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Lampbrush chromosomes of the chaffinch (*Fringilla coelebs* L.)

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Abstract

The seven macrochromosomes of the chaffinch (*Fringilla coelebs* L.) are described in their lampbrush form. The relative lengths of bivalents, the positions and arrangements of chromosomal regions with lateral loops of similar length and appearance, as well as the positions of protein bodies and loops of peculiar morphology have been defined and mapped, so that each of the seven lampbrush macrobivalents may be identified in oocytes from every individual of the species. This morphological analysis has been supplemented by determining the positions of certain loci and objects that are specifically and consistently labelled after immunostaining or fluorescence *in-situ* hybridization with defined molecular probes.

Introduction

The chaffinch (*Fringilla coelebs* L., Aves, Passeriformes, Fringillidae) is common and widely distributed throughout the Old World. The chaffinch karyotype consists of 40 chromosome pairs including seven pairs of macrochromosomes, autosomes 1–6 and sex chromosomes Z and W, and 33 pairs of microchromosomes (Piccinni & Stella 1970, Saifitdinova *et al.* 2000). Avian chromosomes, both macro- and microchromosomes, transform into the typical lampbrush structure during oogenesis (Koecke & Muller 1965, Kropotova & Gaginskaya 1984, Callan 1986, Hutchison 1987, Chelysheva *et al.* 1990, Solovei *et al.* 1993, Myakoshina & Rodionov 1994). Avian microchromosomes are exceedingly small and numerous and, even in their lampbrush form, they can hardly be distinguished from one another and arranged in a regular order.

Nuclear structures in growing oocytes of the chaffinch were first described by Loyez (1906). This account was illustrated with detailed watercolour pictures of histological sections of oocytes in which chromosomes and numerous spherical bodies, termed 'nucleoli', were meticulously depicted. In the 1960–1970s, the dynamics of [^{3}H]uridine incorporation into chaffinch oocyte nuclei was analysed (Gaginskaya & Gruzova 1969) and *in-situ* hybridization with radioactively labelled rRNA was applied to oocyte sections (Gaginskaya & Gruzova 1975). In these experiments, the massive ribosomal DNA amplification that is so characteristic of amphibian oocytes, was not evident in the chaffinch oocytes and chromosomal nucleolus organizers were found to be

inactive during the whole oocyte growth period. On this basis, the spherical structures associated with lampbrush chromosomes in birds were redefined, not as nucleoli, but as ‘protein bodies’ (Gaginskaya & Gruzova 1969, Gaginskaya 1972).

F. coelebs is proving to be a valuable system for the exploration of various aspects of avian cytogenetics, avian genome organization and genome architecture (Solovei *et al.* 1993, Saifitdinova *et al.* 2000, 2001, Liangouzov *et al.* 2002). It is also proving to be an excellent system for the study of avian lampbrush chromosome structure and function, lampbrush centromeric regions and a range of general problems in lampbrushology (Morgan 2002). An essential prerequisite for this kind of research is a reliable system for identifying individual lampbrush chromosomes and defining positions along these chromosomes. In this paper, we offer a characterization of landmarks on chaffinch lampbrush chromosomes and we present a standard lampbrush map for macrobivalents of the species.

Materials and methods

Preparation of chromosomes

Chaffinch lampbrush chromosomes were isolated manually from oocytes of 0.25–0.75 mm diameter according to the standard technique (Solovei *et al.* 1993, 1994) with certain modifications. $MgCl_2$ was added to the solutions for chromosome isolation and fixation to a final concentration of 1 mmol/L. After brief fixation in 2% paraformaldehyde, preparations were post-fixed in 70% methanol overnight. Some preparations were dried from 100% methanol and kept at −20°C before using for fluorescence *in-situ* hybridization (FISH). The preparations for immunostaining were never dried before use.

Mitotic chromosomes were obtained from a fibroblast culture using a standard procedure.

Immunostaining

The monoclonal antibodies H14 (BAbCO) and V22 (produced and kindly provided by U. Scheer) against phosphorylated C-terminal domain of RNA-polymerase II were applied to chaffinch lampbrush chromosomes according to the standard protocol. Signal detection was performed with anti-mouse Cy3-conjugated $F(ab')_2$ (Jackson ImmunoResearch Lab, Inc).

DNA probes for hybridization

Centromeric highly repetitive sequence FCP (Saifitdinova *et al.* 2001; GenBank accession number AF160980) and non-centromeric repeated sequence GS (Liangouzov *et al.* 2002; GenBank accession number AF427997) previously cloned from *F. coelebs* were used as probes. The probes were labelled with biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche) by PCR.

Fluorescence in-situ hybridization (FISH)

The standard protocol for FISH on lampbrush chromosomes (Solovei *et al.* 1994, 1996) was slightly modified. Pretreatments with RNase A (200 μg/ml), pepsin (0.1–0.5 μg/ml), and 1% paraformaldehyde were followed by 0.1% Triton X100 for 30 min at the room temperature. Preincubation of preparations in hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulphate) was performed at 37°C for 1–2 h. The biotinylated probe was then mixed with the hybridization buffer and 50-fold excess of salmon sperm DNA to a final concentration of 5 ng/μl and applied to the slides. The preparations were covered with a coverslip and chromosomes were denatured together with the probe at 81.5°C for 5 min. Hybridization was performed at 37°C for 12–18 h. Biotin was detected by avidin-Cy3 (Jackson ImmunoResearch Lab. Inc.). Chromosomes were counterstained with DAPI.

Microdissection

Microdissection was carried out using an inverted Opton microscope with the help of a micromanipulator. Individual centromeric and non-centromeric PBs were scraped from lampbrush chromosomes separately. Chromosomal fragments dissected from the regions which lacked PBs were taken as negative control samples. The tip of a glass microneedle carrying the scraped material was broken off into a PCR tube. The samples were treated with proteinase K. After inactivation of

