Nucleolar association of pEg7 and XCAP-E, two members of *Xenopus laevis* condensin complex in interphase cells

Rustem Uzbekov[1,2], Elmira Timirbulatova[2], Erwan Watrin[1], Fabien Cubizolles[1], David Ogereau[1], Pavel Gulak[3], Vincent Legagneux[1], Vladimir Ju. Polyakov[4], Katherine Le Guellec[1,*] and Igor Kireev[2,5,‡]

[1]Groupe Structure Dynamique de la Chromatine, CNRS, UMR 6061, Faculte de Medicine, 35043, Rennes, France
[2]Cell Cycle Group, Division of Electron Microscopy, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899, Moscow, Russia
[3]Institute of Agricultural Biotechnology, 127550 Moscow, Russia
[4]Division of Electron Microscopy, A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, 119899, Moscow, Russia
[5]Department of Cell and Structural Biology, University of Illinois at Urbana-Champaign, Urbana 61801, USA
*Deceased June 2001
‡Author for correspondence (e-mail: ikireev@life.uiuc.edu)

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Summary

Cell cycle dynamics and localization of condensins – multiprotein complexes involved in late stages of mitotic chromosome condensation – were studied in *Xenopus laevis* XL2 cell line. Western blot analysis of synchronized cells showed that the ratio of levels of both pEg7 and XCAP-E to β-tubulin levels remains almost constant from G1 to M phase. pEg7 and XCAP-E were localized to the mitotic chromosomes and were detected in interphase nuclei. Immunostaining for condensins and nucleolar proteins UBF, fibrillarin and B23 revealed that both XCAP-E and pEg7 are localized in the granular component of the nucleolus. Nucleolar labeling of both proteins is preserved in segregated nucleoli after 6 hours of incubation with actinomycin D (5 mg/ml), but the size of the labeled zone was significantly smaller. The data suggest a novel interphase function of condensin subunits in spatial organization of the nucleolus and/or ribosome biogenesis.

Key words: Chromosome condensation, Condensin, Chromatin, Nucleolus

Introduction

Chromosomes undergo significant changes in structure twice during the cell cycle. First, at the G_2/M transition, the chromosomes condense to form individual compact structures. Second, at the M/G_1 transition, the chromosomes return to their decompacted interphase state. These structural transformations are believed to be essential for complete segregation of genomes into daughter cells during mitosis and to provide differential access for soluble factors to active genetic loci while keeping inactive ones in the silent compact state.

The high level of compactness is achieved by ordered folding of DNA through its interaction with chromosomal proteins. Interaction of DNA with histones gives rise to nucleosomes and a 30 nm chromatin fiber (Wigler and Axel, 1976). The mode of DNA folding at higher levels of compaction and molecular mechanisms involved in formation and maintaining of higher order chromatin structures remain largely elusive. Recent studies have led to the identification of a new class of chromosomal proteins, which take part in mitotic chromosome compaction (Strunnikov et al., 1993; Hirano and Mitchison, 1994; Saitoh et al., 1994). These proteins, termed SMC (structural maintenance of chromosomes), participate in multiple chromosomal activities, including mitotic chromosome compaction and segregation (Strunnikov et al., 1993), sister chromatid cohesion (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998), recombination and repair (Jessberger et al., 1996; Stursberg et al., 1999) and dosage compensation (Lieb et al., 1998; Meyer, 2000).

Isolation of condensed chromosomes from cell-free extracts enabled biochemical studies of the proteins associated with DNA during mitosis. Proteins identified by this approach using *Xenopus laevis* cell-free extract (Hirano and Mitchison, 1994; Hirano et al., 1997) form complexes sedimenting at 8S and 13S. The former consists of a heterodimer of SMC proteins belonging to the SMC2/4 subfamily (XCAP-E and XCAP-C), whereas the latter contains three additional subunits, XCAP-D2, XCAP-H and XCAP-G. XCAP-D2 was simultaneously identified as pEg7 by differential screening of the *Xenopus* egg cDNA library for genes expressed during oocyte maturation (Cubizolles et al., 1998). On the basis of their chromosome condensation activity, which was demonstrated in immunodepletion/rescue experiments, these complexes were termed condensins (Hirano et al., 1997). It is believed that 13S condensin is involved in active reconfiguration of DNA (Kimura and Hirano, 1997; Kimura et al., 1999). Non-SMC proteins can act as regulators of condensin activity (Kimura and Hirano, 2000). Initiation of complex assembly and/or modulation of its activity during mitosis may be controlled by cell cycle dependent phosphorylation of XCAP-D2, XCAP-H and XCAP-G (Hirano et al., 1997; Kimura et al., 1998).

Recently it became clear that condensin subunits have more

than one function in the cell. To date, the only clear example of such a dual function is given by the *Caenorhabditis elegans* protein MIX-1, which is homologous to XCAP-E and plays an essential role in gene dosage compensation (Lieb et al., 1998). MIX-1 forms a complex with another SMC-protein, DPY-27, and several other proteins. During mitosis, MIX-1 participates in chromosome compaction by interacting with a yet unidentified SMC protein (Lieb et al., 1998). The interphase behavior of other condensin subunits is yet to be determined.

In the present work, we studied ultrastructural localization of two subunits of the *Xenopus laevis* condensin complex, XCAP-E and pEg7, and the level of their expression during the cell cycle.

Materials and Methods

Chemicals

Bromodeoxyuridine (BrdU), primary monoclonal anti-BrdU antibodies, primary monoclonal anti-β-tubulin antibodies, aphidicolin, nocodazole, Taxol, glutaraldehyde, actinomycin D, trypsin-EDTA, secondary anti-rabbit 5 nm gold-conjugated and anti-mouse 10 nm gold-conjugated antibodies were obtained from Sigma. ALLN (N-acetyl-leucyl-leucyl-norleucinal) was from Calbiochem (San Diego, CA). Secondary Texas-Red-conjugated goat anti-human and goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were obtained from Interchim (Montlucan, France). Leibovitz-15 (L-15) cell culture medium and antibiotic-antimycotic solution (penicillin-streptomycin-amphotericin) were from Gibco-BRL. Fetal calf serum was obtained from Biotimes. All components of Epon 812 mixture were obtained from Ernest F. Fullam Inc (Latham, USA).

Xenopus cultured cells

The embryonic *Xenopus laevis* cell line XL2 (Anizet et al., 1981) was a gift from J. Tata (Mill Hill-NIMR Laboratory, London). Cells were grown at 25°C in L-15 medium supplemented with 10% fetal calf serum and antibiotic-antimycotic solution (Gibco-BRL).

Cell synchronization

XL2 cells were synchronized according to the protocol of Uzbekov et al. (Uzbekov et al., 1999) with modifications. After serum starvation for 24 hours, cells were incubated 30 hours in complete medium with 2 μg/ml aphidicolin, then released from the block by several washes with fresh complete medium. Fractions enriched with S phase (max S) and G2 phase cells (max G2) were collected 2 and 10 hours after the last wash, respectively. For preparation of a fraction of mitotic cells (max M), 8 hours after washing out aphidicolin, cells were incubated for 3 hours in complete medium with 0.5 μg/ml nocodazole and then for 4 hours in complete medium with 0.5 μg/ml nocodazole and 40 μg/ml calpain inhibitor I (ALLN). Mitotic cells were collected for 20 minutes after washing off nocodazole and ALLN with fresh medium. The fraction enriched with G1 phase cells (max G_1) was collected 11 hours after removing the nocodazole/ALLN mixture. The fraction of cells in G0 (max G0) was obtained by cultivating cells for 7 days in complete medium at 9°C and then 24 hours in the medium without serum at 25°C.

