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RESEARCH ARTICLE

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Centromeric protein bodies on avian lampbrush chromosomes contain a protein detectable with an antibody against DNA topoisomerase II

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Abstract In the oocyte nuclei (germinal vesicle or GV) of a variety of avian species, prominent spherical entities termed protein bodies (PBs) arise at the centromeric regions of the lampbrush chromosomes (LBCs). In spite of the obvious protein nature of PBs, nothing is known about their composition. We show that an antibody against DNA topoisomerase II (topo II), the DNA unwinding enzyme, recognizes PBs from chaffinch and pigeon oocytes. In later chaffinch oocytes, the PBs fuse to form a karyosphere, which is also labeled by the anti-topo II antibody. Furthermore, we show that proteins characteristic of Cajal bodies and B-snurposomes are not found in PBs, despite morphological similarities among these structures. Using immunoelectron microscopy and immunofluorescent laser scanning microscopy we demonstrated that topo II localizes predominantly in the dense material of PBs. Two antigens of ~170 kDa (which corresponds to topo II) and ~100 kDa were revealed with the antibody against topo II on immunoblots of avian GV proteins. We propose that the smaller protein results from oocyte specific topo II cleavage, since it was not detected in nuclei from testis cells. This represents the first report of a defined protein in the centromeric PBs on avian LBCs.

Introduction

In growing avian oocytes, just as in those of amphibians, intensive widespread transcription results in the formation of giant lampbrush chromosomes (LBCs), which have a characteristic chromomere-loop structure (for reviews see Callan 1986; Morgan 2002). Remarkable spherical entities up to 12 μm in diameter, termed protein bodies (PBs), arise on each of the lampbrush bivalents in a variety of avian species (Gaginskaya 1972; Solovei et al. 1996; Saifitdinova et al. 2003). Protein bodies have been shown to form in association with highly repeated centromeric sequences (Solovei et al. 1996; Saifitdinova et al. 2003) and to consist mainly of poorly soluble proteins (Tsvetkov and Gaginskaya 1983). In spite of their obvious protein nature, specific proteins of the PBs have not yet been identified.

In the course of oocyte growth, the PBs, being associated with centromeric regions of chromosomes, fuse to form the karyosphere—a protein structure with condensed chromosomes attached to its surface. The process of karyosphere formation in the chaffinch germinal vesicle (GV) has been described in more detail (Gaginskaya and Gruzova 1969; Gaginskaya 1972; Saifitdinova et al. 2003). It was proposed that the fusion of PBs into the karyosphere might point to their function in the topological organization of chromosomes during later oogenesis.

The PBs on avian LBCs reveal certain morphological similarities with sphere organelles (Cajal bodies, CBs) and B-snurposomes from amphibian GVs. For this reason it has been repeatedly suggested that avian PBs are homologs of these structures, which are known to contain a variety of RNA processing and transcription factors (Gall and Callan 1989; Gall 2000, 2003). Additionally, avian PBs have a morphological similarity with the so-called axial granules, which have been described on LBCs of some amphibians (Callan 1986) and found to contain DNA topoisomerase II (topo II; Hock et al. 1996).

In the present research the localization of RNA splicing factors, transcription enzymes and topo II was determined in avian GVs. We showed that PBs from chaffinch and pigeon oocytes, as well as karyospheres from later chaffinch oocytes, are recognized by an antibody against amphibian DNA topo II. Splicing factors and transcription enzymes, which are characteristic of CBs and/or B-snurposomes, were not found in the PBs, notwithstanding morphological similarities among these organelles.

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Materials and methods

Lampbrush chromosome preparation

Chaffinch (*Fringilla coelebs*) and pigeon (*Columba livia*) LBCs were isolated manually from oocyte GVs according to the standard technique (Solovei et al. 1993, 1994; Saifitdinova et al. 2003). For chromosome isolation and fixation, $MgCl_2$ was added to the solutions to a final concentration of 1 mM. After brief fixation in 2% paraformaldehyde, preparations were post-fixed in 70% methanol overnight. The preparations were not dried before immunostaining.

Fibroblast fixation

Chaffinch fibroblasts were grown directly on coverslips in DMEM supplemented with 10% fetal calf serum. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After fixation the cell membrane and the nuclear envelope were permeabilized by incubation in 0.5% Triton X-100. The fixed and permeabilized cells were stored in PBS with 0.04% sodium azide at 4°C until use.

