 1984, 1988). These scaffold-attached regions of DNA are characterized by oligo dT or dA stretches that form a double-stranded DNA with an unusually narrow minor groove (Käs et al.,

1989; Amati et al., 1990). A number of proteins bind preferentially to such sequences *in vitro*, including histone H1 (Izaurrealde et al., 1989) and topoisomerase II (topoII)¹ (Adachi et al., 1989). In addition, topoII is the most abundant component of the mitotic scaffold as isolated from human metaphase chromosomes (Gasser et al., 1986), where its copy number is approximately three to four copies per average 70-kb loop. In yeast, the gene encoding topoII is essential for growth, and studies of temperature-sensitive mutants show that topoII is required both for the decatenation of sister chromatids, as well as for the condensation of chromatin before mitotic segregation (Dinardo et al., 1984; Holm et al., 1985; Uemura et al., 1987). The ability of topoII to bind two separate DNA molecules is consistent with its proposed role in mitotic chromosomal structure.

A second abundant nuclear protein that has been identified as a component of the yeast nuclear scaffold is the Repressor Activator Protein (RAP1) (Hofmann et al., 1989). RAP1 binds within a sequence element that promotes transcriptional repression of the silent mating type loci in yeast, HMR α and HML α (Shore and Nasmyth, 1987), and binds upstream of numerous genes, where it acts as a transcriptional enhancer (Huet et al., 1985; Buchman et al., 1988; Devlin et al., 1991; Kurtz and Shore, 1991). This versatile protein has a third type of binding site (ACACCCACAC-ACC), found within the repeated sequence motif that extends

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1. *Abbreviations used in this paper:* RAP1, Repressor Activator Protein 1; r.t., room temperature; SC, synaptonemal complex; topoII, topoisomerase II.

from 300–500 bp at the end of each telomere, i.e., $(C_{1-3}A)_n$ (Berman et al., 1986; Buchman et al., 1988). Roughly 14 RAP1 binding sites have been demonstrated to exist within a 300-bp stretch of the $C_{1-3}A$ repeat (Roberge, M., E. Gilson, and S. M. Gasser, manuscript in preparation). Both temperature-sensitive RAP1 mutants and overexpression of the RAP1 protein alter telomere length *in vivo* (Conrad et al., 1990; Lustig et al., 1990; Sussel and Shore, 1991), providing evidence that RAP1 either binds telomeres directly, or interacts with and regulates a telomere binding protein.

Although extensive study has been made on the role of RAP1 as a potential modulator of transcription and of telomere length, no immunolocalization study has been published. To help understand the diversity of functions ascribed to RAP1, it is important to know how it is distributed in the yeast nucleus, and whether it is in continual association with chromosomes throughout the meiotic and mitotic cell cycles.

Immunolocalization studies of yeast chromosomal proteins are somewhat hindered by the difficulty of visualizing condensed mitotic chromosomes. In fact, the only time in the yeast life cycle during which individual yeast chromosomes can be readily prepared for microscopy is the pachytene stage of the first meiotic division (Dresser and Giroux, 1988; Laroche, T., and S. M. Gasser, unpublished results). Pachytene is the period of meiotic prophase I, where homologue chromosomes are fully paired and synapsed, held together by a proteinaceous structure called the synaptonemal complex (SC). Both ends of the synaptonemal complex appear to be in contact with the inner nuclear membrane in yeast (Byers and Goetsch, 1975) and in most other organisms investigated (reviewed in Moses, 1968; Wettstein et al., 1984; John, 1990).

To date only two well-characterized proteins have been immunolocalized to meiotic chromosomes. An antibody against HOPI1, a 69-kD zinc finger protein, binds to yeast meiotic chromosomes (Hollingsworth et al., 1990), and a serum raised against chicken topoisomerase II was reported to react with meiotic chromosomes in chicken (Moens and Earnshaw, 1989). Using our modification (Loidl et al., 1991) of the spreading method of Dresser and Giroux (1988), we present evidence that yeast topoII is located along the core of meiotic yeast chromosomes and that RAP1 is situated at the ends of meiotic bivalents. In interphase, RAP1 is found in intranuclear aggregates, preferentially located near the nuclear membrane, whereas topoisomerase II is distributed uniformly throughout the yeast nucleus.

Materials and Methods

Strains

The homothallic diploid wild type strain SK1 (Kane and Roth, 1974) was used for meiotic spreads. For immunofluorescence of vegetatively growing cells and for most biochemical studies the following strains were used: 62-5c (MAT a, *leu2*, *his3*, *lys2*, *pep4-3*); MS-27a (MAT a, *trp1-A1*, *ura3-52*, *rho+*, *Kil^o*, *pep4::URA3*); GA-7 (MAT a/ α , *ade2/ADE2*, *his4/HIS4*, *lys2/LYS2*, *pep4-3/pep4-3*, *trp1/TRP1*); GA-138 (MAT a/ α , *cdc13-1/cdc13-1*, *ade2/ade2*, *adel/adel*, *ural/ural*, *his7/HIS7*, *lys2/LYS2*, *tyr1/TYR1*, *gall/GALI*; grown at permissive temperature) and a commercial bakers' yeast. Yeast was grown in YPAD (1% yeast extract, 2% bacto-peptone, 40 mg/l adenine, 2% dextrose; Sherman et al., 1986).

Preparation of SCs in Yeast

SCs were prepared as reported previously (Loidl et al., 1991) using a

modification of the method of Dresser and Giroux (1988). Cells were grown in presporulation medium (1% KOAc, 2% Peptone, 1% Yeast Extract) at 30°C to a density of 2×10^7 cells/ml. Cells were harvested, washed, resuspended in 1/2 volume of sporulation medium (2% KOAc, pH 7), and shaken at 30°C. Yield of pachytene nuclei in SK1 was highest when the cells were taken when 3–5% four-nucleate cells were counted in 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI)-stained (100 ng/ml) aliquots after 4.5 to 6.5 h. Cells of a 10-ml sample were harvested and resuspended in 1 ml of 2% KOAc, pH 7.0, 1 M sorbitol, 10 mM DTT, to which 5 μ l of a 10 mg/ml solution of Zymolyase 20T (Seikagaku Kyogo Co.) was added. Digestion took 20 to 40 min at 37°C. Spheroplasting was monitored by lysis in a 1:1 mixture of cells with 2% wt/vol Sarkosyl. At 90% lysis spheroplasting was terminated by addition of 10 ml ice-cold stop solution (0.1 M 2-[N-morpholino] ethane sulfonic acid [MES], 1 M sorbitol, 1 mM EDTA, 0.5 mM MgCl₂, pH 6.4). Spheroplasts were spun down at 3,000 rpm, resuspended in 1 ml stop solution, and kept on ice. 20 μ l cell suspension, 40 μ l fixative (4% wt/vol paraformaldehyde/3.4% wt/vol sucrose), 80 μ l 1% vol/vol Lipsol, and again 80 μ l fixative were pipetted sequentially onto a clean microscope slide and were mixed. The mixture was evenly spread out on the slide with a glass rod. The slides were then laid flat under a chemical hood and allowed to air dry for at least two hours.

Staining and Microscopy

Silver staining of spread preparations and their transfer to grids for EM was done as reported previously (Loidl et al., 1991). For immunostaining, slides were washed in PBS (0.14 M NaCl, 10 mM NaPi, pH 7.4) for 20 min, preincubated 30 min with 1% BSA, and incubated for 2 h at room temperature (r.t.) in a moisture chamber with affinity purified rabbit anti-RAP1 IgG or anti-topoisomerase II IgG diluted from 1:5 to 1:50 in PBS. Slides were then washed 3 \times 3 min in PBS and incubated again for 2 h at r.t. with the secondary antibody (fluorescein- or rhodamine-conjugated goat anti-rabbit IgG at 1 mg/ml) diluted 1:50 in PBS. During these procedures the slides were never allowed to dry. After washing for 10 min in PBS and mounting in antifade buffer (0.1% *p*-phenyldiamine-dihydrochloride in PBS adjusted to pH 8.0 with 0.5 M Na[CO₃]₂ and 70% glycerol) or 50% glycerol and 2 μ g/ml DAPI in PBS and then the slides were examined by fluorescence microscopy on a Nikon Microphot-FX, a Reichert Polyvar, or a Leitz Labovert using appropriate filters. In some cases the PBS used for the staining included ovalbumin and Triton X-100 as described below for whole cells, without noticeable differences in staining.

