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Precise identification of chicken chromosomes in the lampbrush form using chromosome painting probes

Svetlana Derjusheva,[1] Anna Kurganova,[1] Alla Krasikova,[1] Alsu Saifitdinova,[1] Felix A. Habermann[2] & Elena Gaginskaya[1]*
[1]*Biological Institute of St. Petersburg University, Oranienbaumskoie sch. 2, Stary Peterhof, St. Petersburg, 198504, Russia; Tel: (+7-812)4277311; Fax: (+7-812)4277310; E-mail: chromas@paloma.spbu.ru;*
[2]*Technical University of Munich, Freising-Weihenstephan, Germany*
**Correspondence*

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Abstract

Chromosome painting probes specific for macrochromosomes 1, 2, 3, 4, 5, and Z were applied to both mitotic and lampbrush chromosomes of the chicken (*Gallus gallus domesticus*). Five autosomal macrobivalents and sex chromosome Z in the lampbrush phase were identified and their correspondence to the target chromosomes in the metaphase of mitosis was shown. Nascent transcripts on lateral loops of the target lampbrush chromosome were intensively labelled when the hybridization was performed without RNase A treatment according to the DNA/(DNA + RNA) hybridization protocol.

Introduction

Lampbrush chromosomes (LBCs) are a form of eukaryotic chromosome organization, which is specific to chromosomes at the diplotene stage of meiotic prophase I in oogenesis. They are exceptionally long, being more than 30 times the length of the corresponding mitotic metaphase chromosomes in chicken. They have a distinctive chromomeric organization, with pairs of lateral loops associated with each chromomere. The lateral loops are sites of intensive RNA transcription. The chromomeres consist of compact chromatin (Callan 1986, Morgan 2002).

Notwithstanding many years of investigation into lampbrush structure and function, the biological significance of these chromosomes remains uncertain. Nevertheless, LBCs offer a valuable system for exploration into many problems of genome organization, function and regulation. Because of their giant size, LBCs, and particularly chicken LBCs, are a powerful tool for physical gene mapping (Solovei *et al.* 1994, Hori *et al.* 1996, Ogawa *et al.* 1997, Solovei *et al.* 1998, Itoh *et al.* 2001, Itoh & Mizuno 2002). However, working with LBCs, a researcher frequently encounters the problem of their precise identification and individual correspondence to mitotic chromosomes.

The only bivalent that can be unequivocally identified in its lampbrush form in chicken is the ZW sex bivalent. It looks like a single highly asymmetrical LBC and the W chromosome has only a few distinctive chromomeres and is strongly condensed compared to the Z (Solovei *et al.* 1993). Although Callan & Lloyd (1960) clearly demonstrated strong correlation of relative lengths and centromeric indexes between LBCs and mitotic chromosomes in crested newts, (*Triturus*, Urodela), there is no proper evidence that the ratio

of chromosome condensation/decondensation at the lampbrush stage is equal for all autosomes of the complement in the avian species.

Recent efforts at precise identification of certain avian LBCs were carried out by comparative mapping of some molecular markers. Chicken chromosome 1 in the lampbrush form was identified by fluorescence *in-situ* hybridization (FISH) with a telomeric TTAGGG probe (Rodionov *et al.* 2002) and lampbrush macrobivalents in chaffinch (*Fringilla coelebs*) were identified by FISH with an interstitial repetitive sequence GS (Saifitdinova *et al.* 2003).

In this work, we demonstrate that the problem of LBC identification can be easily solved using chromosome-specific painting probes. We were able to identify all macrobivalents in the chicken lampbrush complement by FISH with chicken macrochromosome paints (Griffin *et al.* 1999) and to show their definitive correspondence to chromosomes in metaphase of mitosis. We found that chromosome-specific probes hybridize intensively with the ribonucleoprotein (RNP) matrix on lateral loops of the target chromosomes when the hybridization is performed without RNase A treatment according to the DNA/(DNA + RNA) hybridization protocol.

Material and methods

Chromosome preparation

Chicken *Gallus gallus domesticus* LBCs were isolated manually from oocytes of 1.0–2.0 mm diameter according to the standard technique (Solovei *et al.* 1993, 1994, Saifitdinova *et al.* 2003). Preparations were dried from 96% ethanol before using for FISH. The preparations for immunostaining were not dried.