The composition of all fractions was controlled for by BrdU labeling (see below). The fraction of cells in G0 was estimated by prolonged BrdU labeling (30 hours); the fraction of cells in S phase was assessed by impulse BrdU labeling (30 minutes). The fraction of mitotic cells was estimated by direct counting in a phase contrast microscope. The percentage of cells in G2 and G1 was calculated as described elsewhere (Uzbekov et al., 1999). Data from more than 22,000 cells were used for estimation of cell fraction composition.

Antibodies

The generation and purification of anti-Eg7G polyclonal antibodies were reported in our previous paper (Cubizolles et al., 1998). Polyclonal anti-XCAP-E antibodies were raised against the last 14 amino acids of XCAP-E and affinity purified on CNBr Sepharose column (Amersham Pharmacia Biotech) coupled to the same peptide.

Human autoimmune sera to UBF and fibrillarin were kindly provided by D. Hernandez-Verdun (I. Jacques Monod, France), and antibodies to B23 were a gift from T. I. Bulycheva (National Center for Hematology RAMS, Moscow, Russia) (Bulycheva et al., 2000). Polyclonal anti-topoisomerase II (anti-topoII) antibodies were provided by D. F. Bogenhagen (State University of New York, Stony Brook, USA) (Luke and Bogenhagen, 1989); monoclonal anti-human topoII antibodies were obtained from Calbiochem; monoclonal anti-Pleurodeles topoII antibodies were a gift from R. Hock (University of Wurzburg) (Hock et al., 1996).

Indirect immunofluorescence microscopy

Xenopus laevis XL2 cells were grown on round glass coverslips in 12-well plates (Corning Inc., Acton, USA) for 48 hours, washed with phosphate-buffered saline (PBS: 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate-buffer, pH 7.2) before fixation. The following fixation protocols were tested: (1) 100% methanol for 6 minutes at –20°C; (2) 100% methanol with subsequent post-fixation with 100% acetone for 6-20 minutes at –20°C; (3) 1:1 mixture of methanol and acetone for 6-20 minutes at –20°C; (4) 3% formaldehyde in PBS for 10-30 minutes at room temperature; (5) mixture of 3% formaldehyde and 0.1% glutaraldehyde for 30 minutes at room temperature with subsequent 'quenching' of free aldehyde groups by two 10 minute washes with 2 mg/ml $NaBH_4$ in PBS. In some cases, cells were additionally permeabilized for 3 minutes with 1% Triton X-100 in PBS at room temperature, either prior to or after fixation. Subsequent immunostaining was essentially the same for all fixation protocols. Following three washes in PBS, cells were blocked in PBS containing 3% BSA for 30 minutes and then incubated with a mixture of primary antibodies: rabbit antibodies to pEg7 or XCAP-E (1:50) and either mouse anti-topoII monoclonal antibody or anti-B23 antibody (dilution 1/100), or human antisera to fibrillarin or UBF (1:100). The antibodies were subsequently revealed by fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1/100) and Texas-Red-conjugated goat anti-mouse IgG (dilution 1/70) or Texas-Red-conjugated goat anti-human IgG (dilution 1/100). All antibody reagents were diluted in PBS containing 1% BSA, and incubations were performed at room temperature for 60 minutes. Cells were rinsed three times in PBS containing 1% BSA after each incubation.

For anti-BrdU labeling, cells grown on glass coverslips were incubated in complete medium with 40 μM BrdU either for 30 minutes (pulse-labeling) or longer. Then cells were briefly washed in warm (25°C) PBS and fixed with 70% ethanol for 30 minutes. The coverslips were rinsed in PBS and immersed in 4 M HCl for 20 minutes, then washed 5 times in PBS and incubated for 60 minutes at room temperature with mouse anti-BrdU (Sigma) and then anti-mouse Texas-Red-conjugated antibodies for 60 minutes at room temperature.

After immunolabeling, cells on coverslips were rinsed in PBS and mounted in Mowiol (Calbiochem). Samples were observed using a Zeiss Axiolab microscope (AXIOVERT 35) equipped with phase contrast and epifluorescence, using 40×/0.65 NA and 100×/1.25 NA achroplan objectives, and photographed using a Nikon 601 camera. For quantitative analysis, images were captured using a CH-250 CCD camera (Photometrics, Tuscon, USA) mounted on a Zeiss Axioscope microscope with a 100×/1.25 NA objective. Digital image processing

was performed using Scion Image software (Scion Corp., Frederick, USA).

Western blot analysis

Electrophoreses on SDS-polyacrylamide gel were performed according to the protocol of Laemmli (Laemmli, 1970) and transferred onto nitrocellulose membranes, as described previously (Towbin et al., 1979). Membranes were blocked in TBST (Tris buffer saline with 0.05% Tween-20, pH 7.5) containing 5% skimmed milk for 2 hours at room temperature and incubated for 3 hours with antibodies diluted in TBST containing 5% skimmed milk. Immuno-complexes were revealed with antibodies coupled with peroxidase or alkaline phosphatase (Sigma) by using either NBT/BCIP (Sigma) or an ECL kit (NEN, Boston, MA) according to the manufacturer's instructions.

Immunoelectron microscopy

Cells were rinsed in PBS permeabilized by incubation in buffer containing 50 mM imidazole, pH 6.8, 50 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM beta-mercaptoethanol and 1% Triton X-100 for 3 minutes at room temperature and fixed in cold absolute methanol (6 minutes, –20°C). After three washes in PBS, preparations were blocked for 30 minutes in PBS containing 3% BSA. Cells were then incubated with a mixture of polyclonal purified antibodies against pEg7 or XCAP-E and anti-topoII monoclonal antibodies, washed and incubated in a mixture of secondary anti-rabbit 5 nm gold and anti-mouse 10 nm gold-conjugated antibodies. Antibody reagents were diluted in PBS containing 1% BSA, 3% temperature inactivated (56°C, 30 minutes) goat normal serum, 0.1% Tween 20 and incubated at room temperature for 60 minutes. Cells were then washed with PBS and fixed for 90 minutes in 0.1 M phosphate buffer at pH 7.2 (KH_2PO_4-Na_2HPO_4) containing 2.5% glutaraldehyde. After being rinsed in 0.1 M phosphate buffer, cells were post-fixed with 1% osmium tetraoxide in 0.1 M phosphate buffer, stained with uranium acetate, dehydrated and embedded in an Epon 812 mixture. Serial ultrathin (70 nm) sections were obtained parallel to the substrate plane using an LKB-III ultramicrotome and mounted on single slot grids. The sections were examined using Hitachi-11 and Hitachi-12 electron microscopes operating at 80 kV and photographed.

Results

Levels of pEg7 and XCAP-E proteins did not change significantly during the cell cycle in XL2 cells

Condensins are thought to act exclusively at the final stages of chromosome compaction in mitosis, so it would be reasonable to expect their intracellular level to rise at the beginning of mitosis and to decline early in G1. Such cell cycle dependent oscillations are characteristic for many other proteins involved in mitotic processes (Jessus and Beach, 1992). To address the question of whether levels rise at the beginning of mitosis and fall early in G1, we analyzed the quantities of pEg7, XCAP-E and topoII present in a synchronized cellular population by using immunoblotting.

Two proteins with known behavior during the cell cycle were used as standards: β-tubulin, whose level remains constant during the cell cycle, and pEg2, whose concentration changes cyclically, rising at the beginning of mitosis (Roghi et al., 1998; Arlot-Bonnemains et al., 2001).