Nuclear Isolation and Subfractionation

Preparation of yeast nuclei and nuclear scaffolds from vegetatively growing cultures was done as described (Amati and Gasser, 1988; Cardenas et al., 1990). To adapt these protocols to meiotic cultures the following modifications were made. After lysis in 18% Ficoll in 10 mM Pipes-KOH, pH 6.8, 2 mM K-EDTA, 20 mM KCl, 0.125 mM spermidine, 0.05 mM spermine, 1% Trasylol (aprotinin; Bayer), 1% thiodiglycol (Pierce Chemical Co., Piscataway, NJ), 0.5 mM PMSF, and 1 mg/ml pepstatin, the cell homogenate was centrifuged at 4,000 rpm in an HB-4 rotor (Sorvall, Newton, CT). The supernatant was then recovered and a crude nuclear fraction was obtained by centrifugation at 12,000 rpm for 12 min, in the same rotor. The crude nuclear pellet was resuspended to 8 ml/g wet weight starting cells in gradient buffer (10 mM Pipes-KOH, pH 6.8, 2 mM K-EDTA, 20 mM KCl, 2.125 mM spermidine, 0.85 mM spermine, 1% Trasylol, 1% thiodiglycol, 0.5 mM PMSF, 1 mg/ml pepstatin, and 10% glycerol) and were then dounced vigorously in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The mixture was then adjusted to final this volume with gradient buffer containing 60% Percoll, such that the final concentration of Percoll was 30%. Percoll gradients were formed in 50-ml tubes by centrifugation at 38,700 g for 45 min at 4°C in a rotor (SS-34; Sorvall, Newton, CT). The cleanest nuclei were recovered as a band in the lower third of the gradient, as judged by DAPI staining and microscopic analysis. These were recovered and washed in the lysis buffer minus Ficoll. The stabilization of a scaffold fraction and extraction of histones from yeast nuclei with Li-2',3'-diiodosalicylate is described in Amati and Gasser (1988) and Cardenas et al. (1990).

Antibody Production and Affinity Purification

The preparation of topoII from an overexpressing yeast strain and preparation of anti-topoisomerase II antibody have been described (Cardenas et al., 1992). The preparation of the anti-RAP1 antibody was done by overproduction either of the amino acids 19 to 502 or of amino acids 19 to 827, which

were cloned downstream of the T7 promoter (Studier and Moffatt, 1986). The fusion protein produced includes 11 amino acids of the T7 phage gene 10 product preceding the RAPI protein. This was purified on SDS gels, eluted, and injected as described for topoII (Cardenas et al., 1992). All antibodies were affinity purified before use as described in Gasser et al. (1986). The RAPI used for affinity purification and competition studies was also made by overexpression in bacteria of amino acids 19–827 of RAPI fused to phage T7 gene 10 protein.

Immunofluorescence on Yeast Spheroplasts

Cells were grown overnight and converted to spheroplasts with lyticase and Zymolyase as described (Verdier et al., 1990). After washing twice in YPAD/1 M sorbitol, the cells were fixed for 20 min at r.t. by incubation with 3.7% paraformaldehyde in the same buffer. The cells were recovered by centrifugation (1,000 g for 10 min) and washed twice in the same buffer without paraformaldehyde. The cells were spotted on polylysine-coated slides and left to air dry 10 min. The slides were immersed in methanol at -20°C for 6 min and then in acetone at -20°C for 30 s, and again air dried. After rinsing in PBS, the slides were incubated in affinity-purified antibody diluted 1:5 in PBS containing 1% ovalbumin and 0.1% Triton X-100 for 1 h in a humid chamber at 37°C . After three washes in PBS containing 1% BSA, the slides were incubated with rhodamine-conjugated secondary antibody (or fluorescein conjugated where indicated) diluted to 20 $\mu\text{g}/\text{ml}$ in PBS/ovalbumin/Triton as above. After three washes in PBS/Triton, the slides were mounted with 50% glycerol in PBS and 2 $\mu\text{g}/\text{ml}$ DAPI, and viewed on a Polyvar (Reichert, Vienna, Austria).

Immunoelectron Microscopy

For immunoelectron microscopy an exponentially growing culture of the haploid yeast strain 62-5c was fixed in its culture medium by addition of 4% (wt/wt) final concentration of freshly dissolved paraformaldehyde. After fixing for 30 min at 30°C , the reaction was quenched by incubation with 0.5 mg/ml NaBH_4 for 10 min. Cells were recovered by centrifugation and spheroplasted as described (Amati and Gasser, 1988). After 20 min of spheroplasting at 30°C cells were refixed with paraformaldehyde as described above. The procedure for immunofluorescence of whole cells was followed except that the secondary antibody was a 1 nm gold-conjugated goat anti-rabbit IgG (Biocell Research Laboratories, Cardiff, UK). After the final washes (four times 15 min in PBS), the labeled cells were fixed with 2% glutaraldehyde for 20 min in PBS. The cells were then encapsulated in agarose beads, basically as described (Cardenas et al., 1990), to facilitate subsequent manipulations and to minimize loss. The labeled cells, embedded in agarose, were dehydrated sequentially by incubation in increasing concentrations of ethanol, embedded in LR White, and sectioned as previously described (Cardenas et al., 1990). Sections were stained for

20 min with uranyl acetate, 10 min with lead citrate, and the 1-nm gold particles were enhanced with a silver stain, all following the Biocell Research Laboratories protocol. This entails formation of a silver metal precipitate around the gold grains, by reduction of a silver salt. Visualization and photography was done on an electron microscope (CM10; Electronic Instruments, Inc., Mahwah, NJ).

Results

Anti-RAPI and Anti-Topoisomerase II Antibodies Are Monospecific in Yeast

Antibodies were raised against purified yeast topoII and against the yeast nuclear protein RAPI, expressed as a fusion protein in *Escherichia coli*. The antigen-specific immunoglobulins were purified by binding and release from strips of nitrocellulose saturated with either topoII or with full-length, bacterially expressed RAPI. To demonstrate that the antibodies recognize only one polypeptide both in vegetatively growing and in meiotic yeast cells, extracts of total proteins were transferred to nitrocellulose membranes and were probed with the antibodies.

Fig. 1 shows the patterns of total protein from SK1 cells in meiosis (odd numbered lanes 1–11) and from a mitotically dividing haploid culture (62-5c, even-numbered lanes 2–12). The Coomassie blue-stained patterns are shown in lanes 1 and 2, and the same protein samples were probed on Western blots using RAPI-specific antibodies (lanes 3–6) and topoII-specific antibodies (lanes 9–12). Anti-RAPI recognizes a polypeptide of 116 kD in both vegetatively growing cultures and in meiotic cells of *Saccharomyces cerevisiae*. There is no yeast-specific reaction with the secondary antibody used (lanes 7 and 8). RAPI is extremely sensitive to proteolysis, and in the diploid SK1 we observe weak reaction with two smaller polypeptides of 92 and 60 kD, the sizes of major breakdown products of RAPI. The appearance and abundance of these is variable (see also Fig. 7), becoming more prominent in the extracts of yeast strains that are not protease deficient (e.g., SK1). Competition for the immunostaining by an excess of bacterially expressed and affinity-purified RAPI

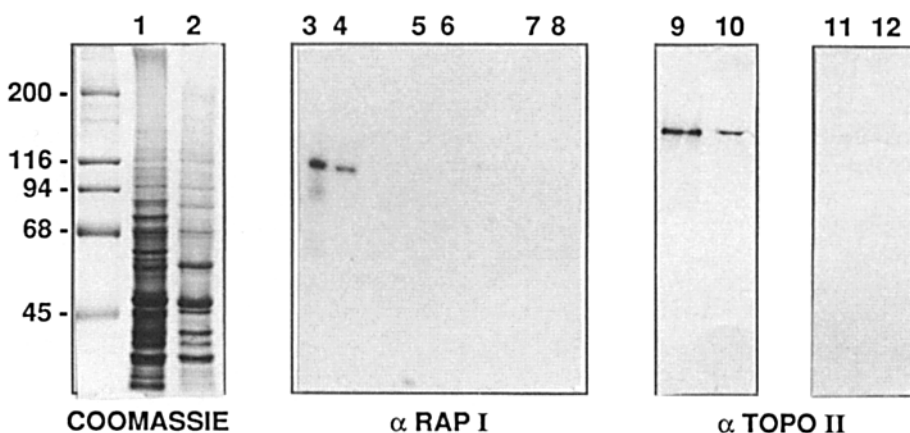


Figure 1. Anti-RAPI and anti-TopoII antibodies are monospecific when tested against both meiotic and mitotic yeast cell extracts. Proteins were extracted from whole cells of the diploid SK-1, which was induced to enter meiosis (lane 1; and see Materials and Methods), and from the haploid 62-5c grown to mid-logarithmic phase on YPAD (lane 2). Proteins were separated on an 8% minigel and stained with Coomassie blue. Odd numbered lanes contain the meiotic SK1 protein and even numbered

lanes have the haploid 62-5c. Western blots were probed with affinity-purified anti-RAPI (lanes 3–6) and anti-TopoII (lanes 9–12), prior to incubation with ^{125}I -labeled donkey anti-rabbit F(ab) $_2$ fragments. In lanes 7 and 8 the primary antibody was omitted. An excess of *E. coli*-expressed RAPI and of purified yeast topoII were included in the incubations with primary antibodies in lanes 5 and 6 (RAPI) and lanes 11 and 12 (topoII). Exposures of the control lanes (5–8, and 11–12) were equal to, or in the case of topoII, longer than those of the positive lanes. Molecular weight markers are indicated in kilodaltons at the far left, and the arrow indicates the migration of full length topoII.