Mitotic metaphase chromosomes were obtained from chicken embryonic fibroblasts using conventional techniques.

Probes and competitors

Whole chromosome paints, generated by Griffin *et al.* (1999) from flow-sorted chicken chromosomes, were re-amplified and labelled with biotin-16-dUTP (Roche) or TAMRA-dUTP (Applied Biosystems) in a DOP-PCR reaction using the 6MW primer (Telenius *et al.* 1992). The labelled probes were dissolved in 50% formamide, 10% dextran sulphate and $2 \times$ SSC to a final concentration of 45–50 ng/μl with a 10–50-fold excess of chicken Cot-1 DNA. A 100-fold excess of chicken Cot-1 DNA was used in hybridization mixes containing painting probes for chromosome 1 applied to LBC preps. A 100-fold excess of plasmid pCZTH8, containing a fragment of the chicken Z chromosome macrosatellite (Hori *et al.* 1996), was used as additional competitor DNA for painting of the Z LBC. The plasmid was kindly donated by Irina Solovei.

Fluorescence in-situ *hybridization (FISH)*

Chromosome painting on metaphase spreads was performed as described elsewhere (Habermann *et al.* 2001). Slight modifications of the procedure were made for painting on the LBCs. LBCs were pretreated with pepsin and Triton X100 as reported previously (Saifitdinova *et al.* 2003). Two variants of *in-situ* hybridization were carried out: (1) according to the DNA/DNA hybridization protocol, chromosomes were pretreated with RNase A and denatured; and (2) according to the DNA/(DNA + RNA) hybridization protocol, chromosomes were denatured without a preceding RNase A treatment. LBCs were denatured in 70% formamide/$0.6 \times$ SSC at 70–72°C for 3 min. Painting probes were denatured and then pre-annealed at 37°C for 20–30 min. In the case of chromosome 1 paint, the pre-annealing time was varied from 20 min up to 3 h. Avidin-Cy3 (Jackson ImmunoResearch Laboratory) was used to detect biotin-labelled probes. Chromosomes were counterstained with DAPI.

Immunostaining

Immunostaining of chicken LBCs was carried out with the following antibodies: mouse mAbs K121 against the trimethylguanosine cap present on most splicing snRNAs (Oncogene Research Products), Y12 against the Sm core proteins found on most splicing snRNPs (Lerner *et al.* 1981), anti-SC35 against the SR splicing factor SC35 (Fu & Maniatis 1990), H14 (BAbCO) and

V22 (kindly donated by U. Scheer) against the phosphorylated C-terminal domain (CTD) of RNA polymerase II, rabbit sera Sat3 and Sat4 against dsRNA adenosine deaminase (ADAR1; Eckmann & Jantsch 1999), and anti-RPC39 against subunit RPC39 of RNA polymerase III (Wang & Roeder 1997).

Slides with LBC spreads were blocked in PBS containing 1% blocking reagent (Roche) for 1 h at room temperature (RT). Then the slides were incubated for 1 h at RT in the primary antibodies listed above. Primary antibodies were applied in dilutions recommended by authors or manufacturers. Slides were washed with PBS and incubated for 1 h at RT in secondary antibody, which was either Cy3-labelled goat anti-mouse IgG + IgM or Cy2-labelled goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted according to manufacturer's recommendations. For double labelling, the same secondary antibodies were used simultaneously. The slides were again washed with PBS and mounted in DABCO antifade solution containing 1 μg/ml DAPI.

Microscopy

Preparations were examined using a Leica DMRXA fluorescence microscope equipped with a black-and-white Cohu CCD camera and appropriate filter cubes. Leica QFISH software was used to acquire and process multicolour images.

Results

The detailed cytological map of the chicken macrochromosomes at the lampbrush phase was constructed by Chelysheva *et al.* (1990). LBCs were designated according to size as A, B, C, etc. The suggested assignment of chicken lampbrush bivalents to mitotic metaphase chromosomes was not conclusive. Recently, the correspondence of lampbrush bivalent A to mitotic chromosome 1 has been confirmed by comparative mapping of TTAGGG sites (Rodionov *et al.* 2002). However, the correspondence between the remaining chicken LBCs and mitotic chromosomes remains uncertain.