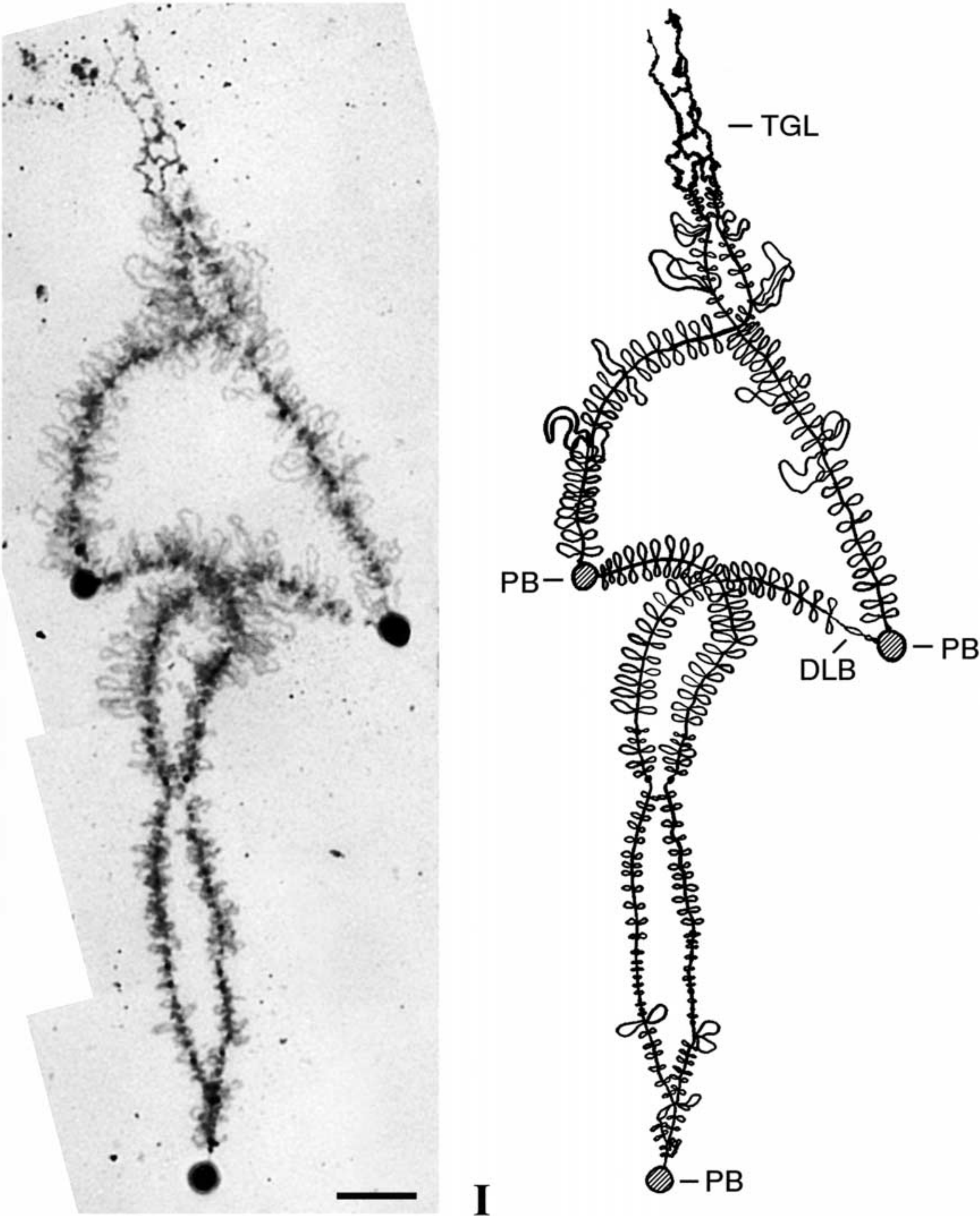
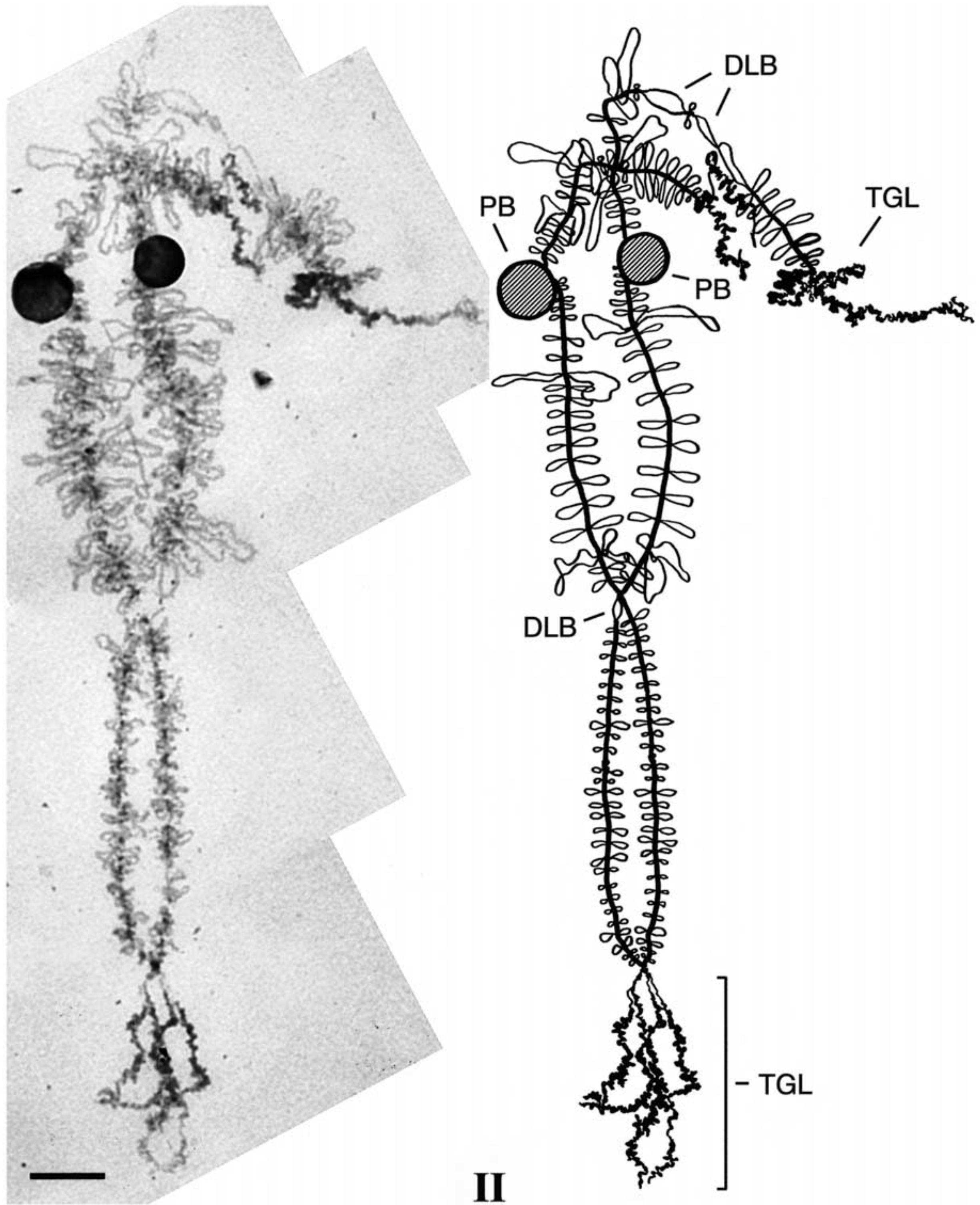


Figure 1. Representative chaffinch chromosomes I–VII, Z-W, and some microchromosomes in the lampbrush phase. Chromosomes are stained with Coomassie blue R250. The light microscopic image of the macrobivalent (left) is accompanied by a drawing of the same chromosome (right). Double-loop bridges (DLBs), protein bodies (PBs), lumpy loop (LL), and telomere giant loops (TGLs) are indicated on the drawings. Scale bars = 10 μm.