Figure 2. Silver stained electron micrograph of a spread yeast pachytene nucleus. SK1 cells were induced to enter meiosis and were spread as described (Materials and Methods). Silver staining reveals the tripartite SC stretches of 15 bivalents. Small arrows show end to end association of some chromosomes. Bivalent number 12 is associated with the large dense staining nucleolus (*N*). Groups of electron-dense structures that resemble nuclear pores in size as well as in shape (*filled arrowheads*) are located near the ends of several bivalents. Empty arrowheads point to connections between the pores and the chromosome ends. Bar, 1 μm .

(Fig. 1, lanes 5–6), suggest that the smaller polypeptides as well as the 116-kD band are derived from RAP1. Anti-topoII reacts specifically with a single polypeptide of 170 kD (lanes 9 and 10), and the interaction can be competed by addition of an excess of purified topoII (lanes 11 and 12). Thus both antibodies are monospecific for their respective targets.

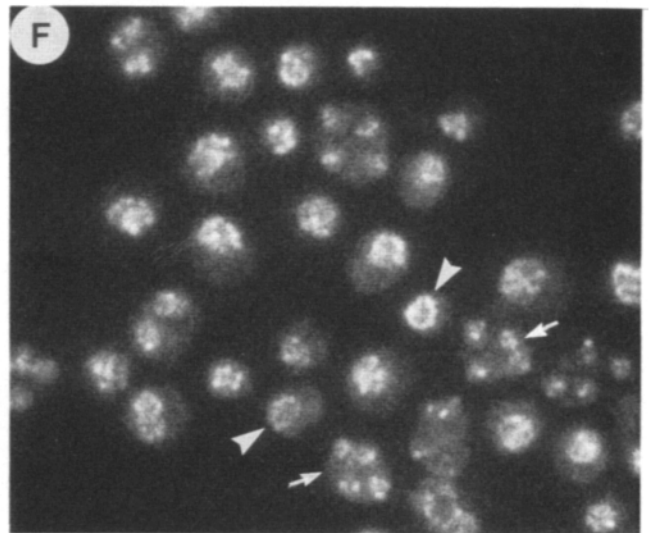
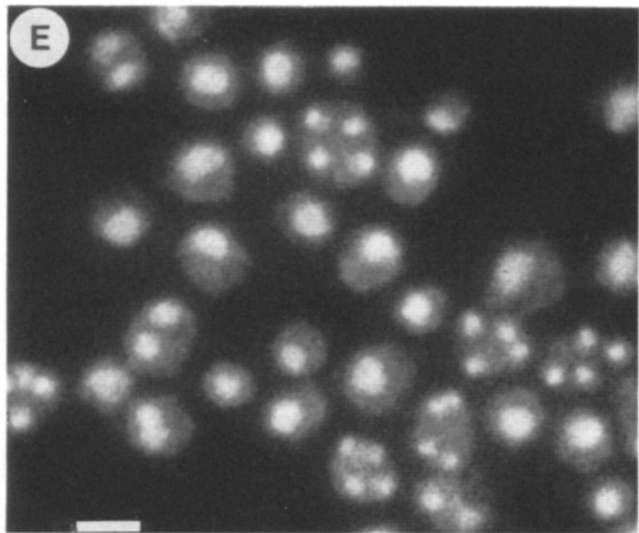
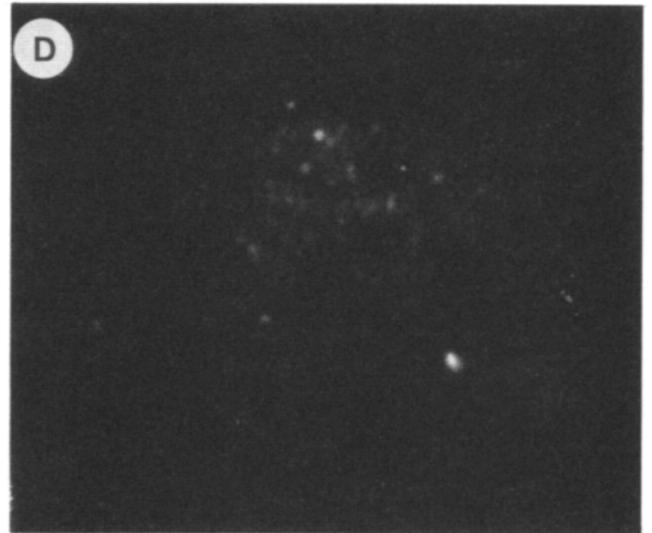
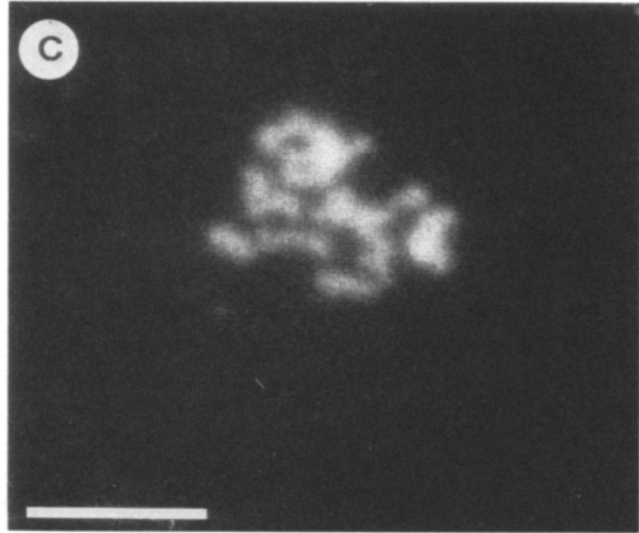
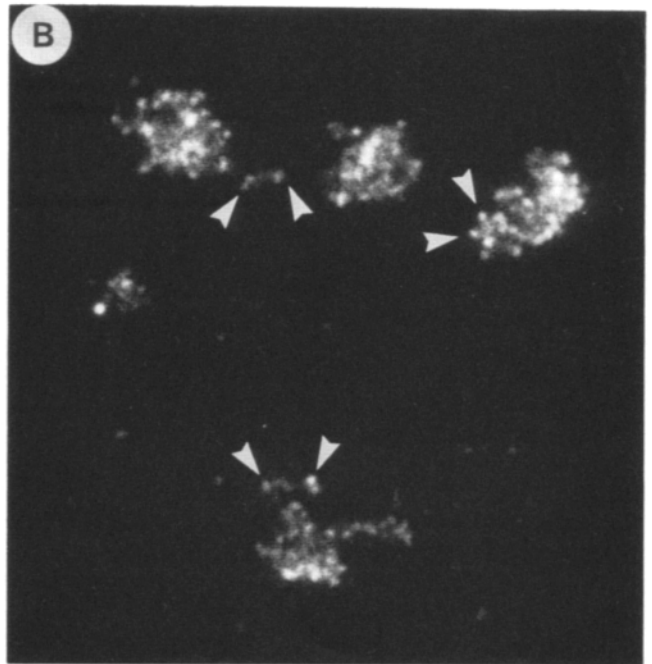
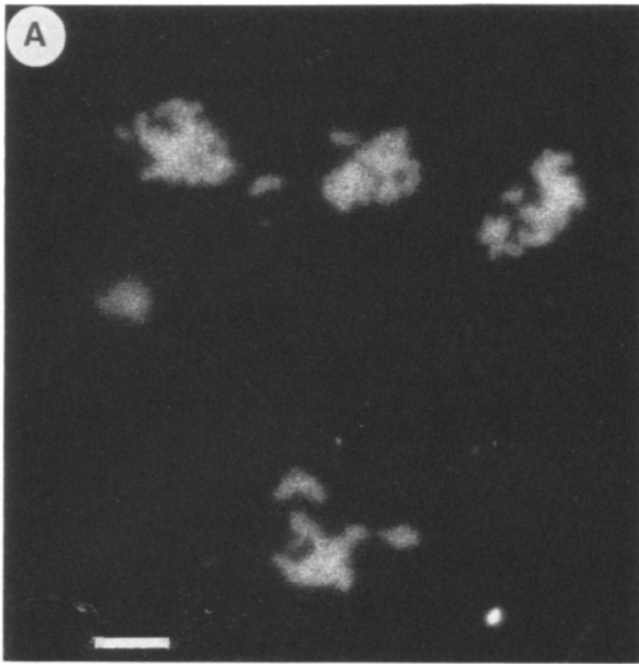
RAP1 Localization at the Ends of Meiotic Chromosomes

As mentioned above, both topoII and RAP1 are very abundant yeast nuclear proteins (roughly 4,000 and 6,000 copies per haploid nucleus, respectively; Verdier et al., 1990). In the case of RAP1, both its abundance and its multiple binding sites at telomeres, as well as at silencers, and promoters along the yeast chromosomes have led to the suggestion that RAP1 may play either a role in general chromatin organization, or in telomere structure. Immunolocalization studies have shown that topoII is localized along an axial core of

metaphase chromosomes from a variety of organisms, supporting its proposed role in chromosome organization. To date no detailed immunolocalization has been performed for RAP1, and no analysis of topoII distribution in yeast has been made.