Antibodies and immunofluorescent staining

Immunostaining of avian LBCs and fibroblasts was carried out with the following mouse monoclonal antibodies (mAbs): 4A6 against amphibian topo II (Hock et al. 1996), K121 against the trimethylguanosine (TMG) cap of most splicing snRNAs (Oncogene Research Products), Y12 against the Sm core proteins of most splicing snRNPs (Lerner et al. 1981), anti-SC35 against the SC35 splicing factor (Fu and Maniatis 1990), H14 (BAbCO) and V22 (kindly donated by U. Scheer) against the phosphorylated C-terminal domain (CTD) of RNA polymerase II, 72B9 and 17C12 against fibrillarin (Pollard et al. 1997), No114 against *Xenopus* Nopp140 and No185 against *Xenopus* NO38 (Schmidt-Zachmann and Franke 1988) as well as with R288 anti-human coilin rabbit serum (Andrade et al. 1993).

The LBC spreads and fixed fibroblasts were blocked in PBS containing 1% blocking reagent (Roche) for 1 h at room temperature. Then they were incubated for 1 h at room temperature with the primary antibodies listed above. Primary antibodies were applied in dilutions recommended by authors or manufacturers. Slides were washed in PBS, 0.05% Tween-20 and incubated for 1 h at room temperature in Cy3-conjugated goat anti-mouse IgG + IgM secondary antibody (Jackson ImmunoResearch Lab). The slides were again washed in PBS, 0.05% Tween-20 and mounted in DABCO antifade solution containing 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI).

Fluorescence microscopy

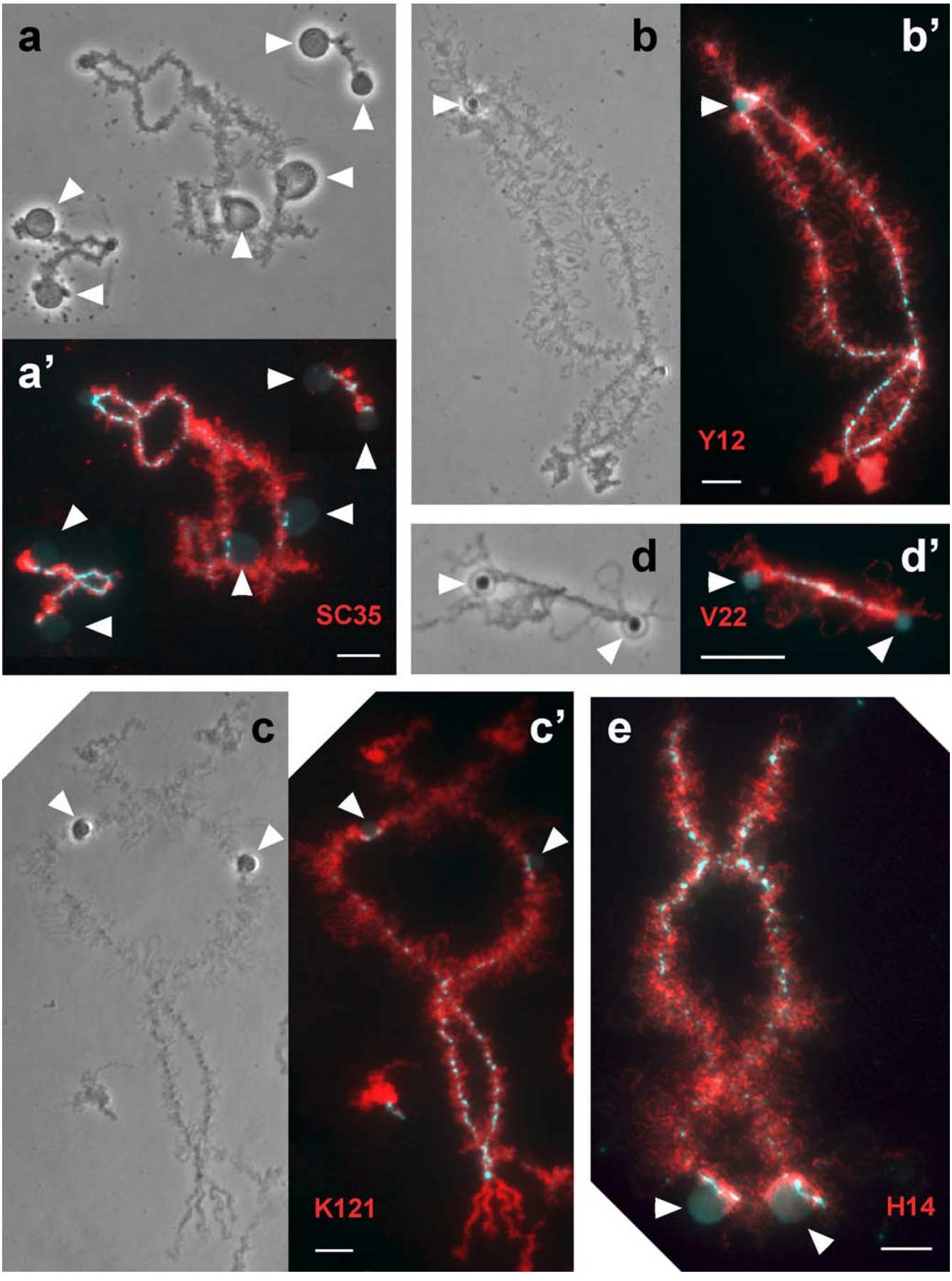
Preparations were examined using a DMRXA fluorescence microscope (Leica Microsystems Wetzlar, Germany) equipped with a FLUOTAR 100/1.30 N.A. objective, appropriate filter cubes, and a black and white CCD camera (Cohu). QFISH software (Leica Cambridge) was used to acquire and process multicolor microscopic images. Confocal laser scanning microscopy was carried out on a Leica TCS SL microscope equipped with a HeNe (543 nm) laser (Leica Microsystems Wetzlar, Germany).