All used antibodies, except monoclonal anti-topoII, detected one single specific band on western blots; purified Eg7G polyclonal antibodies recognized a band of 150 kDa [Fig. 1A, see also Fig. 2C (Cubizolles et al., 1998)], purified XCAP-E antibodies a band of 125 kDa (Fig. 1B), monoclonal antibodies against pEg2 [clone 1C1, see Fig. 4B (Roghi et al., 1998)] a band of 46 kDa, monoclonal antibodies against β-tubulin (clone no. TUB 2.1, Sigma) a band near 50 kDa (Fig. 1Aa). Monoclonal antibodies against human Topoisomerase-IIα (Clone SWT3D1 Calbiochem) detect one major specific band near 180 kDa and one additional small band near 50 kDa (Fig. 1B).

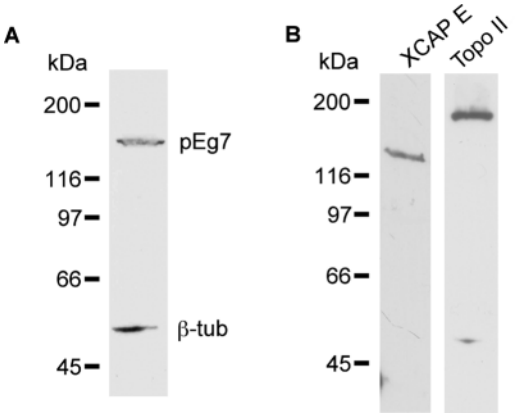

Fig. 1. Specificity of antibodies to Eg7G, XCAP-E1, TopoII and β-tubulin in XL2 cell extract. Proteins in *X. laevis* XL2 cell lysate (2×10^5 cells) were separated onto 7% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes and immunodetected with mixture of purified Eg7G and monoclonal antibodies against β-tubulin (lane1) or XCAP-E1 antibodies (lane 2) or monoclonal anti-topoII antibodies (lane 3).

Table 1. Percentage of cells in different phases of the cell cycle in synchronized XL2 cell fractions (more than 1000 cells were analyzed in each fraction)

	% Cells in G1	% Cells in S	% Cells in G2	% Cells in M
Fraction 'max G1'	**85.6**	11.9	1.9	0.7
Fraction 'max S'	7.7	**92.3**	0	0
Fraction 'max G2'	0	18.2	**77.2**	4.6
Fraction 'max M'	12.1	13.5	3.4	**71.7**

The synchrony of the cell population and its progression through the cell cycle were monitored by BrdU incorporation and immunofluorescent microscopy (Uzbekov et al., 1998; Uzbekov et al., 1999). This approach, although rather time-consuming, has some advantages over FACS analysis, particularly in discriminating between late G1 and early S cells, and seems to be especially useful when working with partially aneuploid or polyploid cells. Table 1 shows the percentage of cells in different phases of the cell cycle in synchronized cell populations, which were used for western blot analysis.

For quantification of protein levels at different cell cycle stages, densitometric data were normalized to that of β-tubulin. In Fig. 2, cell cycle dependent changes in protein level is shown (OD ratio=1 in G1). From G1 to M, the quantity of pEg2 increased more than 15-fold. TopoII level was maximal in G2 (3.5 G1 level) and remained practically the same during mitosis, which is in good agreement with published data for topoIIα (Heck et al., 1988; Drake et al., 1989; Woessner et al., 1991).

Table 2. Labeling of different cell regions by pEg 7G antibodies

	Mitotic chromosomes	Cytoplasm	Karyoplasm	Nucleolus
Met 6 minutes, –20°C	+++	f gr+	f gr –+	+
Met 6 minutes, –20°C, Trit 3 minutes RT	+++	f gr+	dif –+	++
Met 6 minutes, Ac (6 or 10 or 20 minutes), –20°C	+++	f gr+	dif –+	+
Met 6 minutes, Ac 20 minutes –20°C, Tr 3 minutes RT	+++	f gr+	dif –+	++
Met/Ac (6, 10 or 20 minutes), –20°C	++++	l gr +	dif –+	+
Met/Ac (6, 10 or 20 minutes), –20°C, Tr 3 minutes RT	+++	l gr –+	f gr +	++
Tr 3 minutes, RT Met 6 minutes –20°C	++++	f gr –+	f gr +	+++
Tr 3 minutes RT, Met 6 minutes, Ac 20 minutes, –20°C	++++	f gr –+	f gr +	+++
Tr 3 minutes, For 3% 30 minutes RT	+++++	f gr –+	f gr +	+++
Tr 3 minutes, For 3%/Glu 0.1%, $NaBH_4$ 2×10 minutes, RT	++++	–	dif —+	–
Tr 3 minutes, Glu1%, $NaBH_4$ 2×10 minutes, RT	++++	–	dif —+	–
For 3% 10 minutes, Tr 2×5 minutes	++++	–	f gr +	–

Abbreviations: Met, methanol 100%; Tr, Triton X-100 1%; For, formaldehyde; Glu, glutaraldehyde; $NaBH_4$, 2 mg/ml in PBS; f gr, fine granular labeling; l gr, large granular labeling; dif, diffuse labeling.

In contrast, levels of pEg7 and XCAP-E increased slowly as cells progressed from G1 to M and became less than two-fold higher in the mitotic fraction when compared to the G1 fraction (Fig. 1b). Similar results were obtained for human orthologs of XCAP-E and XCAP-C (Schmiesing et al., 1998).

The ratio of pEg7: β-tubulin and XCAP-E: β-tubulin in the fraction 'max G0' (G0, 86.7%; G1, 6.7%; S, 5.9%; G2, 0.8%, M, 0.3%) was even higher than in G1: 1.38 for pEg7 and 1.33 for XCAP-E. This increase apparently correlates with the low level of β-tubulin brought about by cultivation of cells at low temperature, but in any case, these data show that both pEg7 and XCAP-E are continuously expressed in non-dividing cells.

Taken together, the data suggest that not only SMC proteins, representing core subunits of the 13S-condensin complex, but also some non-SMC subunits are expressed throughout the cell cycle.

The effect of fixation on immunolocalization of pEg7 and XCAP-E

In our previous paper (Cubizolles et al., 1998), we reported chromosomal localization of pEg7 during mitosis. With the formaldehyde-glutaraldehyde fixation protocol used in these experiments, immunostaining with anti-pEg7 antibodies produced only weak diffuse nuclear labeling in interphase cells. However, under these conditions, nuclear antigens could be partially masked. In order to test this possibility, in the current work we used some other fixation protocols for both anti-pEg7 and anti-XCAP-E antibodies. Several antibodies were tested after methanol, Triton-methanol, Triton-formaldehyde and Triton-glutaraldehyde fixation protocols (see Materials and Methods). The intensity of mitotic chromosome staining was used as a reference point for estimation of labeling efficiency. Since Eg7G and XCAP E1 antibodies gave the best results on mitotic chromosomes, we used these antibodies for further experiments.

The results of the effect of fixation protocols on pEg7 immunostaining are summarized in Table 2. It appeared that mitotic chromosome staining for pEg7 demonstrates little sensitivity to variations in fixation conditions. Both crosslinking-type and precipitation-type fixatives gave similar results. In contrast, interphase staining was notably different when various protocols were compared.