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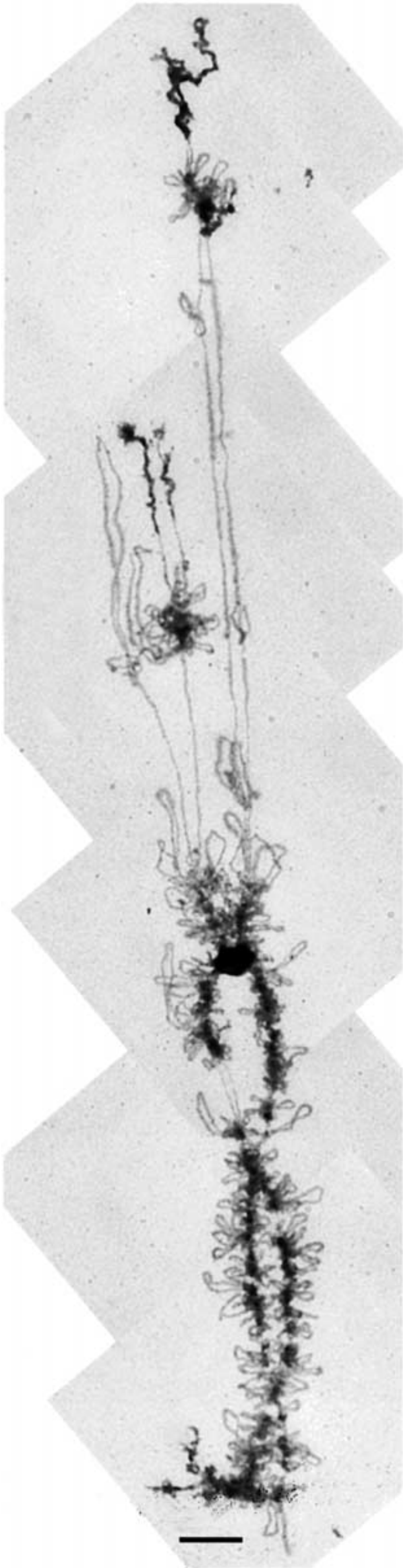

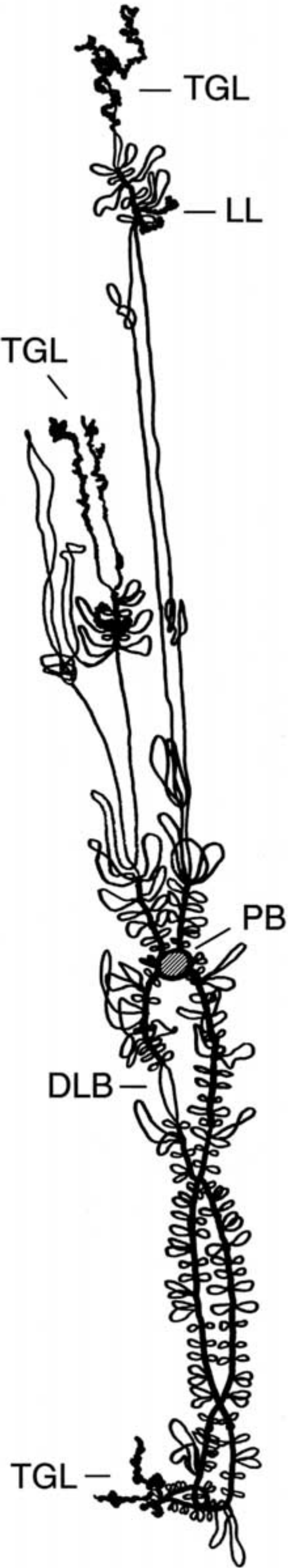

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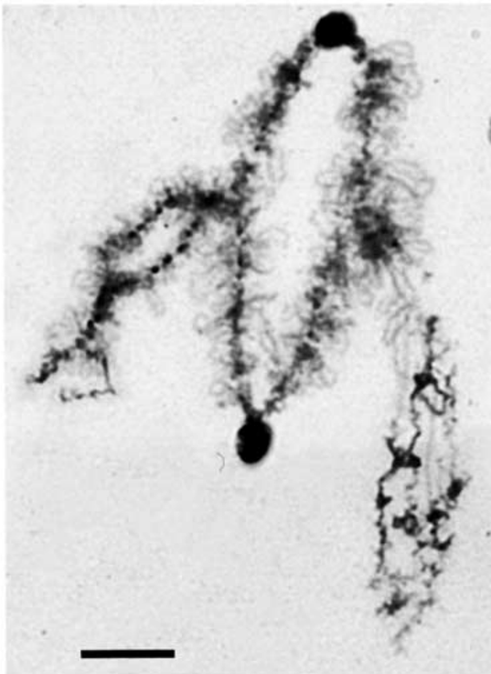