Immunoelectron microscopy

For this study, whole oocytes of 0.2–0.6 mm diameter and GVs isolated manually from oocytes of the same size were used. For the analysis of morphology, oocytes and GVs were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer with 2.5 M sucrose, pH 7.4, and post-fixed in 1% OsO_4. For immunoelectron microscopy analysis, oocytes and GVs were fixed in 4% paraformaldehyde with 0.1% glutaraldehyde in 0.1 M cacodylate buffer with 2.5 M sucrose, pH 7.4. Dehydration was carried out in a series of graded ethanols from 35% to 70%, followed by infiltration and embedding in LR-White resin (Sigma). Polymerization was carried out at 50°C for 36 h. Thin sections were picked up on nickel grids, treated with glycine-HCl in TBS, pH 7.2 for 15 min, washed twice with TBS for 10 min, blocked with TBS containing 0.6% BSA and 0.05% Tween-20 for 30 min and stained with mAb 4A6 diluted in blocking solution for 12–18 h at 4°C. After two washes in blocking solution and two washes in TB, pH 8.2 containing 1% BSA and 0.05% Tween-20, sections were transferred to an incubation medium containing goat anti-mouse IgG coupled with 18 nm gold particles (Jackson ImmunoResearch Lab), which were diluted 1:10 in TB containing 1% BSA and 0.05% Tween-20. Incubation was carried out at room temperature for 1.5 h. Unbound antibodies were washed out twice with the same solution in which the secondary antibodies were diluted, and then were jet-washed in double-distilled water (Newman and Hobot 1993). Thin sections were stained with uranyl acetate and examined using a Tesla-B-500 electron microscope at 60 kV.

Protein preparation and immunoblot analysis of protein extracts

Chaffinch and pigeon GVs were manually isolated and collected in the isolation buffer "5:1" [83 mM KCl, 17 mM NaCl, 6.5 mM Na_2HPO4, 3.5 mM KH_2PO4, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.0] with complete protease inhibitor cocktail (Roche). Nuclei from chicken testis cells were isolated as described by Berezney and Coffey (1974). Purified testis nuclei were extracted for 1 h at 4°C in 10 mM TRIS-HCl, pH 8.0, 0.1 mM $MgCl_2$, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM


a
a'
SC35
b
b'
Y12
d
d'
V22
c
c'
K121
e
H14

◀ **Fig. 1a–e** Immunodetection of splicing factors and RNA polymerase II on lampbrush chromosomes (LBCs) of *Fringilla coelebs* (**a**,**a′**, **c**,**c′**,**d**,**d′**,**e**) and *Columba livia* (**b**,**b′**). **a**,**b**,**c**,**d** Phase contrast images. **a′**–**c′** Immunodetection of splicing factor SC35 (**a′**), the Sm epitope of snRNPs (**b′**) and the trimethylguanosine cap of splicing snRNAs (**c′**) with monoclonal antibodies (mAbs) anti-SC35, Y12 and K121, respectively; the RNP matrix of the majority of lateral loops is stained (*red*), while protein bodies (PBs) are not stained. **d′**,**e** Immunodetection of the phosphorylated form of RNA polymerase II with mAb V22 (**d′**) and mAb H14 (**e**). The axes of the majority of simple lateral loops show prominent labeling (*red*), whereas PBs from pigeon and chaffinch germinal vesicles (GVs) show no immunostaining. The DNA of the lampbrush chromosomes is counterstained with 4′,6-diamidino-2-phenylindole (DAPI, *blue*). *Arrowheads* indicate centromeric PBs. *Bars* represent 10 μm