We have chosen to localize both RAP1 and topoII in meiotic spreads from the pachytene stage, where individual chromosomes can be readily visualized. At this stage of meiosis, chromosomal homologues are stably paired, held together by the synaptonemal complex, which is thought to provide a physical basis for meiotic recombination. A typical spread of a *S. cerevisiae* genome in pachytene stage is shown in Fig. 2. Here, because of its reaction with silver stain, the tripartite synaptonemal complex of each paired bivalent chromosome is clearly seen, as well as the large uncondensed nucleolus (Fig. 2, *N*). The contiguity of the nucleolus with the distal arms of chromosome 12, is obvious in this example. 14 or 15, rather than 16, distinct bivalents are usu-

Figure 3. RAP1 localization on synaptonemal complexes and meiotic cells. Pachytene yeast nuclei were spread as described, stained either with DAPI (*A* and *C*) or with fluorescein-conjugated secondary antibody following affinity-purified anti-RAP1 IgG (*B* and *C*). In *D* an excess of *E. coli*-expressed RAP1 was added as competitor, and exposure to the film was twice as long as for *B*. Arrows indicate staining at the ends of chromosome bivalents. *E* and *F* show DAPI staining and anti-RAP1 staining, respectively, of a culture of SK-1 cells at various stages of meiosis. Arrowheads indicate peripheral, punctate staining patterns. Arrows point to examples of ascospores. Bars, 5 μm .



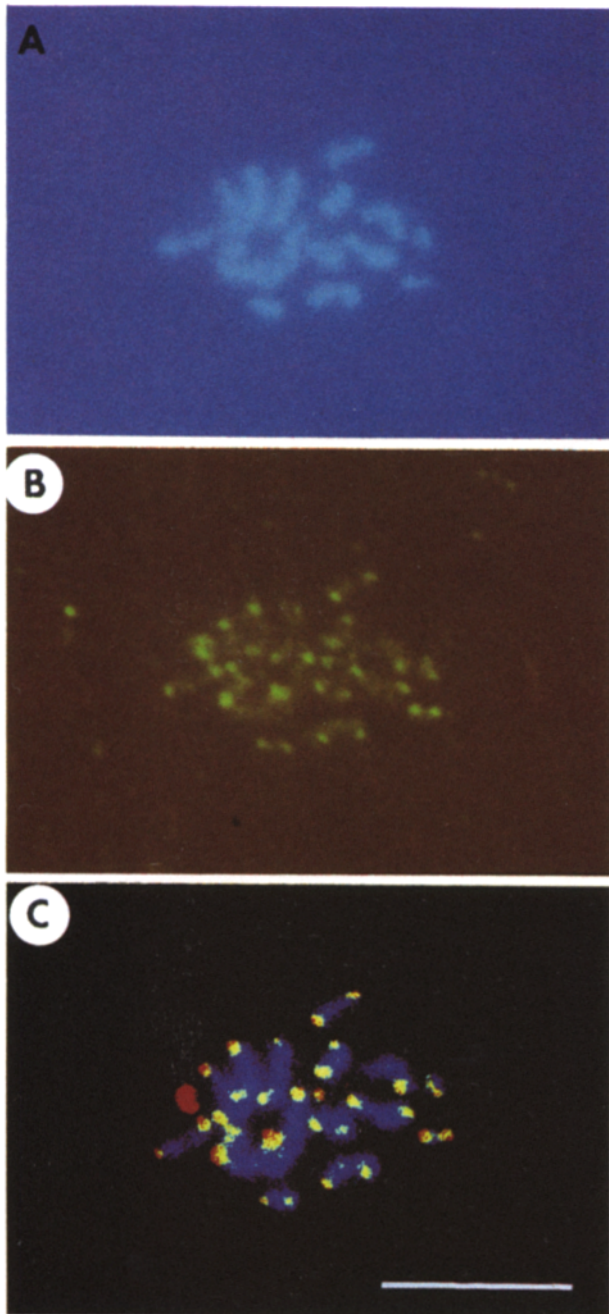


Figure 4. RAP1 localization on a spread pachytene yeast nucleus. (A) SK1 meiotic cells were spread as described in Fig. 2. Bivalents stained with DAPI to visualize chromatin. (B) Indirect immunofluorescence on the same bivalents using rabbit anti-RAP1 as the primary, and fluorescein-conjugated anti-rabbit Ig as the secondary antibody. The anti-RAP1 serum was affinity-purified against RAP1 protein, as stated in Material and Methods. (C) Projection of A on B. Blue areas indicate DNA staining, yellow areas represent anti-RAP1 labeling, and red shows RNA stained with propidium iodide. Bar, 5 μm .

ally seen in these spreads, perhaps because of the tendency of certain chromosomes to associate end-to-end (*small arrows*, Fig. 2). In this example, clusters of round, or disc-like particles are seen near some, but not all telomeres (*filled arrowheads*). These clusters appear to be linked by long filaments to the chromosome ends (*open arrowheads*). The shape, size, and position of these structures suggests that

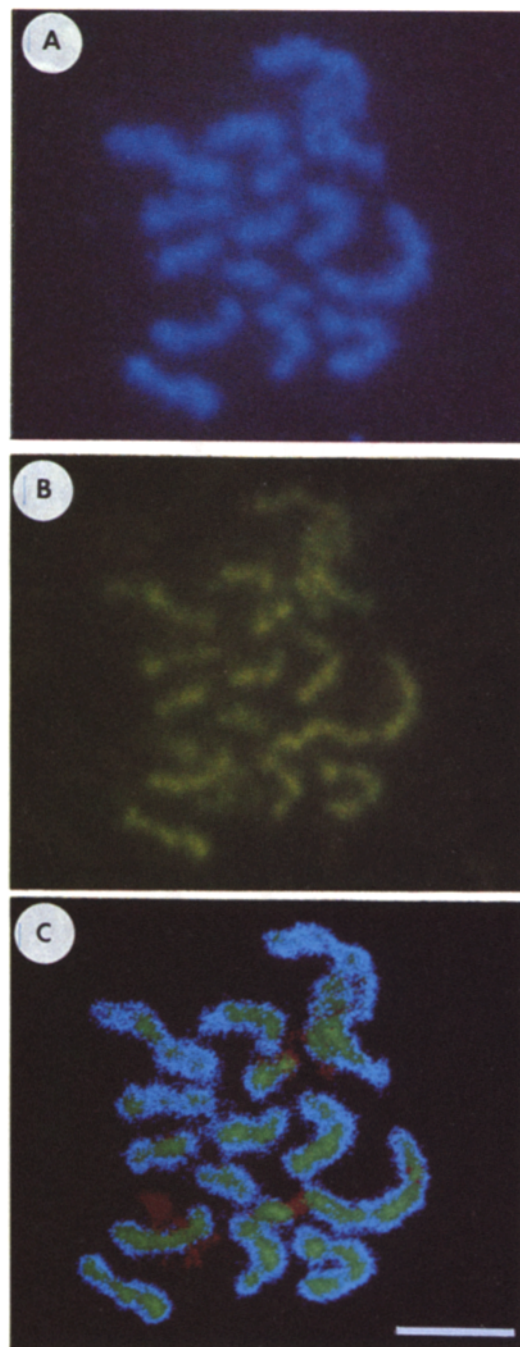


Figure 5. Immunolocalization of topoisomerase II in yeast synaptonemal complexes. Pachytene nuclei were spread as described in Materials and Methods, and were stained with anti-yeast-topoII (A) and DAPI (B). The superposition of the two labelings by confocal microscopy is shown in C. Quantitation of the area showing topoII-staining and the area of DNA staining is given in the text. Bar, 2 μm .

they are nuclear pore complexes, and are thus remnants of the association of telomeres with the nuclear envelope.