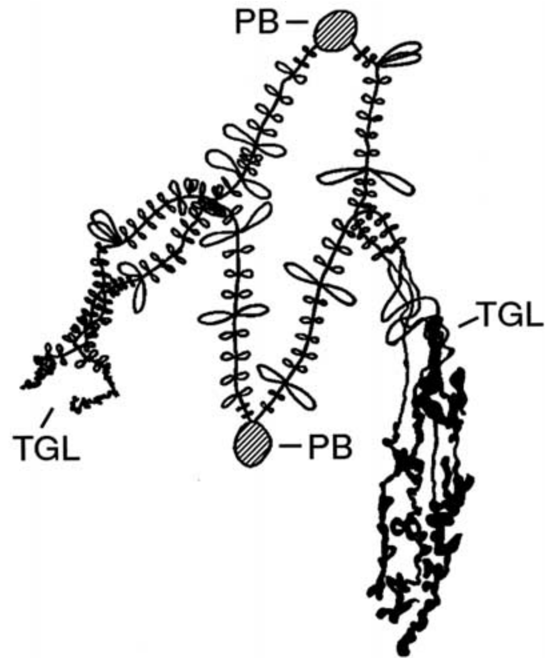

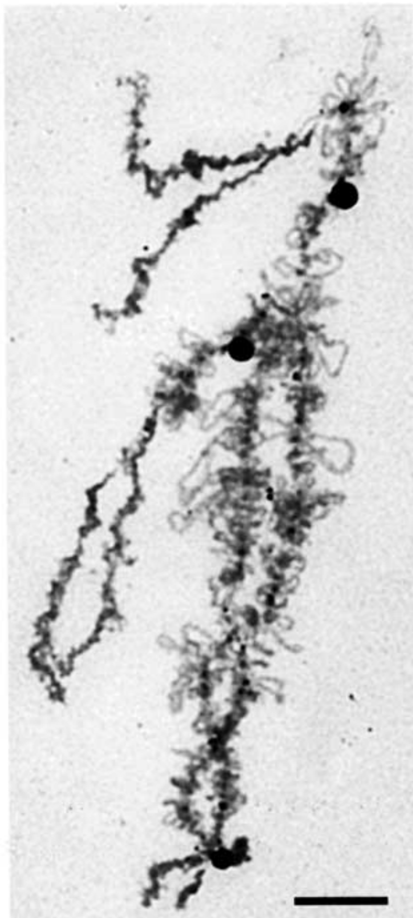

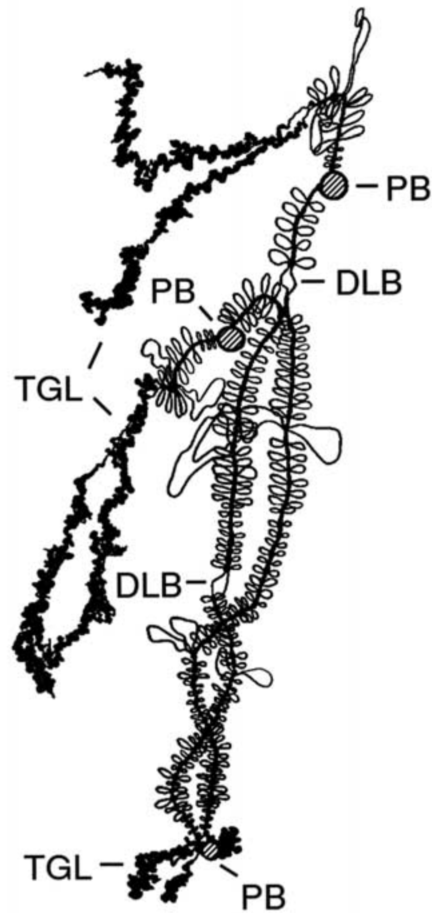

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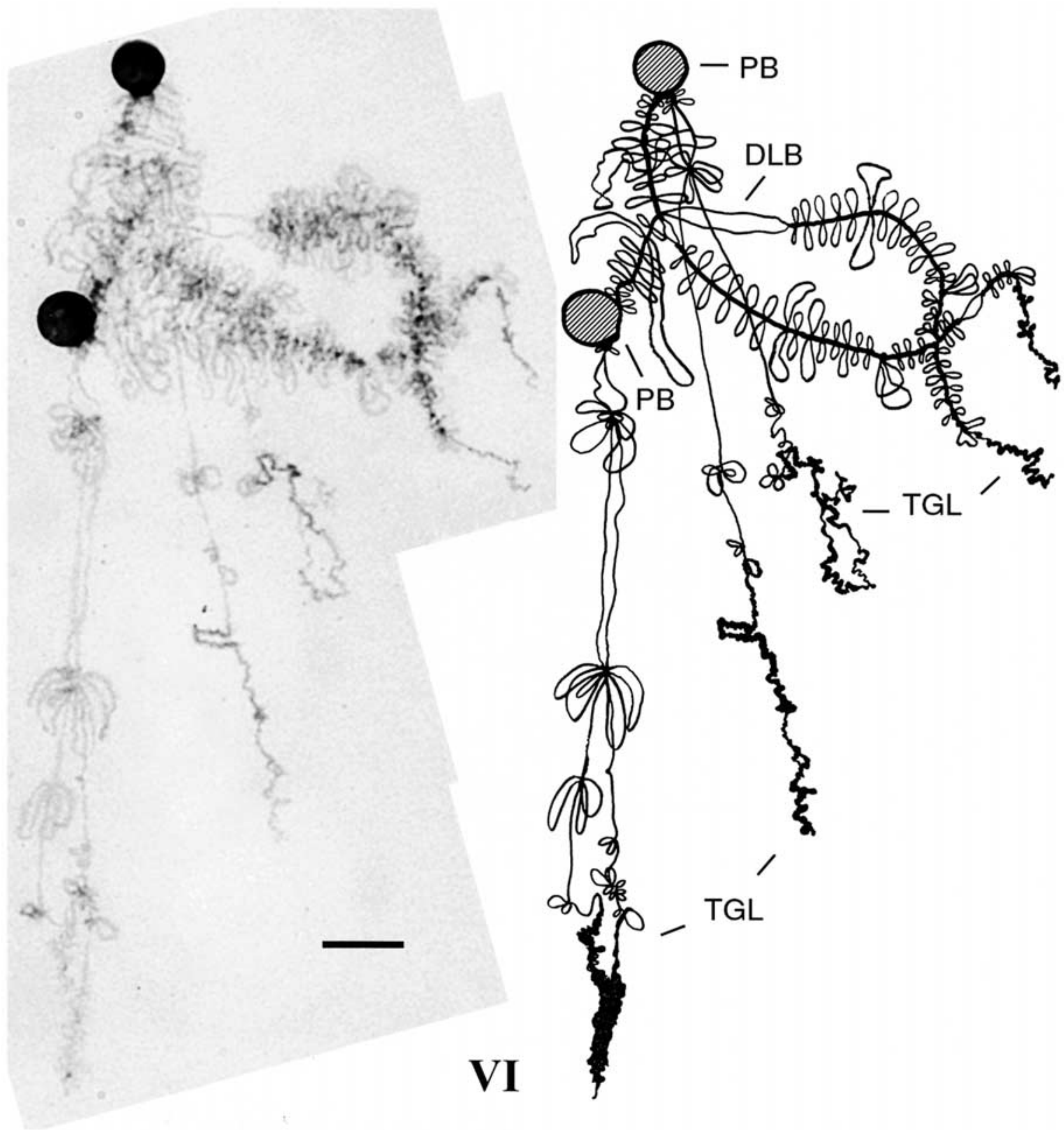