DTT, 5% glycerol and 0.35 M NaCl, centrifuged for 10 min at 5000 *g*, and the supernatant nuclear extracts were collected. Proteins from chaffinch oocyte GVs and from chicken testis cell nuclei extracts were separated on 7% polyacrylamide/SDS gels, and transferred onto Hybond C+ (Amersham, UK), using a mini transfer cell from BIO-RAD (Hercules, Calif.). Membranes were blocked in 5% fat-free dry milk in TBS, washed in TBS, 0.1% Tween-20 and incubated for 1 h at room temperature with primary antibody. The primary antibody was mouse mAb 4A6 diluted 1:100 in blocking solution. After three washes in TBS, 0.1% Tween-20, the membrane was incubated with a 1:10,000 dilution of rabbit anti-mouse Ab (Sigma) conjugated with peroxidase. After several washing steps, bound antibody was visualized using the TMB membrane peroxidase substrate system (Kierkegaard and Perry Laboratories).

Results

In order to compare PBs with CBs and B-snurposomes we analyzed the localization of RNA splicing factors in chaffinch and pigeon GVs by immunocytochemistry. Spreads of GV contents were stained with mAb K121 against the TMG cap of most splicing snRNAs, mAb Y12

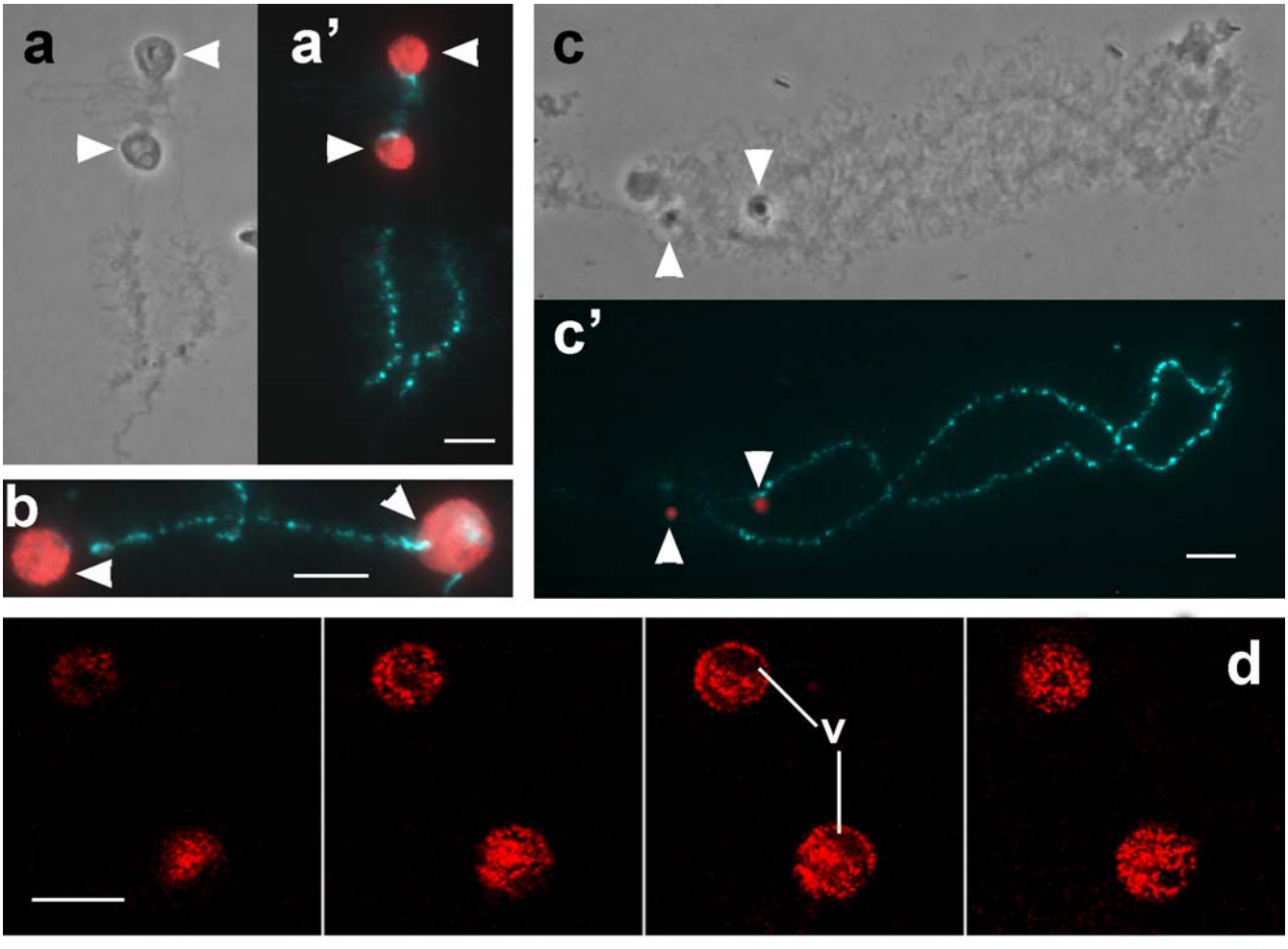


Fig. 2a–d Immunostaining of chaffinch (**a′**,**b**) and pigeon (**c′**) GV spreads with mAb 4A6 against DNA topoisomerase II (topo II). **a**,**c** Phase contrast images of chaffinch (**a**) and pigeon (**c**) LBCs. Protein bodies but not LBCs show a bright signal (*red*). The DNA of the LBCs is counterstained with DAPI (*blue*). *Arrowheads* indicate PBs. **d** Four representative confocal sections through two chaffinch PBs immunostained with mAb 4A6 (*red*). Note that the vacuoles (*V*) of PBs are not stained. *Bars* represent 10 μm

against the Sm epitope found in the majority of splicing snRNPs, and mAb anti-SC35 against splicing factor SC35. Protein bodies on chaffinch and pigeon LBCs were not stained with these antibodies, whereas the majority of simple lateral loops stained strongly (Fig. 1a–c). Simple lateral loops on LBCs (according to the classification of Morgan 2002) are involved in active transcription by RNA polymerase II (pol II). We immunostained chaffinch and pigeon GV spreads with mAbs V22 and H14 against the phosphorylated CTD of pol II. These antibodies recognize pol II during the elongation phase of RNA transcription (Patturajan et al. 1998). Protein bodies were not stained with mAbs V22 and H14, whereas all simple loops on the LBCs