Immunolabeling the spreads of SCs for EM proved difficult because there was no means to identify the pachytene cells other than by silver staining of the synaptonemal complex. This interfered with the visualization of the antibody either by peroxidase-catalyzed precipitates or through gold particles. Therefore spreads of SCs such as the one shown

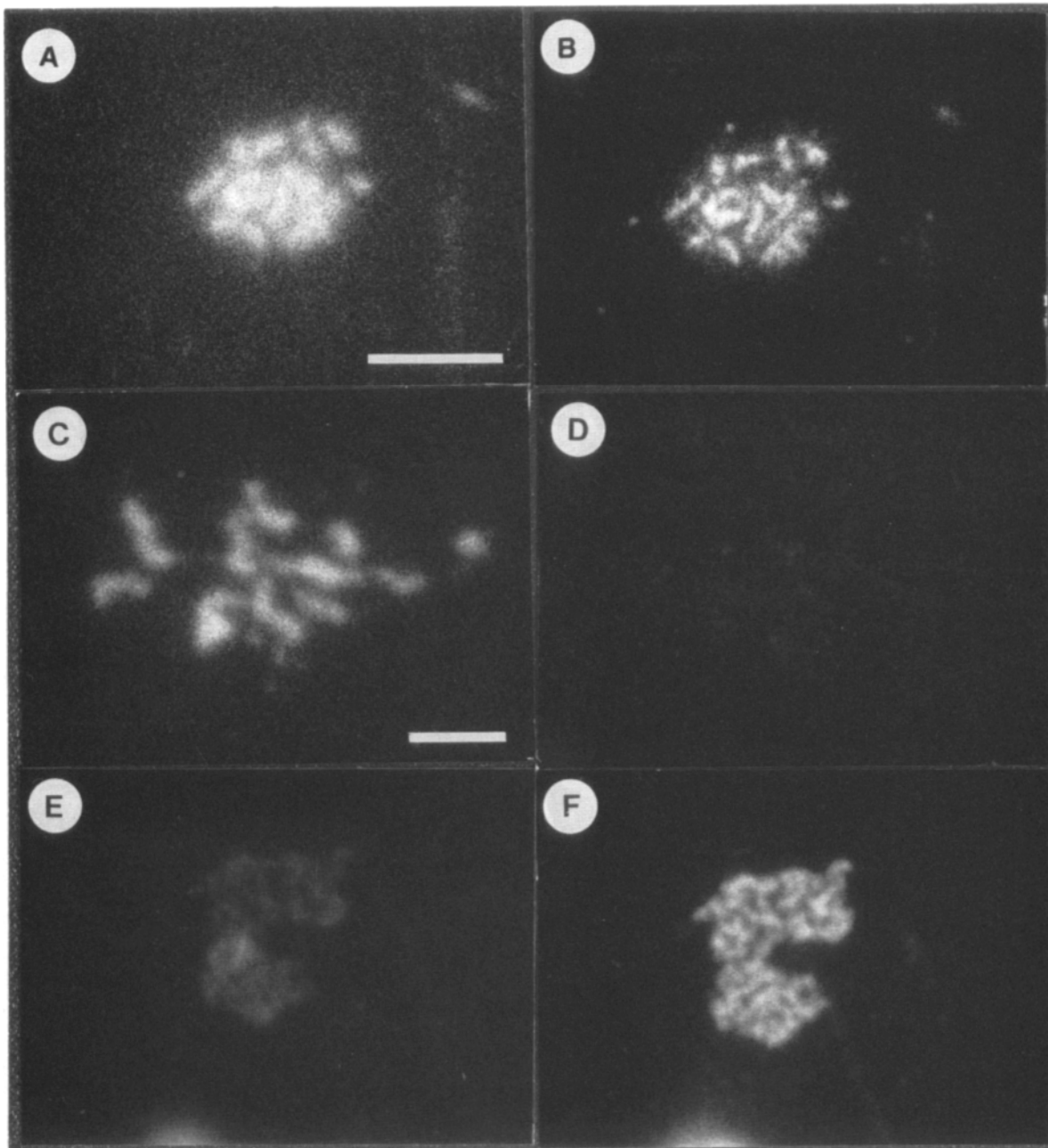


Figure 6. Topoisomerase II resists extraction by DNase 1 digestion. Pachytene nuclei were spread as described in Figure 5. *A*, *C*, and *E* are DAPI-stained. In *E* and *F* the spread was treated with 10 $\mu\text{g/ml}$ DNaseI in PBS with 2 mM MgCl_2 before staining. *B*, *D*, and *F* show reaction with affinity-purified anti-yeast topoII visualized with a rhodamine-conjugated secondary antibody. In *D* an excess of purified topoII (see Material and Methods) was added as specific competitor. Bars: (*A*, *B*, *E*, and *F*) 5 μm ; (*C* and *D*) 2 μm .

in Fig. 2, have been labeled with anti-RAP1 antibody and a fluorescein-coupled secondary antibody (Fig. 3). On a light microscopic level the individual SCs are less clearly defined. Nonetheless, the bright, punctate staining with anti-RAP1 appears to be telomeric, i.e., located at the ends of the bivalent paired chromosomes, when the SCs are spread such that the ends of bivalents are clearly visible (*white arrowheads*, Fig. 3 *B*). In fig. 3 *A*, the corresponding region of the spread is shown stained with DAPI. We demonstrate that the anti-RAP1 staining is antigen specific, since addition of RAP1 protein can compete for the staining (Fig. 3, *C* and *D*).

Staining of a spread of SCs in which the individual bivalents are readily identified is shown in Fig. 4. Superposition of the DAPI fluorescence (Fig. 4 *A*) with the RAP1 immunoreaction (Fig. 4 *B*) is shown in panel *C*. It is noteworthy that in addition to weak irregular staining along the bivalents, every telomere appears to have RAP1 associated with it.

The presence of RAP1 in meiotic nuclei is not unique to the pachytene stage. Immunofluorescence on a population of SK1 cells at other stages of meiosis shows a strong punctate staining present in all nuclei, including the nuclei of asco-

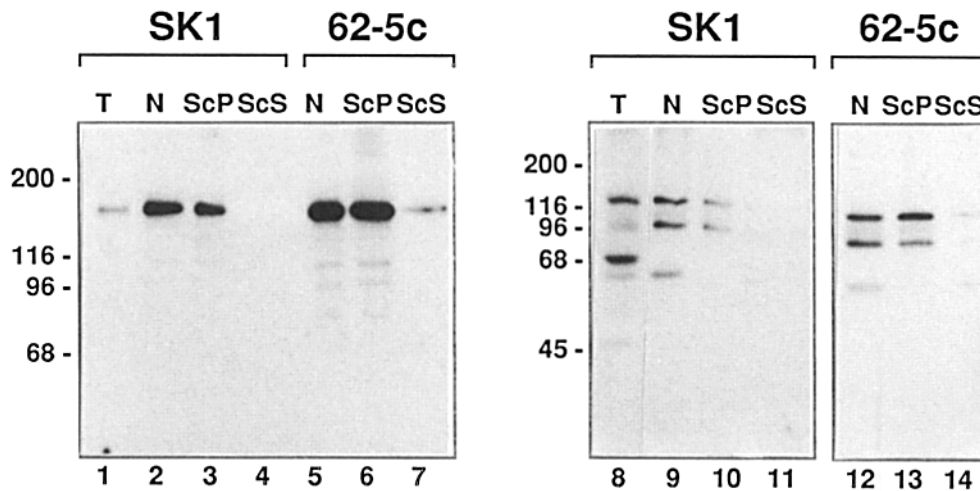


Figure 7. TopoII and RAPI cofractionate with an insoluble nuclear fraction from both meiotic cells and vegetatively cycling cells. Immunoblots of cell fractionations of meiotic SK1 cells and vegetatively growing 62-5c, were probed with affinity purified anti-yeast topoisomerase II (in lanes 1-7) and with anti-RAPI antibody (lanes 8-14). *T*, total spheroplast protein; *N*, nuclear fraction; *ScP*, an insoluble scaffold fraction; and *ScS*, the soluble nuclear proteins (as described in Cardenas et al., 1990). Equal protein is loaded in each nuclear and

total protein lanes, and an equivalent number of nuclei are fractionated into scaffold pellet and supernatant. Molecular weight markers in kD are indicated at the left of each gel.

spores (Fig. 3 *F*). In many cells the majority of the RAPI is at the nuclear periphery (Fig. 3 *F*, arrow heads, compare with DAPI staining in *E*), as is also the case for the nuclei of vegetatively growing cells (see below). In the four-spored asci there are generally only three or four bright spots of RAPI fluorescence per spore nucleus (arrows), although resolution in these structures is limited.

Immunolocalization of Topoisomerase II in SCs of Yeast

TopoII is a major component of the axially located scaffold of mitotic chromosomes (Earnshaw et al., 1985; Gasser et al., 1986), and the arrest of a cold-sensitive top2 mutant suggests an essential role in the first meiotic division in yeast (Rose et al., 1990). Therefore it was of interest to localize this protein in yeast synaptonemal complexes, which form a sort of meiotic chromosomal scaffolding. Immunodecoration of spreads of yeast pachytene nuclei are shown in Figs. 5 and 6. These are counterstained with DAPI (Figs. 5 *A* and 6 *A*). The immune reaction follows the length of the bivalent, and is somewhat less diffuse than the area occupied by the DNA itself.