We applied individual macrochromosome paints to both mitotic and lampbrush chromosomes of the chicken to demonstrate the correspondence between them. The technique allowed us to paint specifically the target chromosomes in both metaphase and lampbrush chromosome sets. The competitor DNAs and conditions of pre-annealing suitable for successful FISH painting of mitotic chromosomes were found to be appropriate for painting of LBCs 2–5 but not of LBC 1 and the ZW bivalent. Figure 1 demonstrates the result of comparative painting of LBCs and mitotic metaphase chromosomes 2, 3, 4 and 5. The bright fluorescence of lateral loops on LBC preparations that have not been treated with RNase A is due to probe hybridization with nascent RNA, which is abundant on lateral loops of LBCs.

Using the protocol for somatic cells without RNase A pre-treatment, the Z chromosome paint hybridized on LBCs only with telomere bow-like loops, lateral loops and chromomeres of heterochromatin regions. These regions were earlier shown to contain macrosatellite DNA transcribed intensively during the lampbrush stage (Hori *et al.* 1996). The pattern of hybridization with Z chromosome paint (not shown) was similar to the Z macrosatellite FISH pattern reported earlier (Hori *et al.* 1996). Increasing the chicken Cot-1 DNA up to 50-fold excess simultaneously with adding a 100-fold excess of the cloned fragment of chicken Z chromosome macrosatellite to the hybridization mix gave uniform painting of the whole Z chromosome in the lampbrush phase (Figure 2).

Without RNase treatment, only one pair of lateral loops on bivalent 1 preferentially hybridized under conditions suitable for metaphase chromosome painting with the probe for chicken chromosome 1, and the fluorescent signal on these loops was extremely bright (Figure 3A). The addition of a 100-fold excess of chicken Cot-1 DNA and increasing the pre-annealing time up to 3 h did not change the FISH pattern. The only way to eliminate the overfluorescence of these loops was to use LBCs pretreated with the RNase A. This procedure gave painted chromomeres along the entire length of bivalent 1 (Figure 3C).

Discussion

Comparative painting of metaphase and lampbrush chromosomes in the chicken definitely shows that lampbrush bivalents previously