stained as expected (Fig. 1d,e). It should be noted that pol II staining is limited to the axes of the loops, whereas the antibodies against splicing factors bind to the entire RNP matrix of the loops (Fig. 1). The localization of these proteins on simple loops was similar to that on the loops of amphibian LBCs (Wu et al. 1991; Doyle et al. 2002). It is worth noting that, in contrast to avian PBs, extrachromosomal and chromosome-associated CBs in amphibian GVs are known to stain strongly with mAb H14 and antibodies against snRNPs (Gall et al. 1999; Morgan et al. 2000).

Other indicators of CBs, such as fibrillarin, B23 and Nopp140 (Gall et al. 1999), were not detectable in PBs on chaffinch LBCs by indirect immunofluorescence cytochemistry. Because these proteins are characteristic of nucleoli (for review see Olson et al. 2000), and nucleolus organizers are known to be inactive in avian growing oocytes (Gaginskaya and Gruzova 1975), we used preparations of fixed monolayers of chaffinch fibroblasts as a positive control in these experiments. Antibodies against fibrillarin, B23 and Nopp140 stained nucleoli strongly in chaffinch fibroblast nuclei (data not shown). The defining CB component, coilin, was not revealed in PBs with anti-coilin serum R288 (Andrade et al. 1993).

In a study of the LBCs of the newt *Pleurodeles waltl*, Hock et al. (1996) demonstrated intense staining of the so-called axial granules with mAb 4A6, which recognizes amphibian topo II. Topoisomerase II was not detectable in other parts of the LBCs (Fisher et al. 1993). Because of the morphological resemblance between avian PBs and amphibian axial granules, we used mAb 4A6 to stain GV spreads from both chaffinch and pigeon previtellogenic oocytes. We saw strong staining of centromeric PBs, but no reaction in the chromosomal axis or lateral loops (Fig. 2a–c).

The localization of topo II was also studied in chaffinch GV spreads during chromosome condensation and karyosphere formation. It was found that PBs were stained by mAb 4A6 at all stages of their fusion into a karyosphere (Fig. 3). It is worth noting that the condensing chromosomes in the karyosphere, like the axes of the earlier LBCs, show no reaction with the antibody. Strong staining of PBs with mAb 4A6 was also observed on LBCs of other birds, namely greenfinch (*Carduelis chloris*, Passeriformes), sparrow (*Passer domesticus*, Passeriformes) and duck (*Anas platyrhynchus*, Anseriformes) (data not shown).