Because the loops of DNA emanating from yeast synaptonemal complexes are relatively small, the "halo" of DNA around the central tripartite core is minimal. Nonetheless the topoII staining is clearly restricted to a central core (Figs. 5 and 6, compare panels *A* (DAPI) and panels *B*, showing anti-topoII). A superpositioning of the two staining patterns shows that the DNA halo extends beyond the topoII-containing region (Fig. 5 *C*) and quantitation of the two fluorescence patterns in the two spreads shows that the topoII signal covers from 53-71% of the width of the DAPI (DNA) signal. Similar results were obtained when an FITC-coupled antibody was used to stain the DNA, ruling out an artifactually broad fluorescence due to DAPI. The topoII staining area, while less than the total DNA, is nonetheless broader than that stained by anti-HOP1, a protein implicated directly in the synaptonemal core structure (Klein, F., unpublished results).

To demonstrate that the topoisomerase II is not randomly associated with the chromatin of meiotic chromosomes, we

digested the spread of pachytene chromosomes with DNaseI and then visualized topoII with immunodecoration. Fig. 6 *F* shows that the topoII staining remains despite the loss of the bulk of the chromatin (see DAPI staining, Fig. 6 *E*). This suggests that the enzyme interacts with other proteins or with itself, precluding extraction after DNase 1 digestion. This is consistent with an association of topoII with the synaptonemal complex. To demonstrate that the staining of the synapses with anti-topoII is antigen specific, an excess of purified topoII was added during the incubation with the primary antibody, which eliminated the fluorescent signal (Fig. 6 *D*).

Topoisomerase II and RAPI Cofractionate with an Insoluble "Scaffold" Fraction from Pachytene Nuclei

As independent, biochemical evidence that topoII and RAPI are structural components of meiotic chromosomes, we have isolated nuclear scaffolds from a population of yeast cells synchronously undergoing meiosis. The nuclei were purified from the same population used for the pachytene nuclear spreads, assuring that a significant population of the cells is in a pachytene stage with stable SCs. From these nuclei, we have isolated a copper-stabilized, insoluble fraction using the same protocol used for the isolation of nuclear and metaphase chromosomal scaffolds. The majority of nuclear proteins (in yeast roughly 60%) are solubilized and recovered as a supernatant fraction; the scaffold fraction contains a subset of nuclear proteins (Cardenas et al., 1990).

These samples, and scaffolds from vegetatively growing yeast cells, were probed with anti-topoII and anti-RAPI antibodies. As previously shown, both topoII and RAPI cofractionate quantitatively with the nuclear scaffold from interphase nuclei of a haploid strain (62-5c, Fig. 7, compare lanes 6 and 13 with 7 and 14). The same co-fractionation is seen with scaffolds from meiotic cells (Fig. 7, compare lanes 3 and 10 with 4 and 11).

RAPI Is Nuclear Localized in Vegetatively Growing Cells

We next examined the localization of RAP-1 and topoII in in-

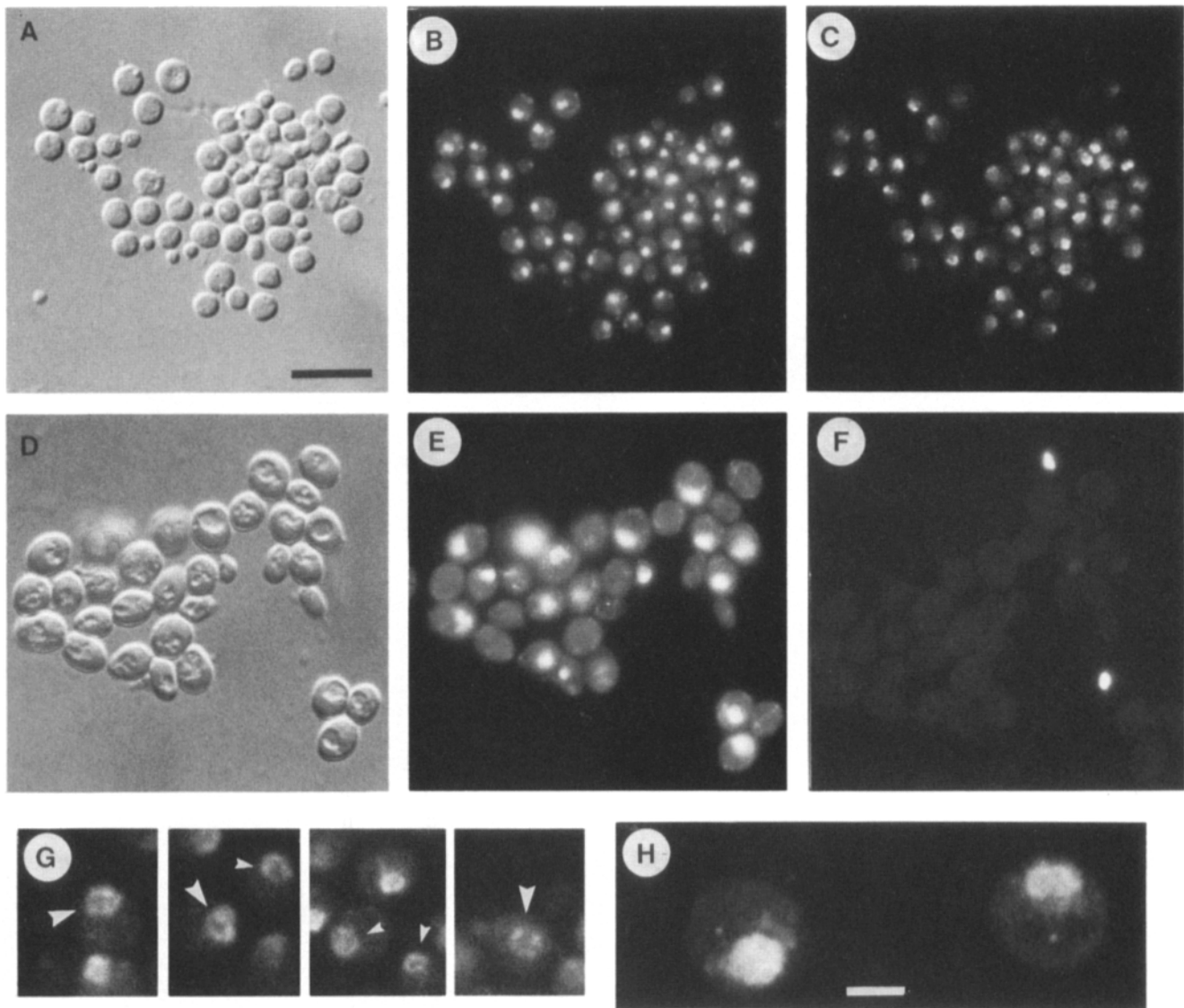


Figure 8. RAP1 is nuclear localized in vegetatively growing yeast cells. Photographs show phase (A), DAPI-fluorescence (B), and anti-RAP1 immunofluorescence (C), on a culture of mitotically growing yeast. Anti-RAP1 is clearly nuclear localized and shows a faintly punctate staining, largely peripheral (see also the 2.5-fold enlargements in G). The specificity of RAP1 is demonstrated by the complete competition for staining by the addition of an excess of purified *E. coli*-expressed RAP1 in F. H shows staining of a vegetatively growing diploid yeast with anti-topoII. The results shown in D-F and H use the diploid strain GA-7, while other manipulations were done on the related haploid, 62-5c. Bars: (A-F) 10 μ m; (H) 2 μ m.

terphase nuclei of vegetatively growing yeast cells. Consistent with cell fractionation studies (Hofmann et al., 1989), RAP1 is nuclear localized in mitotically dividing cells, as shown by its co-localization with the fluorescent DNA probe DAPI (Fig. 8, A-C). The nuclear staining is competed by an excess of purified RAP1 (Fig. 8, D-F). On closer examination of the staining the fluorescence can be seen to be punctate and largely perinuclear (Fig. 8 G), which is not the case with anti-topoII (Fig. 8 H).

The punctate staining seen with anti-RAP1 is more pronounced in larger, diploid yeast cells, and is particularly visible if cells are swollen in PBS after spheroplast formation and fixation. In Fig. 9 the DAPI staining (Fig. 9, A and C) and immunofluorescence using anti-RAP1 antibodies (Fig. 9, B and D) demonstrates more clearly the punctate pattern of localization characteristic of RAP1. Accurate quantitation

of the fluorescent spots was made in 57 such examples. The mean and standard deviation shows 15.3 ± 2.8 spots per diploid nucleus. Assuming that RAP1 binds to telomeres in mitotic, as well as meiotic, chromosomes, this number suggests that either, on the average, four telomeres are grouped together in each brightly staining spot, or that only some of the 64 telomeres in a diploid cell react with anti-RAP1. We consider this latter possibility unlikely, since all telomeres in meiotic spreads react with anti-RAP1 antibodies, and all have $C_{1,3}A$ repeats.