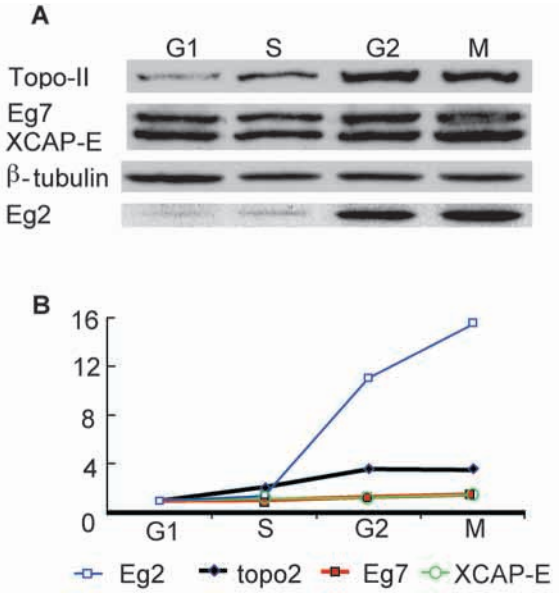

Fig. 2. Western blot analysis of the quantity of pEg7, topoII and XCAP-E in the synchronized cells of different stages of cell cycle. (A) After electrophoresis and transfer onto nitrocellulose, the membrane was cut, the upper part was incubated with antibodies to topoII, the second part with a mixture of pEg7 and the third part with monoclonal antibodies against β-tubulin and the last part with monoclonal antibodies against pEg2 (clone 1C1). Immunocomplexes were revealed using an enhanced chemoluminescence system. (B) Relative quantities of protein during the cell cycle (protein:β-tubulin ratio, with the ratio in G1 equal to 1). The level of cycle-dependent protein kinase pEg2 increased from 1 at G1 to 15.44±3.21 at M phase; the quantity of topoII was maximal in G2 phase (3.61±1.22) and practically the same in mitosis (3.52±1.04). The quantity of pEg7 and XCAP-E increased from 1 at G1 to 1.48±0.09 and 1.48±0.04 times at M phase, respectively. Average data from four measurements from two experiments are presented. Quantitative analysis was performed using Image Quant computer program.

While most methanol- or aldehyde-based protocols gave fine granular staining of the cytoplasm and weak diffuse staining of the nucleoplasm, pretreatment of cells with detergent prior to fixation revealed the concentration of antigen in the nucleolus. Introduction of glutaraldehyde in fixation mixture completely

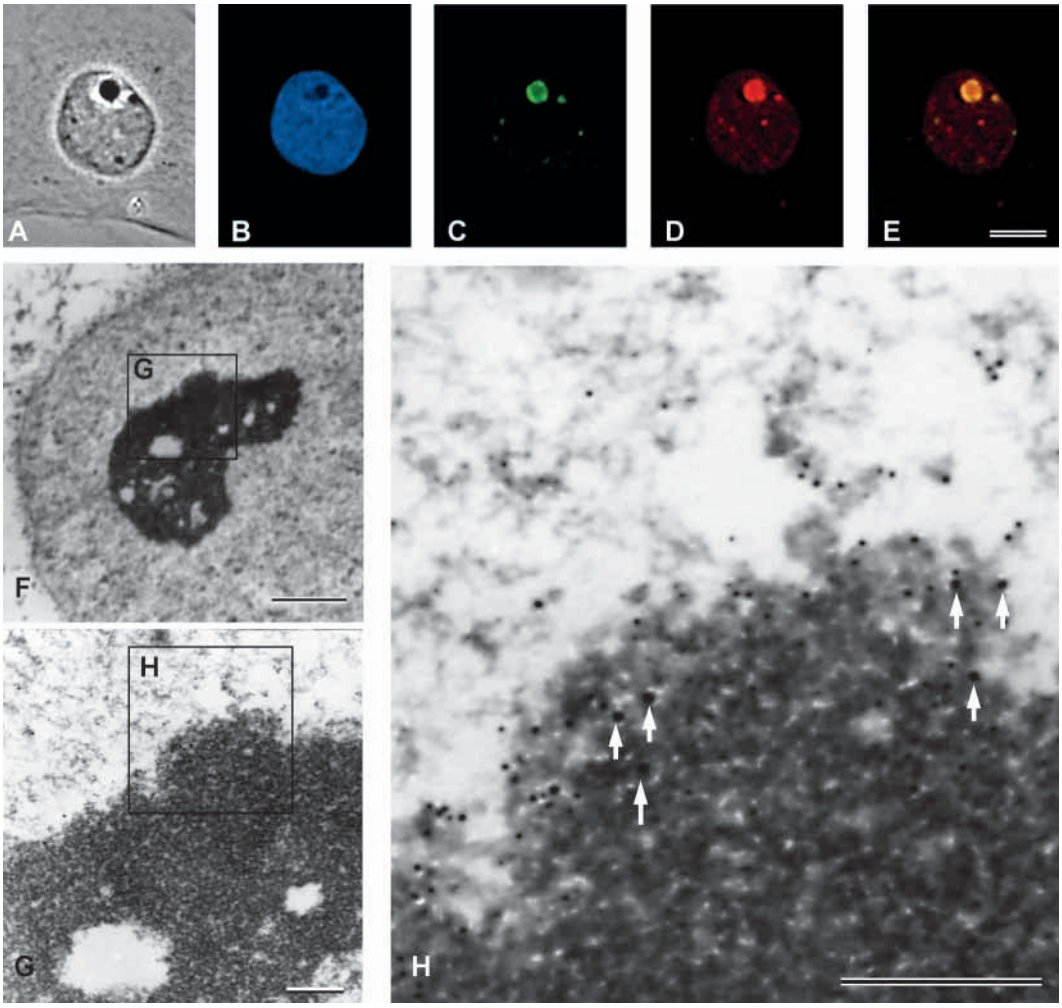

Fig. 3. Nucleolar localization of pEg7 and topoII in the interphase nucleus. XL-2 cells were grown and fixed as described under Materials and Methods. Cell were then processed for double immunofluorescence staining (panels A-E) with an anti-pEg7G affinity-purified polyclonal antibody (C) and an anti-human topoII monoclonal antibody (D). The merged picture is shown in e. Cells were observed by phase contrast microscopy (A) and stained with DAPI (B). For electron microscopy (F-H) cell were fixed as described under Materials and Methods and then labeled for double immunogold electron microscopy with anti-pEg7G affinity-purified polyclonal antibody and anti-human topoII monoclonal antibody. Anti-Eg7 and anti-topoII antibodies were revealed with a secondary anti-rabbit antibody conjugated to a 5 nm gold particle (for pEg7) and an anti-mouse antibody conjugated to 10 nm gold particle (for topoII, arrows), respectively. Bar, 5 μm (A-E), 1 μm (F) and 0.2 μm (G,H).

Table 3. Labeling of different cell regions by XCAP E1 antibodies

	Mitotic chromosomes	Cytoplasm	Karyoplasm	Nucleolus
Met 6 minutes, –20°C	++	f gr ++	–	+
Tr 3 minutes, RT Met 6 minutes –20°C	+++	f gr ++	–	+++
r 3 minutes RT, Met 6 minutes, Ac 20 minutes, –20°C	+++	f gr -+	dif +	++++
Tr 3 minutes, For 3% 30 minutes RT	+++	f gr ++	–	++++
Tr 3 minutes, Glu 1%, $NaBH_4$ 2×10 minutes, RT	+	–	dif -+	–
For 3% 10 minutes, Tr 2×5 minutes	+++	–	f gr +	++++

Abbreviations: Met, methanol 100%; Tr, Triton X-100 1%; For, formaldehyde; Glu, glutaraldehyde; $NaBH_4$, 2 mg/ml in PBS; f gr, fine granular labeling; dif. diffuse labeling.

abolished nucleolar staining despite detergent pretreatment. The data suggest that the inability to visualize nuclear localization of pEg7 in previous reports may be caused by the inaccessibility of antigen to antibodies. Similar experiments performed using antibodies against XCAP-E (Table 3 and Fig. 3) demonstrate that same tendency. However, in contrast to anti-pEg7, antibodies XCAP-E1 labeled the nucleolus also after the formaldehyde-Triton fixation procedure.