To determine the distribution of topo II within PBs, we carried out immunostaining followed by confocal laser scanning microscopy or electron microscopy. Protein bodies have a sphere-like appearance at all stages of their development. At the light microscope level, staining with mAb 4A6 may be homogeneous, or there may be internal regions of lighter stain, which are clearly demonstrable by laser scanning microscopy (Fig. 2d). At the ultrastructural level, PBs may have a homogeneous fibrillar consistency in earlier oocytes (Fig. 4a) or there may be multiple cavities or vacuoles of different sizes within a dense fibrillar matrix in the oocytes of later stages (Fig. 4b). The content of the vacuoles has a structure similar to that of nucleoplasm. After immunostaining with mAb 4A6 the dense fibrillar material was enriched with gold particles relative to the nucleoplasm and the vacuoles (Fig. 4c), whereas LBC chromomeres appeared unlabeled (not shown).

In amphibians, mAb 4A6 reacts with a nuclear antigen of ~182 kDa in oocytes and eggs and ~170 kDa in somatic

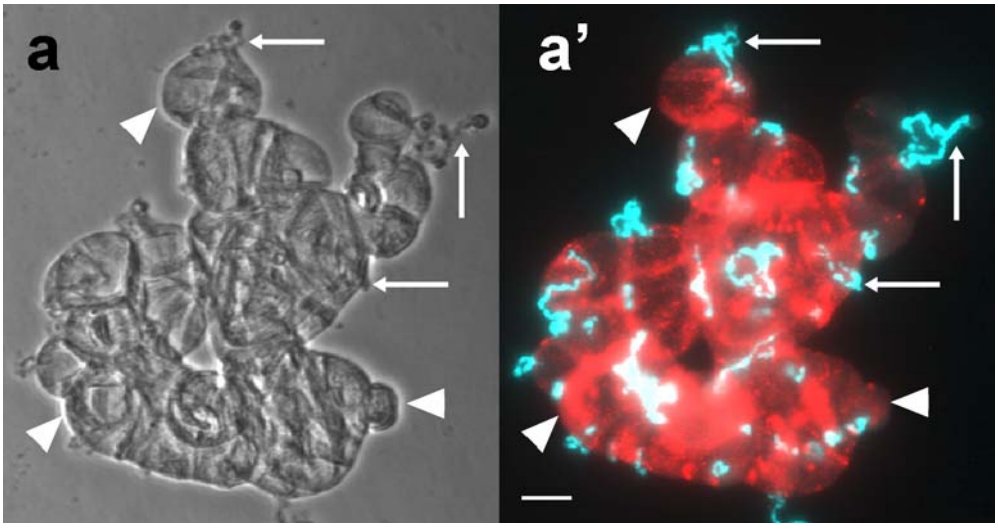


Fig. 3a,a′ A karyosphere from a chaffinch oocyte nucleus: immunostaining with mAb 4A6 against topo II (*red*) (**a′**) and corresponding phase contrast image (**a**). *Arrowheads* indicate PBs that fuse at the stage following the lampbrush phase. The DNA of the condensing chromosomes (*arrows*) is counterstained with DAPI (*blue*). mAb 4A6 against topo II stains the fusing protein bodies but does not stain inactive chromosomes. *Bar* represents 10 μm