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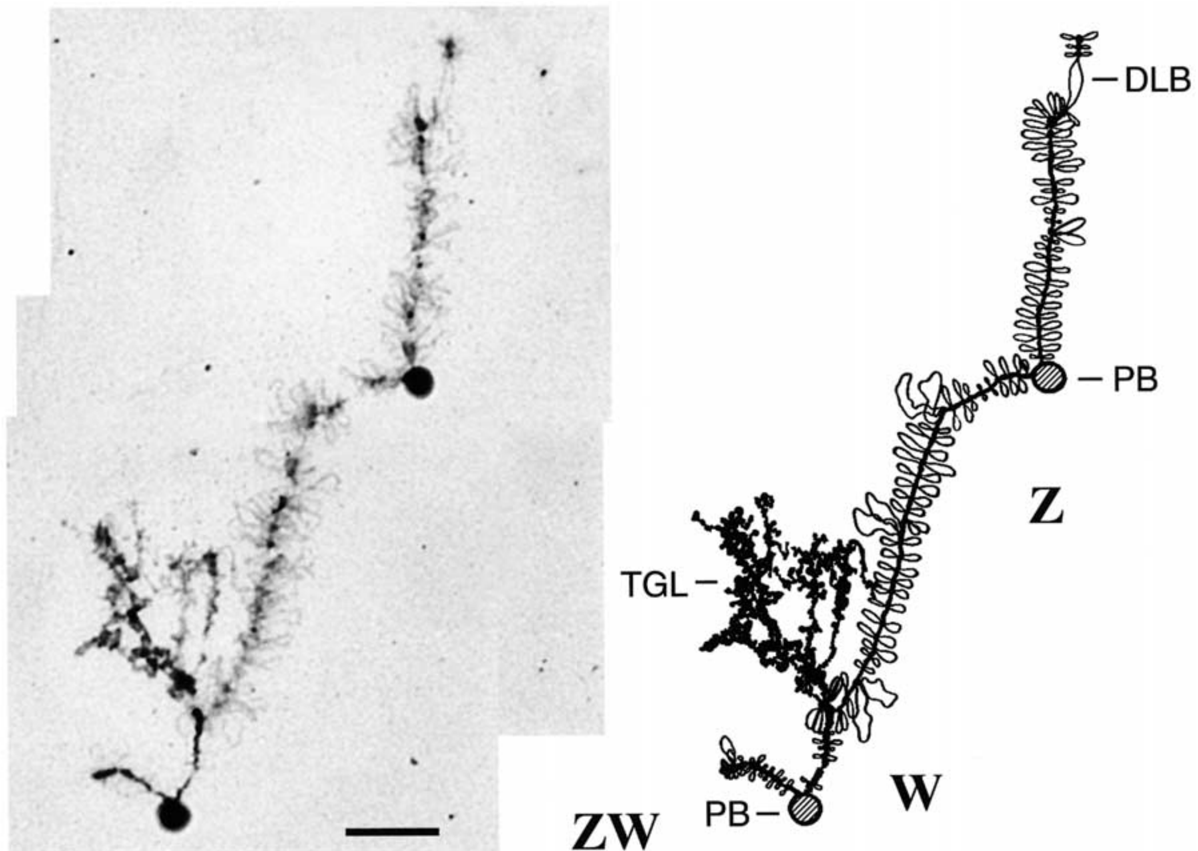
proteinase K by heating, DNA amplification was performed in 20 μl of reaction mix containing 1 U of Taq DNA polymerase, 2.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, and 20 pmol of FCP specific primers ZF1 and ZR2 described earlier (Saifitdinova *et al*. 2001). The reaction was performed in a thermocycler GENIUS (Techne) using the following settings: an initial DNA denaturation at 95°C for 5 min, then 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and the last elongation step at 72°C for 10 min. PCR products were analysed by electrophoresis through 1%



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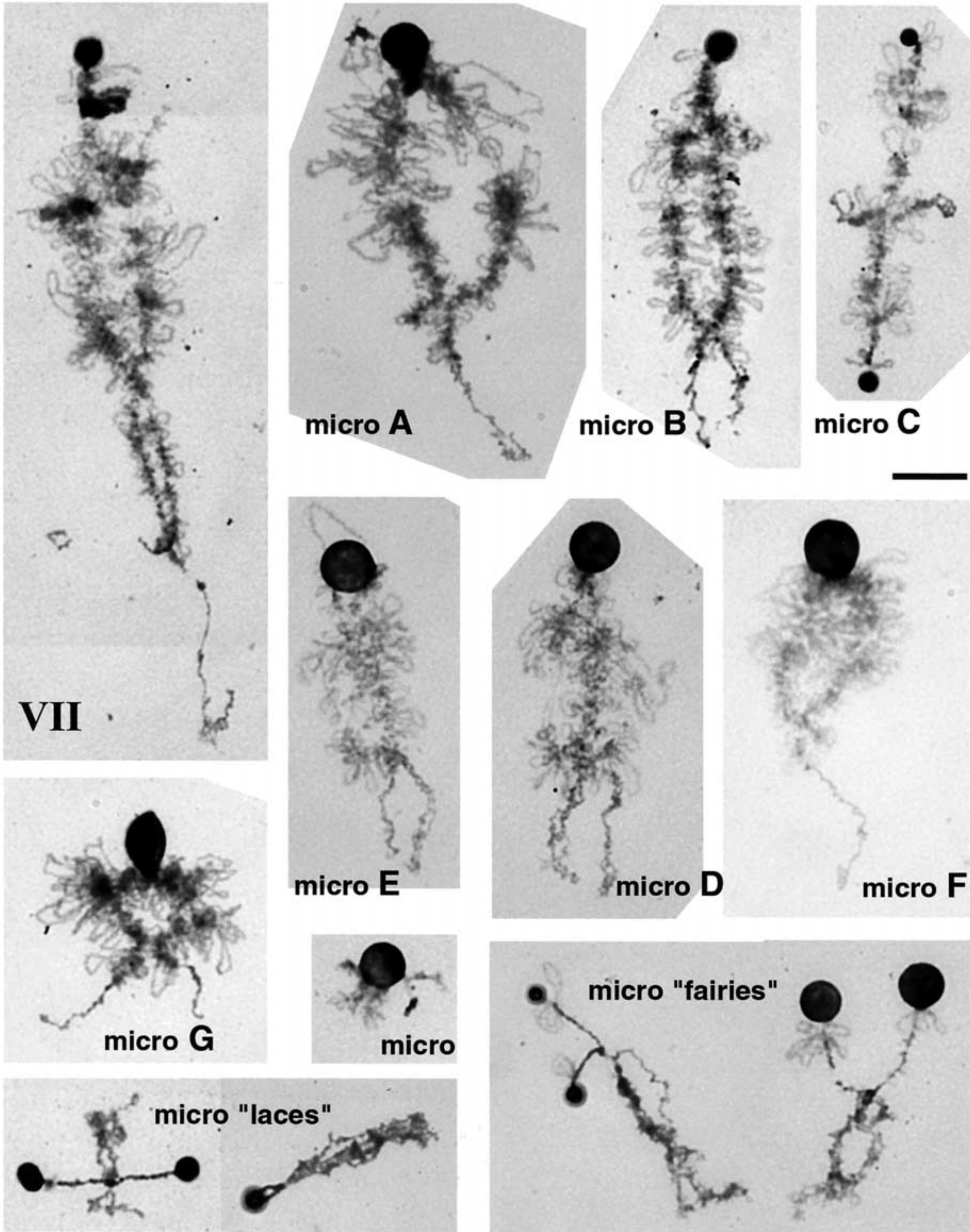
agarose gel. DNA was transferred from the gel onto Hybond N+ membrane (Amersham Pharmacia Biotech) and hybridized with digoxigenin-labelled FCP probe according to the standard protocol for Southern-blot hybridization. Digoxigenin was detected with antidigoxigenin conjugated to alkaline phosphatase (Roche) and the colorimetric detection reagents, NBT and BCIP.