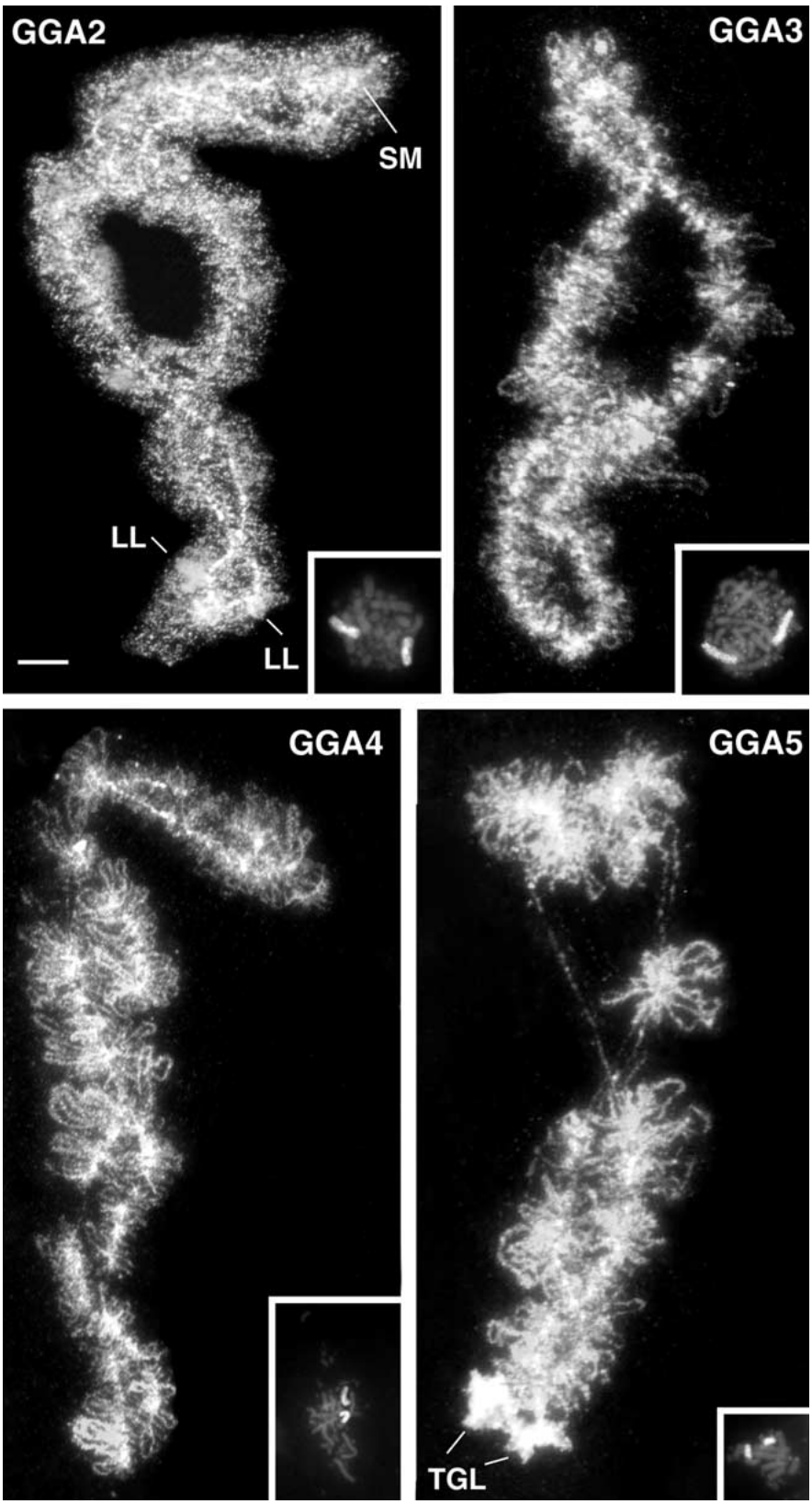


Figure 1. Chicken chromosomes 2–5 in the mitotic metaphase (inserts) and in the lampbrush phase painted with chicken chromosome-specific probes. Chromosome painting of LBCs was performed without RNase A treatment. Metaphase chromosomes are counterstained with DAPI. LL, lumpy loop; TGL, telomere giant loop; SM, spaghetti marker. Scale bar = 10 μm.