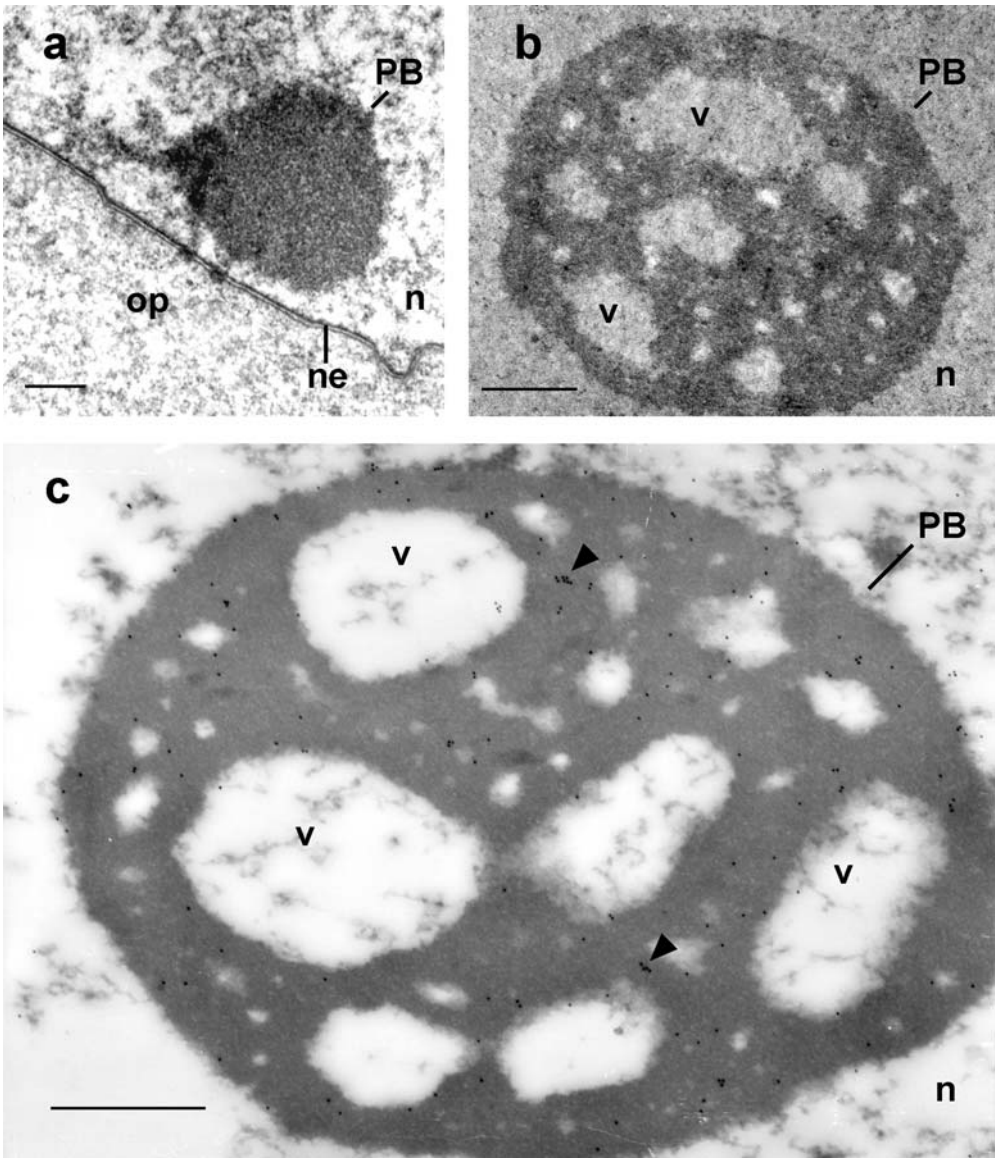


Fig. 4 **a**,**b** The ultrastructural morphology of protein bodies from a chaffinch oocyte of 0.2 mm (**a**) and 0.6 mm (**b**) in diameter. Multiple vacuoles are visible in the dense material of protein bodies from later oocytes (**b**). **c** Electron microscopic immunolocalization of the mAb 4A6 antigen on ultrathin sections of the chaffinch GV. The dense material of the PB is enriched with gold particles. *Arrowheads* indicate conglomerates of gold particles. (*n* nucleoplasm, *ne* nuclear envelope, *op* ooplasm, *PB* protein body, *v* vacuole) *Bars* represent 100 nm in **a**, 1 μm in **b** and **c**

cells, which was shown to be topo II (Hock et al. 1996). To define the proteins that mAb 4A6 recognizes on avian GV spreads, immunoblotting of chaffinch GV proteins was carried out. Two bands of ~170 kDa and ~100 kDa were detected (Fig. 5a). On the basis of its size, the protein of ~170 kDa probably corresponds to DNA topo II. Chicken homologs for both isoforms of topo II were shown to migrate similarly: 170 kDa for the topo II alpha isoform, and 172 kDa for the topo II beta isoform (Niimi et al. 2001). The protein of ~100 kDa recognized by mAb 4A6 in chaffinch GVs may correspond either to a different protein sharing an epitope with topo II, or to a product of oocyte specific cleavage of topo II. Since topo II cleavage to a product of ~100 kDa has been shown recently in mouse ovary (St Pierre et al. 2002), and proteasomal degradation is known to be involved in the regulation of topo II expression during the cell cycle (Heck et al. 1988; Salmena et al. 2001), the second assumption seems to be more plausible. Moreover, in chicken testis cells, we did not observe a lower molecular weight antigen with mAb 4A6. Only the ~170 kDa protein was detected on immunoblots (Fig. 5b) and precipitated from nuclear extracts of testis cells (data not shown).