Localization of RAP1 in Interphase Cells by Immunoelectron Microscopy

To map the subnuclear location of these large RAP1 aggregates, a nonsynchronized population of haploid yeast

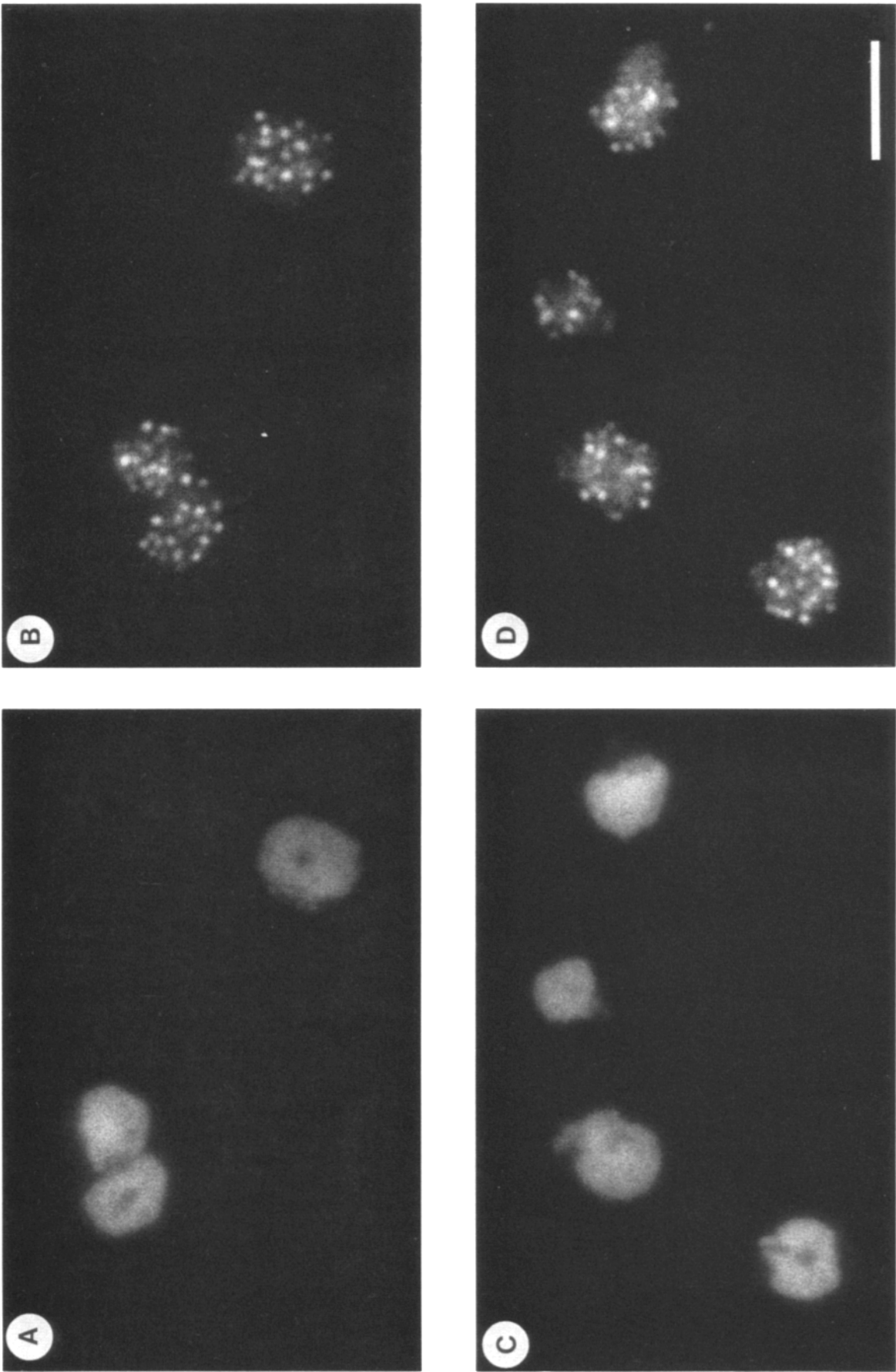
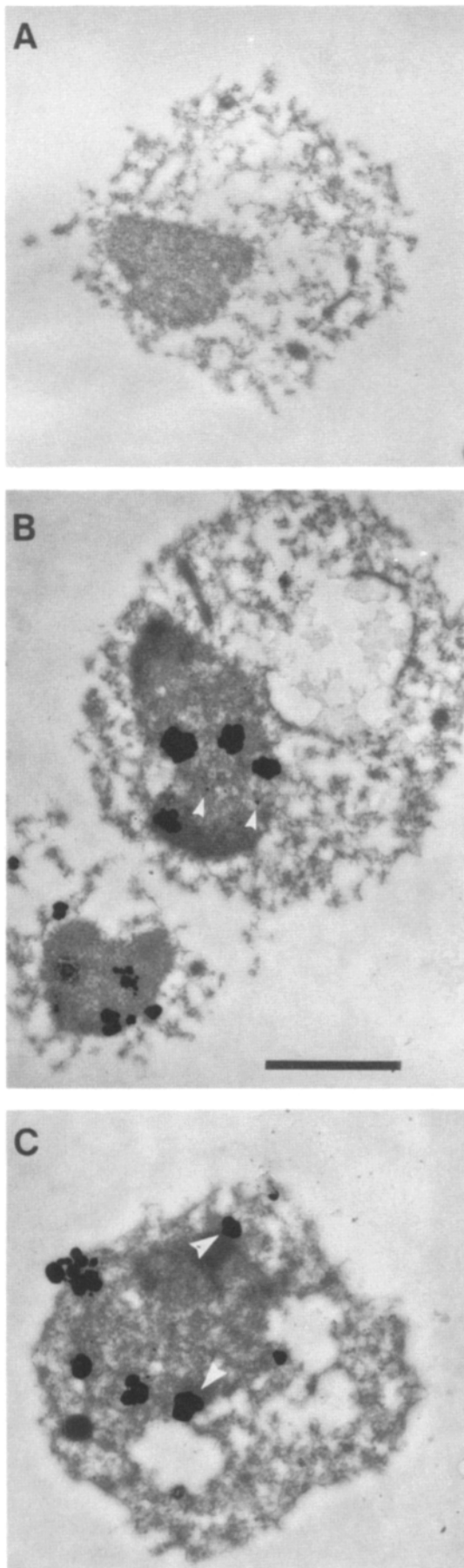


Figure 9. RAP1 gives distinct punctate staining of yeast interphase nuclei. Immunofluorescence using affinity-purified anti-RAP1 was performed on spheroplasts of diploid strain GA-138, or on a commercial diploid bakers' yeast. The cells lysed partially during the incubation with antibodies, leaving the nuclei expanded but intact. *A* and *C* show DAPI fluorescence and *B* and *D* show rhodamine immunofluorescence. Bar, 5 μ m.



cells was fixed, converted to spheroplasts, and labeled with anti-RAP1 for immunoelectron microscopy. An anti-rabbit immunoglobulin conjugated to 1 nm gold was used as secondary antibody, because the 1-nm gold particles have improved penetration. They can be visualized by reaction with silver after embedding and sectioning. Two sections typical of yeast cells reacted with anti-RAP1 antibodies are shown in Fig. 10, *B* and *C*; the control in which primary antibody was omitted from the procedure is shown in Fig. 10 *A*.

Two classes of silver precipitates are found in the thin sections: large black, irregularly shaped precipitates (*large arrows*), and small spots (*small arrows*). Neither of these is seen in control reactions and thus both appear to represent sites of antibody-antigen interaction. The diameter of the large precipitates is 20 to 50 times larger than that observed after reaction of a single 1-nm gold particle with the silver stain for a similar amount of time (data not shown), and we assume this to mean that there is a high concentration of anti-RAP1 antibodies, and the corresponding antigen, at these sites. Such precipitates appeared to be mainly peripheral within the nucleus (see Fig. 10, *B* and *C*).

The localization of over 600 gold grains was quantified on sections like those shown in Fig. 10 (see Table I). The frequency of grains per micron² is nearly 70 times higher in the nucleus than in the cytoplasm. To determine sublocalization within the nucleus, the precipitates found within 0.14 micron of the nuclear membrane, (i.e., within a zone on either side of the membrane 1/10 the diameter of the nucleus) were counted and compared with the number occurring in the center of nuclei. Numbers per unit area are given for both large and small silver precipitates and for the large aggregates alone. The calculation shows a strong, although not exclusive localization of RAP1 at the nuclear periphery in interphase cells with large precipitates fourfold more frequent at the nuclear periphery than in the core of the nucleus (see Table I).

Discussion

We have performed immunolocalization of two essential nuclear yeast proteins, RAP1 and topoII, in interphase nuclei and on meiotic chromosomes. Both proteins share the common features of being abundant, multifunctional nuclear proteins that bind to specific DNA sequences, and cofractionate with the nuclear scaffold. However, the subnuclear localization of RAP1 differs significantly from that of topoII. This observation underscores the fact that the nuclear

Figure 10. Large RAP1 clusters are preferentially located at the nuclear periphery. Formaldehyde-fixed spheroplasts from a haploid culture of *S. cerevisiae* (MS-27) were incubated with anti-RAP1 IgG and with 1 nm gold particles conjugated to goat anti-rabbit IgG. In the control *A* the primary antibody was omitted. After embedding in LR White (see Materials and Methods) and thin sectioning, the sections were reacted with a silver intensifying stain. In the electron micrographs two sizes of silver precipitates were observed: large aggregates (*large arrows* in *C*), presumably representing clusters of RAP1 and small spots of 10-nm diam (*small arrows*, in *B*), presumably representing one or a small number of RAP1 polypeptides. Quantitation of this labeling is presented in Table I. Bar, 1 μ m.