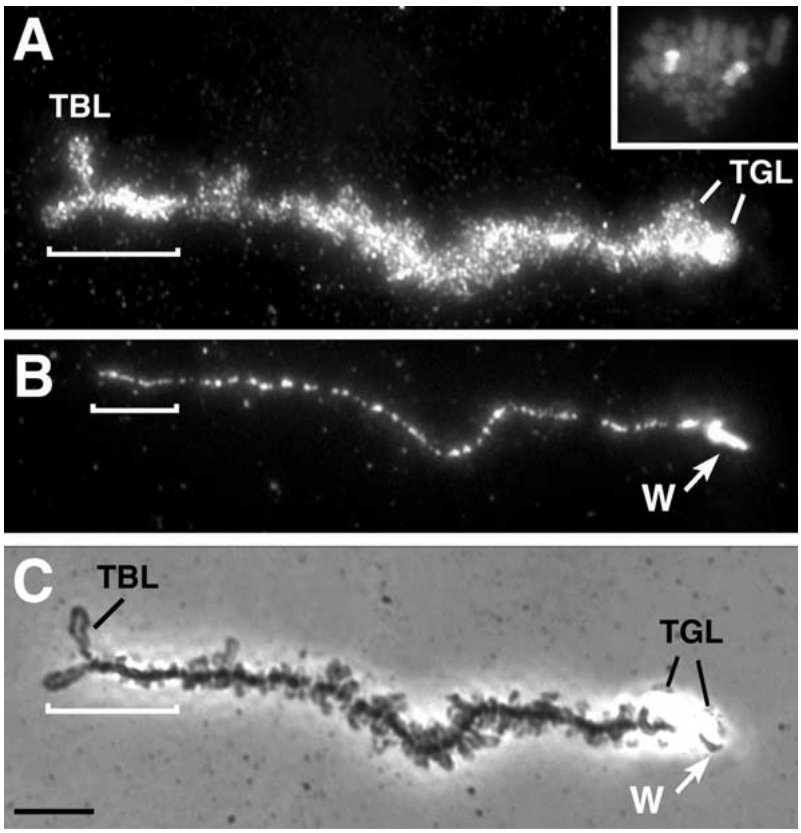
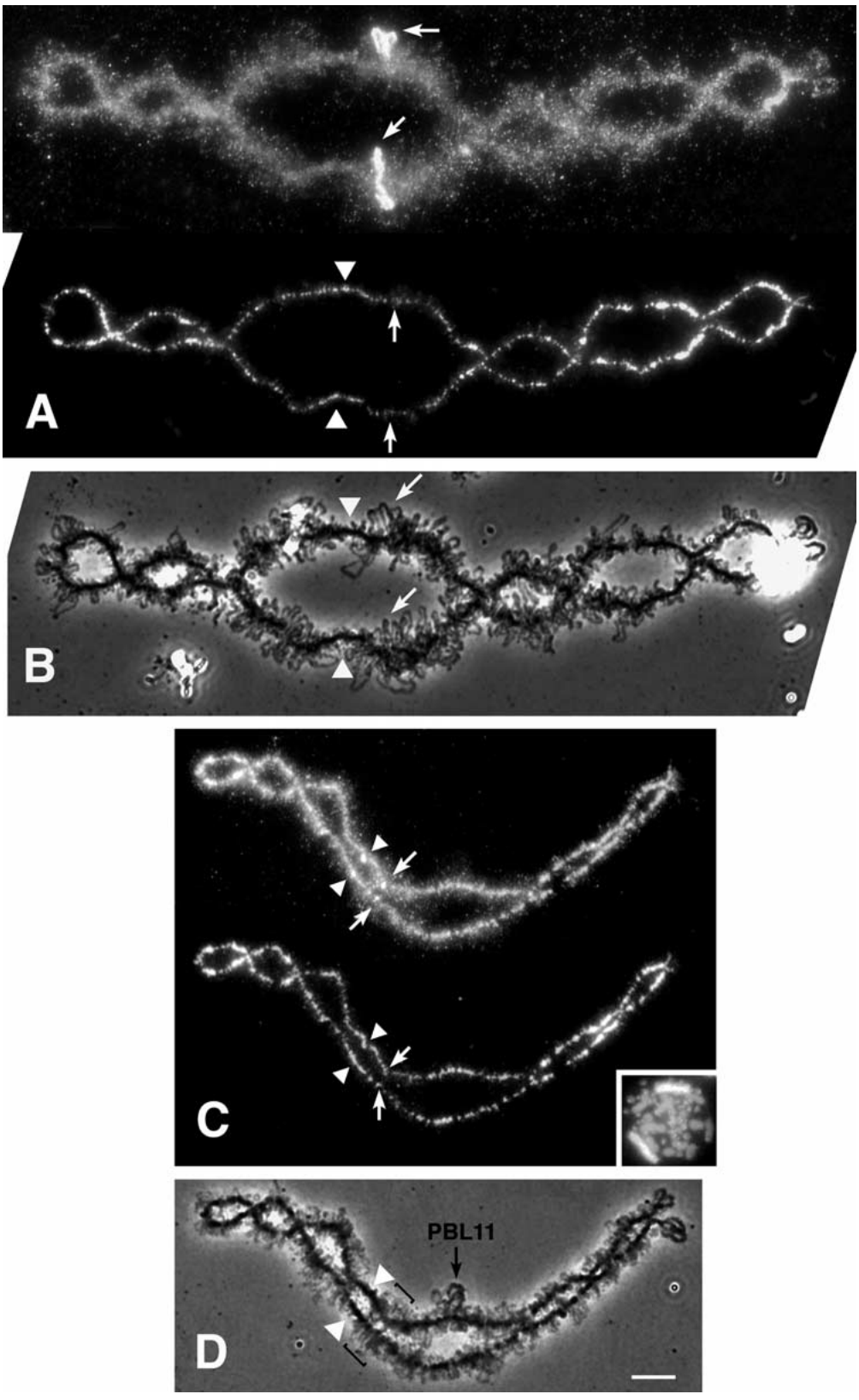


Figure 2. Chicken chromosomes Z in the male mitotic metaphase (insert) and in the ZW bivalent in the lampbrush phase painted with chicken chromosome Z probe. (**A**) Hybridization signal in the Cy3 channel. (**B**) DAPI staining. (**C**) Phase contrast. Chromosome painting of Z LBC was performed without RNase A treatment. Cot-1 DNA and a 100-fold excess of the cloned fragment of chicken Z chromosome macrosatellite were added to the hybridization mix applied to LBCs. All lateral loops and chromomeres intensely painted on the Z LBC. Telomere giant loops (TGLs) and the pseudoautosomal region on the W LBC also painted with chromosome Z probe. Chromosome painting of metaphase chromosomes was performed without additional competitor DNA. Metaphase chromosomes are counterstained with DAPI. Arrow indicates the chromosome W; bracket, the region containing the Z chromosome macrosatellite; TBL, telomere bow-like loops. Scale bar = 10 μm.