Discussion

Protein bodies arise at the centromeric regions of the chromosomes at the very beginning of the lampbrush phase in certain species of birds (Gaginskaya and Gruzova 1969; Gaginskaya 1972; Solovei et al. 1996; Saifitdinova et al. 2003). Until now, there has been no information about protein components of avian PBs. In the present study we describe immunostaining of PBs at the light microscopic level with an antibody against topo II. Ultrastructural localization analyses showed that the dense component of PBs is enriched with this protein. Immunostaining data are also presented that suggest that

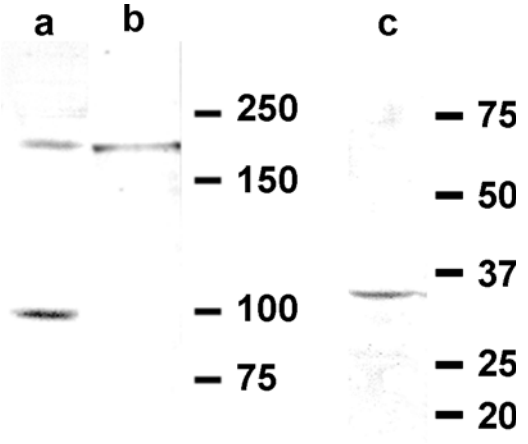


Fig. 5a–c Immunoblot analysis of proteins from chaffinch GVs and testis nuclear extracts with mAb 4A6 against amphibian DNA topo II. **a** Sixty GVs manually isolated from chaffinch oocytes. **b** Testis nuclear extracts. **c** Analysis of the same extract with anti-SC35 mAb is shown. Sizes of protein standards are indicated in kilodaltons

PBs in chaffinch and pigeon GVs do not contain splicing factors or phosphorylated RNA pol II and therefore are not involved in processes related to splicing or transcription. We assume that PBs are unique structures, and are not similar in protein composition and function to CBs or B-snurposomes from amphibian GVs.

Earlier it was shown that PBs on avian LBCs cannot be completely dissolved in high concentration salt and thus might contain nuclear matrix proteins (Tsvetkov and Gaginskaya 1983). The presence of topo II in PBs is in accordance with these data, since topo II is one of the major nuclear matrix components (Berrios et al. 1985).

DNA topo II is a multifunctional protein with decatenating enzymatic activity (for review see Swedlow and Hirano 2003). It was found to be one of the components of the chromosome scaffold (Earnshaw et al. 1985; Earnshaw and Heck 1985; Adachi et al. 1989). Topoisomerase II was considered to be necessary for resolving DNA catenations during chromosome condensation. Its activity is required for the transition from meiotic prophase to meiotic metaphase I in mammalian spermatocytes (Cobb et al. 1999). At the same time, it has been recently demonstrated by RNAi analysis that DNA topo II is not required for global condensation of chromosomes, but is necessary for their segregation (Chang et al. 2003). In avian oocytes, we do not see topo II in the chromomeres of the LBCs and in the condensed chromosomes in karyosphere, but we cannot exclude the existence of another pool of topo II that does not react with the antibody used in this work.

The formation of PBs in association with centromeric regions and their subsequent fusion before the first meiotic division to produce a karyosphere is thought to be related to the spatial organization of chromosomes in oocyte nuclei (Saifitdinova et al. 2003). The presence of topo II in centromeric PBs is especially interesting, since topo II is known to associate preferentially with active centromeres in mitotic and in male meiotic cells at certain stages of the cell cycle (Andersen et al. 2002; Cobb et al. 1999). Throughout most of meiotic prophase, the centromeric regions of mouse male meiotic chromosomes exist as large blocks of heterochromatin, in which large amounts of topo II alpha are found (Cobb et al. 1999). It has been suggested that topo II may have a new function at these regions, distinct from chromosome condensation and scaffolding (Paliulis and Nicklas 2003). In the case of chaffinch GVs, the centromeric PBs that fuse to form the karyosphere contain topo II. Thus, the integration of centromeric regions of the chromosomes into conglomerates that contain DNA topo II seems to be general in the first meiotic prophase of both male and female cells.

Topoisomerase II is known to be responsible for topological reorganization of chromosome DNA in nearly all processes requiring its relaxation, including transcription. Relaxation activity of topo II is essential for RNA polymerase II transcription on chromatin templates (Mondal and Parvin 2001). Thus, it is reasonable to expect topo II to be involved in the process of active pol II transcription on the lateral loops of the LBCs. However, immunofluorescence analysis did not reveal direct association of topo II with lateral loops in avian (our data) or in amphibian GVs (Fisher et al. 1993; Hock et al. 1996). Immunoelectron microscopy studies performed on thin sections of chaffinch GVs showed the presence of topo II in the nucleoplasm. This pool of topo II may be recruited to the DNA of the lateral loops as it is needed.

The function of DNA topo II in centromeric PBs on avian LBCs is not clear and is still to be explored. On the one hand, topo II activity could be essential for condensation and cohesion of centromeric regions in meiotic cells, as well as for proper segregation of centromeres during meiosis. On the other hand, the PBs may be structures that participate in the dynamics and storage of topo II, regulating its concentration in the nucleus. To elucidate the functional role of PBs and the karyosphere, other components of these structures must be found and their dynamics investigated.

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