Microscopy

Slides were examined using a DMRXA fluorescence microscope (Leica Microsystems Wetzlar GmbH, Germany) equipped with a FLUOTAR 100/1.30 objective, appropriate filter cubes and a black–white CCD camera (Cohu). QFISH software (Leica Cambridge Ltd.) was used to acquire and process multicolour microscopic images.

Results and discussion

During oocyte growth, the chromosomes of the chaffinch change to the typical lampbrush form as in other birds. The change affects both micro- and macrobivalents alike (Figure 1). The majority of the lateral loops on these lampbrush chromosomes (LBCs) conform to the 'simple' (according to Morgan 2002) or 'normal' (according to Callan 1986) type. Some of the simple loops may be very short measuring less than 1 μm from one end to the other (contour length) while some loops are exceptionally long (up to 60 μm in contour length). The most common size of simple lateral loops in chaffinch is about 15 μm contour length. Simple loops are enriched with the phosphorylated form of RNA-polymerase II (Figure 2A, D, F) and consist of units of polarized ribonucleoprotein (RNP) matrix corresponding to units of active RNA transcription.



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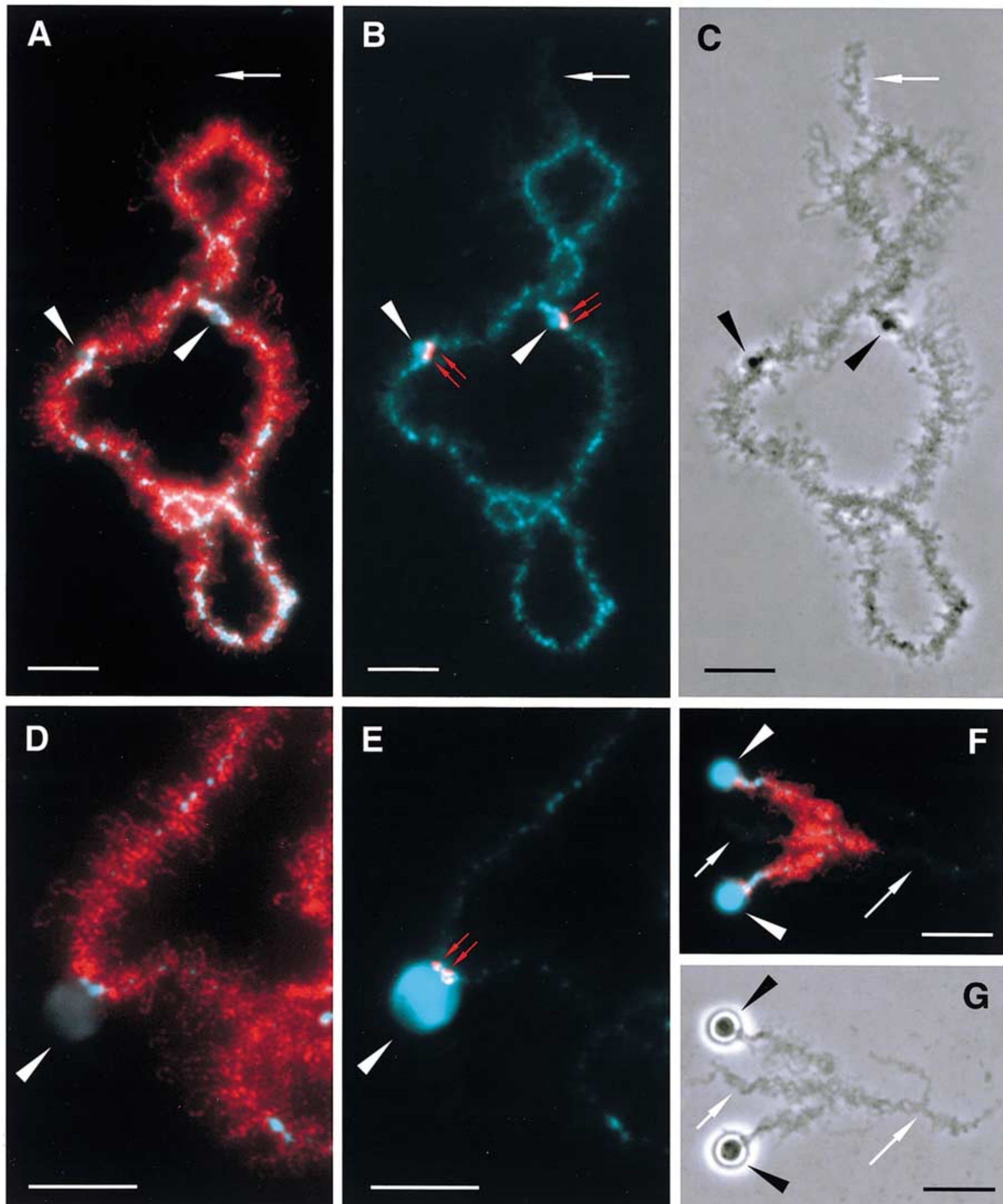

Figure 2. Chaffinch lampbrush bivalent I (**A**–**C**), fragment of chromosome I (**D**, **E**), and a microbivalent (**F**, **G**). (**A**, **D**, **F**) Immunostaining with mAb V22 (red fluorescence). (**B**, **E**) Fluorescent *in-situ* hybridization of FCP on the same preparations after washing off V22 immunofluorescent complexes. Chromosomes are counterstained with DAPI. (**C**, **G**) Phase contrast. Arrowheads indicate protein bodies; white arrows – TGLs; red arrows – FCP hybridization signals. Scale bars = 10 μm.

Telomere bow-like loops (TBLs), which are characteristic of LBCs in representatives of Galliformes (Chelysheva *et al*. 1990, Solovei *et al*. 1994), were not found on chaffinch LBCs.