These results partially explain the inconsistencies between previous reports, where condensins were immunolocalized to nuclear (Saitoh et al., 1994; Schmiesing et al., 2000) or cytoplasmic compartments of interphase cells (Steen et al., 2000).

pEg7 and XCAP-E are associated with nucleolus in interphase

Comparison of the effects of the fixation protocol on condensin immunolocalization permitted us to establish the most reliable conditions for studies of nucleolar localization of pEg7 and XCAP-E in cultured *Xenopus* cells. Although some recent data suggested localization of condensin subunits to the nucleolus, no detailed data on subnucleolar compartmentalization of these proteins have been presented. However, our analysis could provide a clue to the possible role of condensin subunits in the interphase nucleus. In the present study, we addressed the function of the condensin subunits by performing

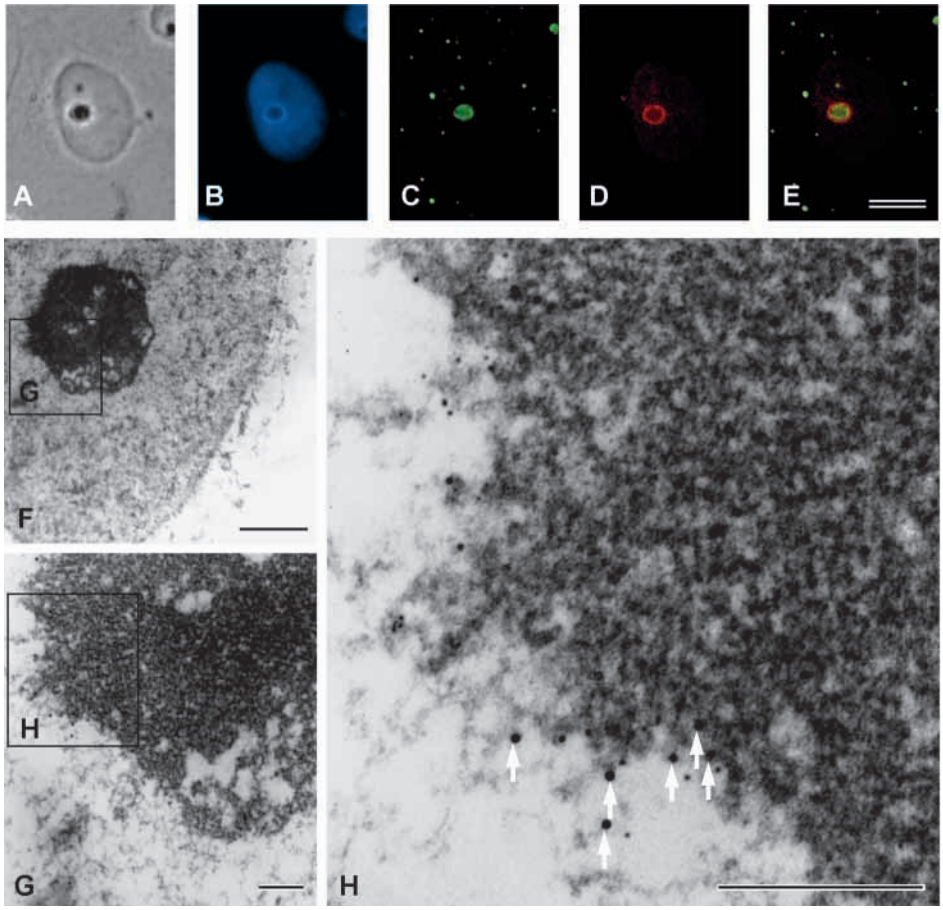

Fig. 4. Nucleolar localization of XCAPE and topoII in an interphase nucleus. XL-2 cells were grown and fixed as described in Materials and Methods. Cell were then processed for double immunofluorescence staining (A-E) with anti-XCAP-E affinity-purified polyclonal antibody (C) and anti-human topoII monoclonal antibody (D). Double staining is shown in e. Cells were observed by phase contrast microscopy (A) and stained with DAPI (B). For electron microscopy (F-H), cells were fixed as described under Materials and Methods and then labeled for double immunogold electron microscopy with an anti-XCAP-E affinity-purified polyclonal antibody and an anti-human topoII monoclonal antibody. Anti-XCAP-E and anti-topoII antibodies were revealed with a secondary anti-rabbit antibody conjugated to 5 nm gold particle (for XCAP-E) and an anti-mouse antibody conjugated to a 10 nm gold particles (for topoII, arrows), respectively. Bar, 5 μm (A-E), 1 μm (F) and 0.2 μm (G,H).

colocalization of pEg7 and XCAP-E with marker proteins of subnucleolar domains. We used UBF for labeling fibrillar centers; fibrillarin for the dense fibrillar component, and B23 for the granular component [see (Scheer and Hock, 1999; Olson et al., 2000) and references therein]. We also studied the colocalization of condensins with topoisomerase IIα. It had been shown previously, in *Drosophila*, that topoII interacts with Barren, a homologue of another member of condensin complex – XCAP-H (Bhat et al., 1996) – and also partially colocalized with condensins during mitosis. By contrast, nucleolar localization of topoII has been demonstrated in a number of studies (Zini et al., 1994; Mo, Beck, 1999; Christensen et al., 2002).

The nucleolar labeling by anti-pEg7 antibodies was not homogeneous; rather, the peripheral part of the nucleoli were preferentially stained, leaving the central part unstained. Depending on the orientation of the nucleolus in different cells, single or double cap-like regions could be observed (Fig. 3). This type of labeling was especially visible in cells with large nucleoli. In small nucleoli or in nucleolus-like bodies, which are often seen in some nuclei, homogeneous staining prevailed.

Anti-XCAP-E antibodies also stained the nucleolus (Fig. 4), but, in contrast to anti-pEg7, labeling was more intense and less sensitive to the fixation protocol used. In interphase cells, besides nucleolar staining, bright spots in the cytoplasm and fine granular staining of nucleoplasm were also observed with anti-XCAP-E antibodies (Fig. 4).

Fig. 3C-E shows double staining of interphase XL2 cells with anti-pEg7 and anti-topoII. The distribution of topoII at the nucleolar periphery perfectly matches that of pEg7. Double staining of topoII and XCAP-E also revealed obvious colocalization of these two proteins at the nucleolar surface, although XCAP-E was localized more towards the inside of the nucleolus (Fig. 4D,E).

For studying the ultrastructural localization of pEg7 and XCAP-E proteins in interphase cells, we performed immunogold labeling using affinity-purified antibodies against these proteins, in combination with monoclonal antibodies against topoII. Fig. 3F-H and Fig. 4F-H show typical staining patterns of interphase nuclei. For all three antibodies, the most intense labeling was detected at the nucleolar surface. The nucleoplasmic and cytoplasmic labeling was very weak. These observations are in agreement with our immunofluorescence data, with the only exception that, at the electron microscopic level, localization of all three proteins is restricted to the surface of the nucleolus, whereas immunofluorescent labeling shows deeper penetration into the nucleolus. This is possibly because of some sterical constraints that limit the diffusion of gold-conjugated antibodies into the densely packed nucleolus in the case of a pre-embedded immunogold staining procedure.