Table I. Distribution of Anti-RAP-1 in Interphase Yeast Cells

	Number of grains			Density (grains/ μm^2)		
	Cytoplasm	Nuclear periphery [§]	Nuclear center	Cytoplasm	Nuclear periphery [§]	Nuclear center
Aggregates*	37	178	83	5	289	67
Small grains [‡]	37	135	134	5	219	107
Aggregates + small grains	74	313	217	10	508	174

The number and location of over 600 large and small silver-enhanced gold grains representing the binding of anti-RAP1, were determined from thin sections of yeast spheroplasts, reacted with primary and secondary antibodies and embedded for EM as described in Materials and Methods. The term aggregates (*) signifies large silver precipitates ($>0.05 \mu\text{m}$; see large arrows, Fig. 8) that appear to represent many RAP1 molecules clustered together, while small grains ([‡]) represents the small precipitates ($<0.02 \mu\text{m}$; small arrows in Fig. 8). The nuclear periphery ([§]) is defined as a zone on either side of the membrane 1/10 the diameter of the nucleus, that is within $0.14 \mu\text{m}$ of the nuclear membrane. Nuclear center (^{||}) is the entire nucleus without this peripheral zone. Total grains, and grains per unit area, are given for both large and small silver precipitates and for the large aggregates alone.

scaffold is operationally defined and encompasses proteins that localize to very different parts of the nucleus. What proteins cofractionating with the scaffold have in common is the tendency to remain insoluble under the conditions used to extract histones.

Meiotic spreads were used in these studies because they allow visualization of individual paired bivalents and identification of components of the synaptonemal complex. Anti-topoII stains a core region of the paired bivalents at pachytene stage, and the antigen is resistant to elution by DNase I digestion. These results are consistent with the observations of Moens and Earnshaw (1989), who observed chick topoII on and near the synaptonemal complex of meiotic chicken cells, and with genetic results that implicate topoII in meiosis I in yeast (Rose et al., 1990). We have also found that anti-topoII antibodies stain human and mouse synaptonemal complexes specifically in testes (Klein, F., and S. M. Gasser, unpublished observations). Both topoII and RAP1 cofractionate with an insoluble protein fraction from meiotic nuclei, which may indicate either association with the SC, or participation in other relatively insoluble protein complexes.

When preparations of meiotic chromosomes were incubated with anti-RAP1 antibodies, signals at chromosomal termini could be observed. This confirms that RAP1 binds in vivo to the multiple binding sites (ACAC₁ACAC₂) observed in vitro in the (C₁₋₃A)_n telomeric repeat of the yeast chromosome. This also suggests that the influence of temperature-sensitive mutations in RAP1 on the length of yeast telomeres, could be a direct reflection of an altered interaction between RAP1 and telomeric DNA (Lustig et al., 1990; Conrad et al., 1990). It is interesting to note that unlike topoII, the RAP-1 immunostaining is not resistant to DNase I treatment, suggesting that the presence of RAP1 at the ends of the synaptonemal complex requires its high affinity interaction with telomeric DNA (Laroche, T., and S. M. Gasser, data not shown).

It has been noted from microscopic studies that the ends of SCs appear to be connected with the nuclear envelope from leptotene to diplotene phases of meiosis in most organisms investigated (reviewed in Moses 1968; Wettstein et al., 1984; John, 1990), including yeast (Byers and Goetsch, 1975). This is confirmed by the perinuclear immunostaining with anti-RAP1 that we have observed in meiotic nuclei (Fig. 3 F). Additionally, in many of our spreads, some SC ends appear to be connected with clusters of spherical structures resembling nuclear pores. While immunostaining with an

anti-pore protein mAb was not successful, these observations are consistent with the association of at least some meiotic telomeres with the nuclear membrane.

Indirect immunofluorescence on vegetatively growing yeast cells reveals that RAP1 is present in globular structures that preferentially map to the nuclear periphery, whereas topoII appears to be distributed evenly throughout the nucleus (Figs. 8-10). In all nuclear spreads reacted with anti-RAP1 sera, including various interphase, mitotic, premeiotic, and meiotic stages, the staining resulted in fluorescent spots with little general nuclear staining. This tells us that much of RAP1 is concentrated in defined aggregates at most stages of cell growth. The presence of a second, less-abundant population of RAP1 molecules binding throughout in chromatin is suggested both by the presence of RAP1 binding sites in regulatory sequences upstream of many genes, as well as by the single silver grains distributed through the nucleus in our immunoelectron microscopy studies. This putative RAP1 population does not, however, form a significant signal in the immunofluorescence of meiotic spreads or of spread interphase nuclei, either due to the limited sensitivity of immunofluorescence or the solubility of this sub-population under the conditions used.

The peripheral and globular distribution of the RAP1 protein in interphase nuclei was observed by both light and electron microscopy. The fluorescein label of RAP1-stained nuclei often shows a dark center with the signals concentrated at the nuclear periphery, as if the label forms a hollow sphere that can be cut equatorially by the optical plane. Neither DAPI staining of the DNA nor anti-topoII staining yields a similar pattern. The globular appearance of the RAP1 stained structure is most evident in spread interphase nuclei, where individual signals are separated from each other, due to flattening of the nucleus and possibly because of extraction of less-tightly complexed RAP1 from the nucleoplasm. In the diploid cells used around 16 RAP1-positive signals were observed, rather than the 64 that would represent one signal per telomere. This is consistent with interactions between groups of telomeres, perhaps those of homologues or those from opposite ends of an individual chromosome. This interaction may be mediated by a membrane-associated factor, since we have observed that larger RAP1 positive aggregates break up into multiple smaller RAP1-positive spots in the presence of nonionic detergents (Laroche, T., and S. M. Gasser, manuscript in preparation). The clustering of telomeres in interphase has also been reported for *Trypanosoma brucei* (Chung et al., 1990). Be-

cause of its characteristic staining pattern in interphase and due to its involvement in telomere metabolism, it seems likely that RAP1 is associated with telomeres throughout the mitotic and meiotic cell cycle, although this question can only be resolved definitively with the development of a double in situ labeling technique for telomeric DNA and RAP1.

These observations in yeast lend strong support to observations made in both meiotic and mitotically dividing higher eukaryotic cells. Telomere association with the nuclear membrane during interphase has been observed in both plant and *Drosophila* cells (Rabl, 1885; Ashley and Pocock, 1981; Gruenbaum et al., 1984; Foe and Alberts, 1985), and end-to-end association of mitotic chromosomes has been well characterized in higher plants, including *Allium cepa* and *Secale cereale* (reviewed by Hilliker and Appels, 1989). During homologue pairing in meiotic prophase, telomeres are concentrated at a small area of the nuclear envelope, with the chromosomes looping out into the interior of the nucleus. This so-called bouquet stage has been described for yeast (Dresser and Giroux, 1988) and is thought to be of importance for proper pairing of chromosomes in many organisms (John, 1990; Loidl, 1990; Wettstein et al., 1984). During this work we have observed bouquet-like chromosome structures, and a strong reaction of anti-RAP1 at the ends of the chromosomes, presumably representing large aggregates of RAP1 (Klein, F., unpublished results). The positioning of RAP1 aggregates would suggest a role in the positioning of chromosomes in the yeast nucleus. Other examples of end-to-end associations among meiotic chromosomes have been observed (Dresser and Giroux, 1988; Loidl et al., 1991) and could be documented in the present study by the presence of two closely situated, seemingly interstitial RAP1-dependent signals (see Fig. 4, A-C).

In summary, we observe that most of the antibody-accessible RAP1 protein throughout the yeast life cycle is concentrated in aggregates. These aggregates are located mainly at the nuclear periphery, and are associated with telomeres during meiotic pachytene stage and probably throughout the mitotic cell cycle. Both genetic and in vitro binding evidence confirm that RAP1 is associated with telomeres in vegetative cells. Thus, we speculate that the large complexes of RAP1 and telomeric repeats provides a means for chromosome positioning and perhaps also mediate telomere-telomere interaction throughout vegetative and sexual life cycles. This interaction may play a role in mitotic stability of chromosomes and/or in the recombination of chromosome ends (reviewed in Zakian, 1989). It is possible, moreover, that the function of RAP1 at certain promoters or at the silencers is also reflected by the RAP1 clusters at the nuclear periphery. One role for RAP1 might be to bring a given transcriptional domain into a particular nuclear context, e.g., near the nuclear envelope, to facilitate either the repression or activation of the gene in question. The role of subnuclear localization mediated by RAP1 and the SIR gene products, in silencing the mating type loci, is currently under investigation.

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