Conspicuous pairs of complex loops are usual at the extreme ends of chaffinch lampbrush chromosomes. They have a very special morphology, which may be compared with that of telomere giant loops (TGLs) on chicken macrobivalents E, F, ZW and on the majority of microbivalents (Chelysheva *et al*. 1990, Solovei *et al*. 1994) and on lampbrush chromosomes of the pigeon (Solovei *et al*. 1996) as well as giant loops (GLs) on amphibian lampbrush chromosomes (Callan & Lloyd 1960). In contrast to simple loops, the TGLs of chaffinch lampbrush chromosomes (Figures 1 & 2C, G) do not bind antibodies H14 and V22 to the phosphorylated form of RNA-polymerase II (Figure 2A, F). This result suggests that they are not actively involved in RNA synthesis. In this respect, the TGLs resemble the so-called sequentially labelling loops described in amphibians (Snow & Callan 1969, Callan 1986, Eckmann & Jantsch 1999). These peculiar terminal loops are characteristic of practically all chaffinch bivalents, both macro- and microbivalents.

Other striking landmark structures on the LBCs of chaffinches are the so-called protein bodies (PBs), which are spherical entities of variable size, attached to the chromosomal axis (Figure 1). The PBs first appear on the chromosomes right at the beginning of chromosome transformation into the lampbrush form and they increase in size up to about 12 μm diameter during the lampbrush phase. At the end of the lampbrush stage, they fuse with one another but remain attached to the axes of the condensing chromosomes. This fusion results in the formation of a karyosphere (Figure 3). Similar PBs were also observed on lampbrush chromosomes in some other birds (Gaginskaya 1972, Solovei *et al*. 1993, 1996). In pigeon, the PBs are known to co-localize with the centromeric highly repeated sequence PR1 (Solovei *et al*. 1996). The centromeric location of PBs on chaffinch LBCs was verified by FISH using a centromeric highly repeated sequence FCP from the chaffinch genome as a probe (Saifitdinova *et al*. 2001). The FCP repeat was found to occupy a part of each chromatin block adjacent to PBs on every chromosome (Figure 2B, E). The formation of PBs in association with the centromeric repeat and their subsequent fusion to produce a karyosphere may

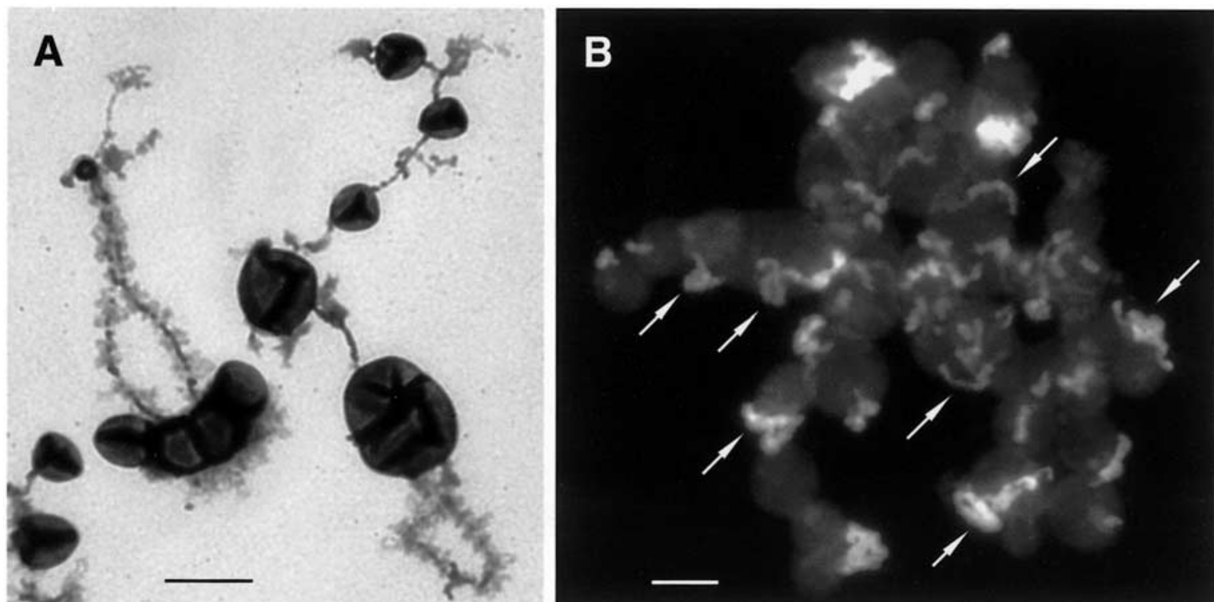

Figure 3. Sequential steps of PB fusion and chromosome condensation in chaffinch oocytes at the karyosphere formation. (**A**) the nucleus from late previtellogenic oocyte, Coomassie blue R250. (**B**) The nucleus from vitellogenic oocyte, DAPI. Arrows indicate condensed chromosomes on the surface of fusing PBs. Scale bars = 10 μm.

point to a special hitherto-unidentified function of centromeric regions concerned with the spatial organization of chromosomes in an oocyte nucleus.

Besides the obligatory centromeric PBs, facultative ones are occasionally observed in non-centromeric regions (Figure 1; bivalents I and V). As a rule, they are present at terminal regions of LBCs and have a smaller size than centromeric PBs. The additional PBs do not occur in all females and may be associated with only one homologue in a bivalent. Using microdissection followed by PCR analysis and blot hybridization of the PCR products with digoxigenin-labelled FCP we were able to prove that both centromeric PBs and facultative, non-centromeric, ones on chaffinch