Counterstaining with DAPI shows no apparent colocalization of DNA with condensin subunits, indicating that these proteins are localized in the nucleolus itself, rather than in surrounding perinucleolar heterochromatin. In order to localize the condensins in the nucleolus precisely, double immunostaining with antibodies to UBF, fibrillarin and B23 was performed. As

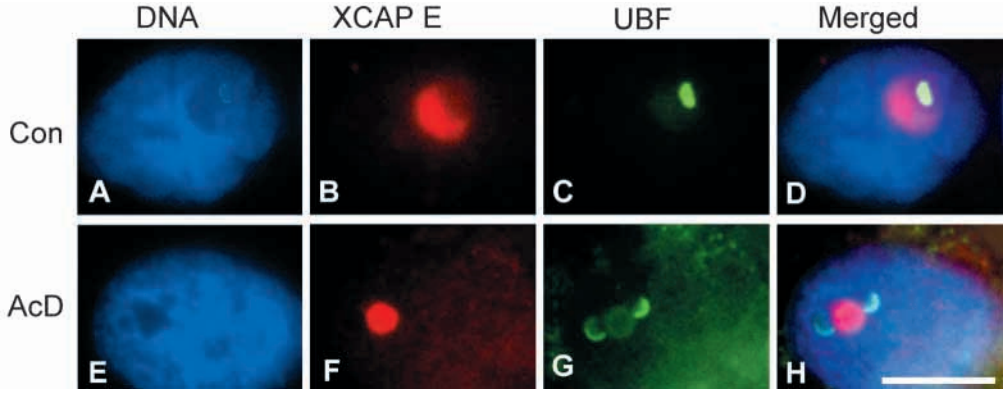

Fig. 5. Immunolocalization of XCAP-E and UBF in control cells and after actinomycin treatment. XL2 cells were incubated for 6 hours in the medium with 5 μg/ml actinomycin D (E-H) or without the drug (A-D). After fixation, cells were processed for immunofluorescence staining with polyclonal anti-XCAP-E1 antibodies (B,F) and human autoimmune serum to UBF (C,G). Cells were stained with DAPI for DNA visualization (A,E). Triple DAPI/XCAP-E/UBF labeling is shown (D,H). Bar, 5 μm.

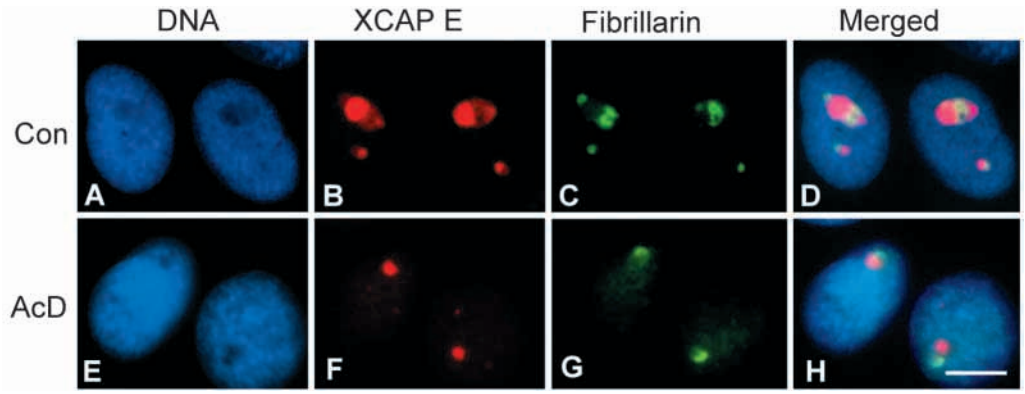

Fig. 6. Immunolocalization of XCAP-E and fibrillarin in control cells and after actinomycin treatment. XL2 cells were incubated for 6 hours in the medium with 5 μg/ml actinomycin D (E,H) or without the drug (A-D). After fixation, cells were processed for immunofluorescence staining with polyclonal anti-XCAP-E1 antibodies (B,F) and human autoimmune serum to fibrillarin (C,G). Cells were stained for DNA visualization with DAPI (A,E). Triple DAPI/XCAP-E/fibrillarin labeling is shown (D,H). Bar, 5 μm.

shown on Fig. 5, UBF is located in series of small dots that reside outside the condensin-positive zone. XCAP-E and fibrillarin also occupy mutually exclusive regions in the nucleoli (Fig. 6A,B). The same distribution of these proteins was seen in smaller nucleolus-like bodies, where distinct domains occupied by XCAP-E and fibrillarin were also visible (Fig. 6). pEg7 labeling was the same as that of XCAP-E (data not shown). By contrast, the distributions of B23 and XCAPE are practically identical (Fig. 7). These data strongly indicate that both SMC-protein XCAP-E and the non-SMC component of condensin are localized to granular component of the nucleolus. However, some differences in intranucleolar distribution of these proteins suggest the existence of a subpopulation of nucleolar XCAP-E that does not interact with pEg7.

Taken together, these observations indicate that, during interphase, two of the condensin subunits, pEg7 and XCAP-E, are targeted to the nucleolus, where they are localized in the granular component.

The effect of rRNA transcription inhibition on the nucleolar localization of condensins

In order to address the question of what is the functional meaning of nucleolar localization of condensins during interphase, we determined the distribution of pEg7 and XCAP-E in cells treated with actinomycin D at a concentration of 5 μg/ml for 2, 4 and 6 hours. Under these conditions, the transcription by RNApol I is completely blocked (Schofer et al., 1996). Indeed, morphological changes in nucleoli in actinomycin-D-treated cells, which result in nucleolar segregation, clearly indicate that rRNA synthesis is severely affected (Figs 5-9). After 6 hours of treatment, there was a notable decrease in the nucleolar size (from 4.5 to 1.8 μm in diameter) as judged by phase contrast microscopy. At the electron microscopic level, segregation of nucleolar material into two distinct compartments was clearly seen (data not shown). These segregated nucleoli were often in close contact with small blocks of condensed chromatin, which were clearly visible after DAPI staining.

Immunolocalization of pEg7 and XCAP-E showed that both proteins were found in the segregated nucleoli as homogeneously stained spherical regions. These regions occupy only a part of the segregated nucleolus, since they are smaller than the entire nucleolus. Again, comparison of the localization of pEg7 and XCAP-E and DNA demonstrates that condensins do not reside in condensed perinucleolar chromatin (Figs 5-9). Double staining for XCAP-E and UBF and fibrillarin does not show any colocalization of these proteins (Fig. 5). As in control nucleoli, these proteins occupy distinct spatially separated nucleolar domains. By contrast, B23 staining displays perfect overlap with XCAP-E in segregated nucleoli (Fig. 7).

After 6 hours of treatment with actinomycin D, the size of the nucleolar compartment containing these proteins decreased several-fold, on average, in treated cells, compared with control untreated cells. The local concentration of pEg7 and

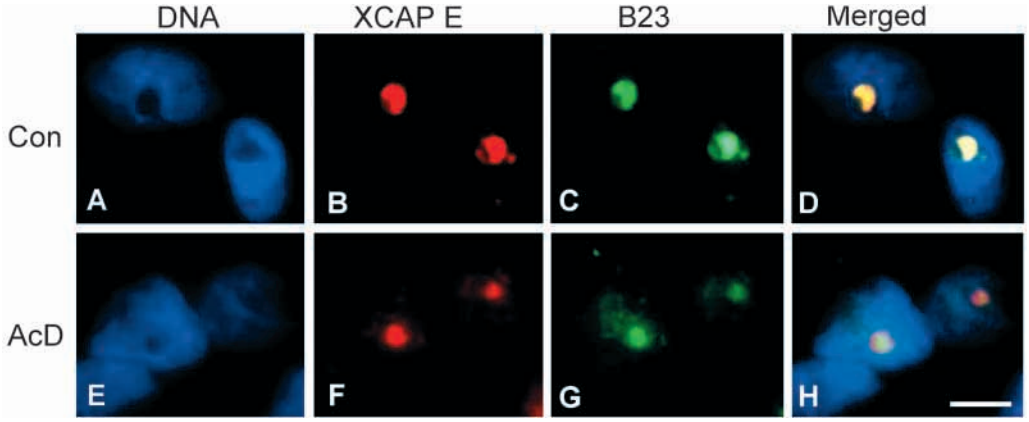

Fig. 7. Immunolocalization of XCAP-E and B23 in control cells and after actinomycin treatment. XL2 cells were incubated for 6 hours in the medium with 5 μg/ml actinomycin D (E-H) or without the drug (A-D). After fixation, cells were processed for immunofluorescence staining with polyclonal anti-XCAP-E1 (B,F) and monoclonal anti-B23 (C,G) antibodies. Cells were stained for DNA visualization with DAPI (A,E). Triple DAPI/XCAP-E/B23 labeling is shown (D,H). Bar, 5 μm.

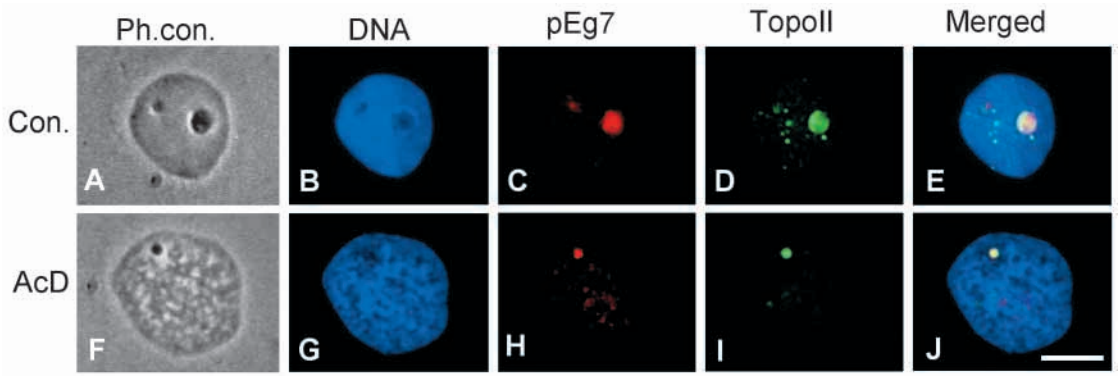

Fig. 8. Immunolocalization of pEg7 and topoII on the nucleolus in control cells and after actinomycin treatment. XL2 cells were incubated for 6 hours in the medium with 5 μg/ml actinomycin D (E-H) or without the drug (A-D). After fixation cells were processed for immunofluorescence staining with polyclonal anti-pEg7 (C,G) and monoclonal anti-topoII (D,H) antibodies. Cells were observed by phase contrast microscopy (A,E) and stained for DNA visualization with DAPI (B,F). Bar, 5 μm.

XCAP-E did not change significantly. Thus, the overall decrease in the nucleolar level of pEg7 and XCAP-E occurs under these conditions.

In actinomycin-D-treated cells, nucleolus-like bodies also decrease in size, but, in contrast to large nucleoli, fibrillarin staining is undetectable, whereas XCAP-E remains detectable (Fig. 6).

Discussion

Condensins were postulated to be the key chromosomal components driving, in cooperation with topoII, the final steps of mitotic chromosome condensation (Gasser, 1995; Hirano, 1995). This conclusion was derived from an in vitro immunodepletion assay of chromosome compaction and from mutational analysis of individual condensin subunits (Hirano and Mitchison, 1994; Cubizolles et al., 1998; Freeman et al., 2000; Lavoie et al., 2000; Ouspenski et al., 2000). The localization of condensin subunits to condensed chromosomes during mitosis also supports this idea. Moreover, decondensation of in vitro preassembled chromosomes after the addition of anti-XCAP-C antibodies implies that this protein, another subunit of the condensin complex, is also required for the maintenance of compact chromosome structure (Hirano and Mitchison, 1994).

Condensin function during interphase is not so obvious. Taking into account the fact that intracellular levels of condensin subunits remain practically constant throughout the cell cycle [this study; (Schmeising et al., 1998; Cabello et al., 2001)], one should expect condensins to exert some yet unknown function(s) during interphase. It is a plausible hypothesis that condensins act as regulators of gene expression by controlling the accessibility of chromatin loci to transcription factors via local condensation/decondensation of interphase chromosomes. The only example, so far, of dual function of SMC proteins in mitosis and interphase is the participation of *C. elegans* protein MIX-1 in both mitotic chromosome compaction and dosage compensation of the X chromosome in XX hermaphrodite worms (Lieb et al., 1998). Recently it was shown that Cnd2, a non-SMC subunit of fission yeast condensin, the analog *Drosophila* Barren (Lavoie et al., 2000), is required for mitotic chromatin condensation and is important for correct DNA reparation in interphase (Aono et al., 2002). Thus, the participation of condensin subunits in some related activities in interphase cannot be ruled out.

It is generally agreed that condensins are nuclear proteins, but some controversial observations were made concerning their subnuclear localization. Most authors demonstrated diffuse distribution of SMC proteins throughout the nucleus (Saitoh et al., 1994; Hirano and Mitchison, 1994), whereas

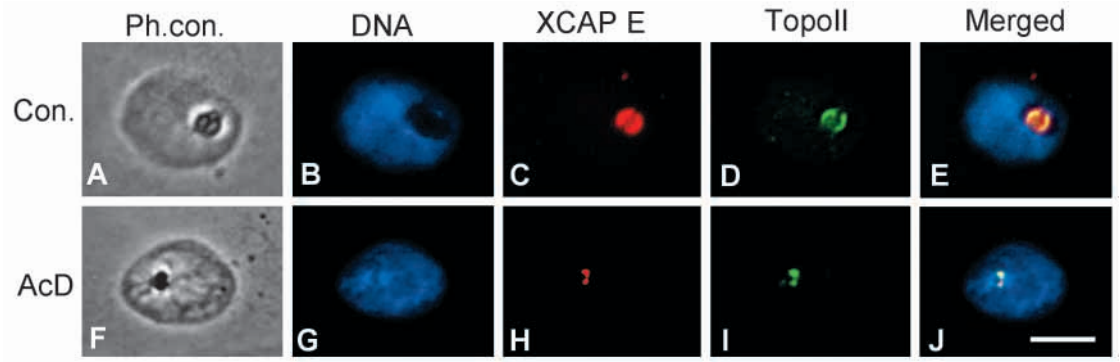

Fig. 9. Immunolocalization of XCAP-E and topoII on the nucleolus in control cells and after actinomycin treatment. XL2 cells were incubated for 6 hours in the medium with 5 μg/ml actinomycin D (E-H) or without the drug (A-D). After fixation, cells were processed for immunofluorescence staining with polyclonal anti-XCAP-E1 (C,G) and monoclonal anti-topoII (D,H) antibodies. Cells were observed by phase contrast microscopy (A,E) and stained for DNA visualization with DAPI (B,F). Bar, 5 μm.

others reported concentration of these proteins in discrete subnuclear domains of an unknown nature (Schmiesing et al., 1998). Recently, nucleolar localization of the human condensins hCAP-H has been reported (Cabello et al., 1999; Cabello et al., 2001). Similar results were obtained for *S. cerevisiae*, where GFP-tagged pSmc4 was shown to accumulate in the nucleolus prior to entry into mitosis (Freeman et al., 2000).