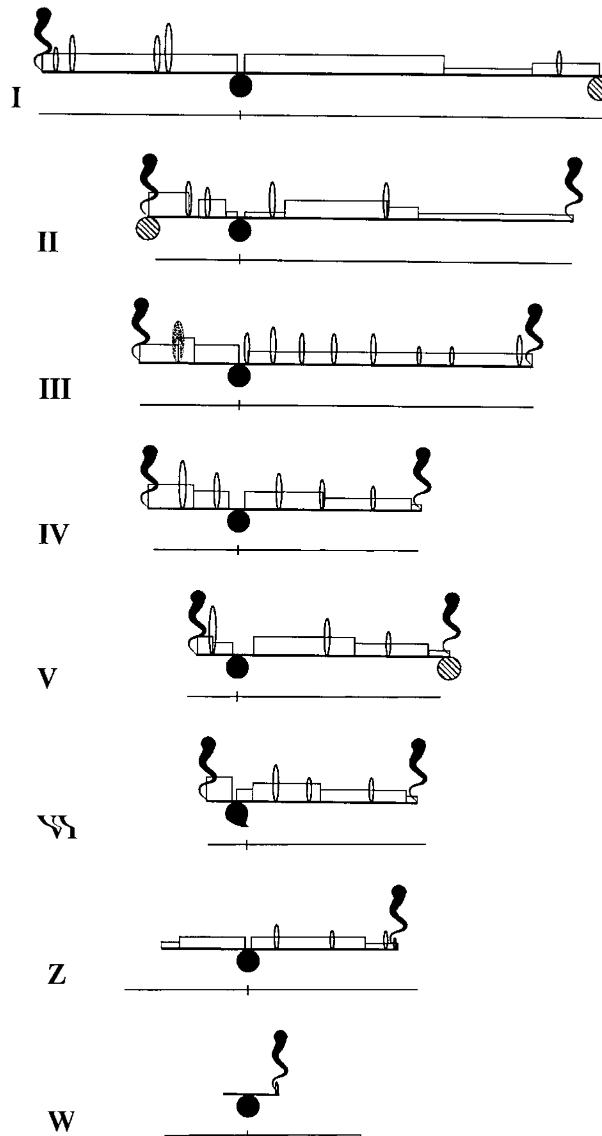

Figure 4. Working map of chaffinch lampbrush chromosomes representing macrobivalents I–VI and ZW bivalent. (●) centromere protein body; (◍) additional protein body; (ʃ) telomere giant loop; (◊) lumpy loop; (0) marker simple loop; step line reflects average regional L-factor. The line under each of the LBC schemes shows the corresponding mitotic metaphase chromosome. Mitotic chromosome 1 is shown as extended to the length of the lampbrush chromosome I; other mitotic chromosomes are drawn to scale with mitotic chromosome 1; vertical bar on the line shows position of centromeric region.

LBCs are co-localized with the FCP repeat (data not shown).

In morphology, the PBs on chaffinch LBCs resemble sphere organelles (Cajal bodies) on amphibian LBCs (Wu *et al.* 1991, Gall 2000, Morgan 2002). However immunocytochemical investigations did not reveal snRNPs and other indicators of Cajal bodies within chaffinch PBs (unpublished observations). Organelles corresponding to the spheres on amphibian LBCs were not found in chaffinch oocytes.

Figure 1 is a set of photomicrographs of fixed and stained chaffinch lampbrush macrobivalents and some microbivalents, together with a diagram of each macrobivalent constructed according to the conventions established by Callan & Lloyd (1960) and Chelysheva *et al.* (1990), with due regard to the relative lengths of each of the chromosomes, the arrangement of regions bearing lateral loops of comparable lengths, the positions of protein bodies, loops of peculiar morphology and other landmark structures. It is worthy of note that, in birds, only macrochromosomes can be properly arranged in order according to their morphology and lengths. The microchromosomes are just small and too numerous for this to be possible. Nevertheless, we can distinguish some of them due to characteristic appearance. A few typical lampbrush microbivalents are shown in Figure 1. The microchromosomes' general features are as follows: most of them are acrocentrics with PBs on one end, PBs of homologues are often fused, and a few of the longer microbivalents have two chiasmata while the majority of micros have only one.

A 'working map' of chaffinch lampbrush macrobivalents is shown in Figure 4. As a general indicator of the state of 'loopiness' of chromosome regions, we use L factor, which is the average of distances from the chromosome axis to the loop inflexions over the regions (Vlad & Macgregor 1975). The chromosomes are arranged and numbered in order of their decreasing length. Correspondence in relative lengths of lampbrush macrobivalents and mitotic metaphase chromosomes in chaffinch was confirmed using FISH mapping of a repetitive sequence GS. The GS location pattern was previously shown to be a characteristic of each individual macrochromosome (Liangouzov *et al.* 2002). Figure 5 demonstrates the result of comparative mapping of GS on mitotic and meiotic chromosomes 1, 2 and 3. The macrochromosomes retain the proportions of mitotic metaphase counterparts when they change to the lampbrush form. The finding relates to both the chromosome arms and the whole chromo-

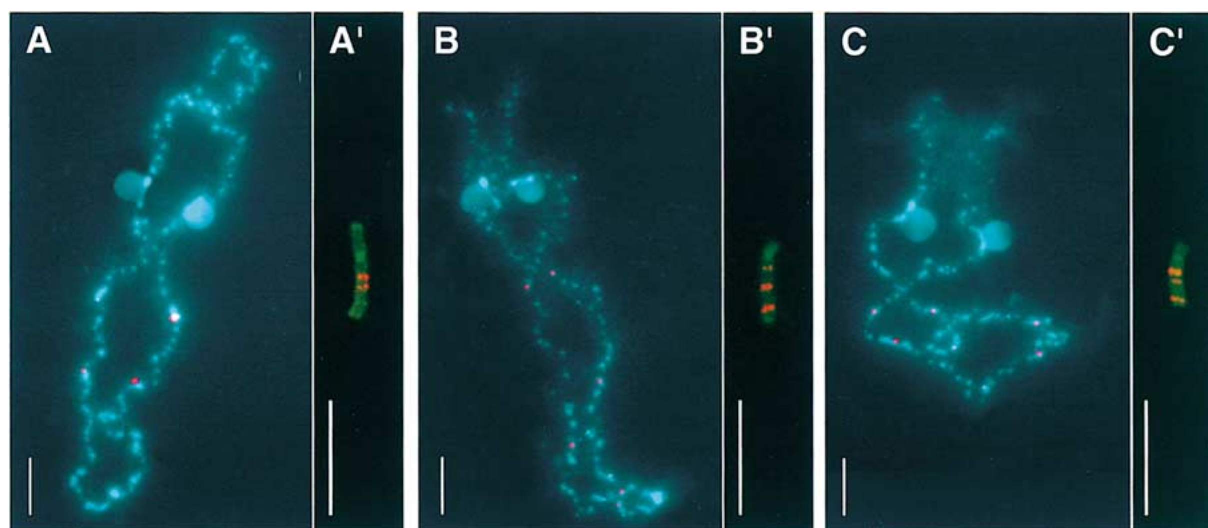

Figure 5. Fluorescence *in-situ* hybridization of GS sequence on chaffinch lampbrush chromosomes I (**A**), II (**B**), III (**C**) and corresponding mitotic chromosomes 1 (**A′**), 2 (**B′**), 3 (**C′**). FISH-signal is red (Cy3 fluorescence), lampbrush chromosomes are counterstained with DAPI, and mitotic chromosomes are counterstained with Chromomycin A_3. Scale bars = 10 μm.

somes (Figure 4). The only exceptions are sex chromosomes Z and W. They, in the lampbrush form, remain more condensed than other macrochromosomes although their centromere indexes appear to be about the same during mitosis and meiosis. This is especially defined on the W chromosome (Solovei *et al.* 1993, Figures 1 & 4).

It is shown here, on the sample of the chaffinch, that, notwithstanding the occurrence on avian LBCs of extended chromosome regions carrying either long loops and small chromomeres or short loops and large chromomeres, at least autosomal macrobivalents in the lampbrush form reveal an equal range of condensation/decondensation of chromosomal arms and whole chromosomes so that the LBC numbering corresponds to that of their mitotic counterparts.

Acknowledgements

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