In the present study, we demonstrate, for the first time, a nucleolar localization of both a SMC protein (XCAP-E) and a non-SMC member of the *X. laevi*s condensin complex (pEg7) throughout interphase. In our previous work (Cubizolles et al., 1998), a diffuse signal was detected in the interphase nucleus when using more robust formaldehyde-glutaraldehyde fixation. Careful comparison of various fixation protocols showed that interphase staining is indeed very sensitive to fixation procedure (Tables 2 and 3). So, it could be hypothesized that the differences in interphase localization of condensins, reported by other authors, is based on variations of antigen preservation and/or accessibility, depending on the protocol of fixation. This explanation seems most acceptable when comparing our data on XCAP-E localization with those of Hirano and Mitchison (Hirano and Mitchison, 1994) and for its ortholog proteins hCAP-E (Schmeising et al., 1998) and ScII (Saitoh et al., 1994). The same holds true for the immunolocalization of pEg7 or its homologs (Cubizolles et al., 1997; Steen et al., 2000; Schmiesing et al., 2000). However, immunoblot analysis of subcellular fractions indicated that human orthologs of XCAP-E and pEg7 are predominantly cytoplasmic during interphase (Schmiesing et al., 2000; Steen et al., 2000). This inconsistency may be explained by the 'leakiness' of nucleolar condensins (Saitoh et al., 1994), which exit the nucleus during fractionation procedure.

The functions, if any, of condensins in the nucleolus during interphase remain unclear. As was shown previously by many authors, the 13S condensin complex and its core subunits – SMC proteins – display DNA-binding activity in vitro (Akhmedov et al., 1998; Sutani and Yanagida, 1997; Kimura and Hirano, 1997) and interaction with condensing chromosomes both in vivo and in vitro (Hirano and Mitchison, 1994; Freemen et al., 2000). Therefore, in agreement with the generally accepted hypothesis, it may be speculated that condensin subunits bind to perinucleolar or intranucleolar chromatin. In the *X. laevis* nucleolus, two related explanations can be proposed. On the one hand, in *X. laevis* cells about 500 rRNA cistrons per haploid set (Wallace and Birnstiel, 1966) are located on the short arm of chromosome 12 (Kahn, 1962), close to the centromere (Graf and Kobel, 1991), so that during interphase, the centromeric region of chromosome 12 appears in close proximity to the nucleolus. Non-random association of centromeres with the nucleolus has also demonstrated in other cell types (Stahl et al., 1976; Cerda et al., 1999; Chou and DeBoni, 1996; Haaf and Schmid, 1989; Lee et al., 1999; Leger et al., 1994; Park and DeBoni, 1992). These associations were thought to be important for spatial arrangement of the genome or regulation of rDNA transcription. On the other hand, during mitosis, accumulation of condensins in the centromeric regions was detected. Since blocks of heterochromatin maintain their highly compacted state throughout the cell cycle, one should expect condensins to keep interacting with these loci during interphase. However, such interaction has not been clearly demonstrated so far.

Alternatively, the nucleolar localization of condensins could reflect a direct and specific interaction with rDNA in interphase. In this case, condensin-driven compaction of ribosomal gene loci might serve as a mechanism of controlling rRNA synthesis at the level of higher-order chromatin structure. In situ hybridization with specific rDNA probes often demonstrates the presence of rDNA in clumps of nucleolus-associated chromatin both at microscopic and ultrastructural levels (Thiry et al., 1988; Thiry and Thiry-Blaise, 1991; Kaplan et al., 1993). The retraction of rDNA out of the nucleolus into perinucleolar chromatin upon inhibition of transcription by actinomycin D (Schofer et al., 1996) confirms the idea that the balance between compacted (perinucleolar) and extended (intranucleolar) rDNA is correlated with the level of rRNA synthesis. Strong correlation between condensin and topoII localization, observed in nucleoli, implies the cooperative action of these proteins in transcription-related reconfiguration of the rDNA template (Kimura et al., 1999). However, treatment with teniposide (stabilizing covalent catalytic DNA intermediates of topoII) causes relocation of topoII (both α and β) from the nucleoli to nucleoplasmic granules (Christensen et al., 2002), thus indicating that nucleolar subpopulation of this proteins seems to be catalytically inactive.

In the present work no colocalization of XCAP-E and pEg7

with DNA was detected during interphase. The lack of colocalization was especially clear after actinomycin treatment, when nucleolar DNA and XCAP-E occupied different non-overlapping domains. Moreover, XCAP-E is distributed throughout the granular component of nucleolus, where little or no DNA was detected (Thiry and Thiry-Blaise, 1989; Derenzini et al., 1990; Wachtler et al., 1992; Jimenez-Garcia et al., 1993).

Recent findings on the essential role of condensins in rDNA segregation during mitosis in *S. cerevisiae* (Freeman et al., 2000) provide another clue to the function of condensins in the nucleolus. The authors demonstrated accumulation of condensins in the nucleolus at the G2/M transition and blocked segregation of rDNA loci in *smc2* and *smc4* mutants. These observations were considered as evidence for a special role of condensins in proper segregation and/or folding up the arrays of repetitive DNA. This idea is further supported by the apparent concentration of condensins in centromeric and telomeric regions of mitotic chromosomes. In *Xenopus* cells, however, nucleolar localization of condensins is cell cycle independent and does not correspond to localization of rDNA during interphase, which is indicative of some additional function for condensin subunits. Although the existence of a minor subfraction of condensins bound to nucleolar DNA cannot be completely ruled out, nucleolar localization of the majority of XCAP-E and pEg7 cannot be explained exclusively by their association with DNA.

Another possible role of condensins in the nucleolus might be related to rRNA synthesis and/or processing. Localization of condensins in the granular component and their behavior upon the inhibition of transcription by actinomycin D indicate that condensins could be associated with released rRNA transcripts. This association is apparently maintained in hypotonically treated cells, where the granular component is redistributed to aggregates of RNP particles containing nucleolar proteins scattered throughout the nucleoplasm (Kireev et al., 1988; Dudnik and Zatsepina, 1995; Zatsepina et al., 1997). Under the same conditions, XCAP-E displays dynamics similar to that of B23 (Timirbulatova et al., 2002). The mechanisms of condensin action in nucleolar function remain largely unknown. Since heterodimers of cut3/cut14 (S. pombe homologs of XCAP-E and XCAP-C) possess a DNA-renaturing activity (Sutani and Yanagida, 1997), it seems possible that condensins (or the XCAP-E/C dimer only) facilitates the process of adoption of specific secondary structure in rRNA.

Condensins may also play a scaffolding role in spatial organization of the nucleolus. Structural motifs of SMC protein rod domain, reminiscent of those in intermediate filament proteins, may favor cooperative self-association and filament formation. This speculative mechanism of condensin-driven chromosome compaction (Strunnikov, 1998) may also take place during interphase.

The authors dedicate this manuscript to the memory of Katherine Le Guellec, who died suddenly in June 2001. She was a good friend and colleague, and it was her initiative that made this work possible. We miss her dearly.

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