described as A, B, C, D and E (Chelysheva *et al.* 1990) represent chromosomes 1, 2, 3, 4 and 5, respectively. Our results demonstrate that the relative lengths of the autosomal macrochromosomes are the same in the lampbrush phase as in mitosis. The same conformity has been found recently in the chaffinch (Saifitdinova *et al.* 2003) and seems to be common in birds.

The special pair of loops on bivalent 1 revealed by FISH with the painting probe for chicken chromosome 1 requires special discussion. It is worth noting that these loops are not the so-called PBL11 loops (Figure 3D), which were mapped at the same region and shown to bind C-rich single-stranded nucleic acids(Solovei *et al.* 1995). Our data suggest that the painting of LBC loops in general is determined by RNA. Thus we suggest that the special loop on chicken LBC 1 must contain an unusually high concentration of RNA, perhaps due to the transcription of repetitive sequences, which dramatically raises the target for the hybridization probe, as in the case of Z chromosome macrosatellite noted above. Indirect evidence for this suggestion is the pattern of painting of chromomeres after RNase A treatment (Figure 3C). The brightness of hybridization signals on chromomeres corresponds directly to the intensity of DAPI staining of the same chromomeres. Exceptions are two chromomeres in the presumed centromeric region of LBC 1 and the small chromo-


A
B
C
PBL11
D

mere from which the special loops extended. Because these chromomeres hybridize more intensively than expected on the basis of their DAPI staining (Figure 3C), we presume they contain repetitive DNAs. Unfortunately, no repetitive DNA has been mapped so far to the region of chicken chromosome 1 marked with the special loop.

The transcriptional activity of the special loops on LBC 1 was confirmed by immunostaining with mAbs H14 and V22 directed against the phosphorylated CTD of RNA polymerase II (Pol-II). The delicate signal seen along the axis of the special loop on chicken LBC 1 suggests that Pol-II-dependent transcription occurs on this loop (Figure 4B). On the other hand, knowing that RNase treatment removes both RNAs and RNA-associated proteins from LBC loops, we cannot exclude that paint 1 binds also with a specific protein component of RNP on the special loops when a hybridization procedure is carried out without RNase treatment.

Although the loops on chicken LBC 1 display quite unusual painting characteristics, they hardly differ from the majority of transcribing loops when viewed by phase contrast microscopy (Figure 3B, D). At the same time, they are the only loops on chicken LBC 1 that stain brightly with sera Sat3 and Sat4 against double-stranded RNA adenosine deaminase (ADAR1) (Figure 4A, C). To confirm the identity of the special loops, we performed FISH with the chromosome 1 painting probe on the same preparation after each immunostaining experiment.

The pattern of ADAR1 localization reminded us of the 'special loop' on *Xenopus* bivalent 3 (Eckmann & Jantsch 1999). To test the suggested similarity of the special loops on chicken and *Xenopus* LBCs, we performed double immunofluorescence staining with Sat3 serum and mAbs K121, Y12 or anti-SC35, all three of which are directed against splicing components and stain most lateral loops, including brilliant staining of the ADAR1 'special loop' in *Xenopus* (Eckmann & Jantsch 1999). In our experiments, mAbs K121, Y12 and anti-SC35 labelled the special loops on chicken LBC 1 with the same intensity as they labelled all regular loops (Figure 4D). Additionally, in contrast to the *Xenopus* special loop, which shows no detectable signal with antibodies against Pol-II, the special loop on chicken LBC 1 revealed the presence of a phosphorylated form of Pol-II (Figure 4B). Surprisingly, the special loop on chicken LBC 1 also showed bright staining with an antibody against subunit RPC39 of RNA polymerase III (Figure 4E). However, typical Pol-III LBC loops are well-known to have an undetectable RNP matrix (Morgan 2002, Murphy *et al.* 2002). The special loops on chicken LBC 1 have a thin-to-thick RNP matrix (Figures 3B & 4) which contains snRNPs and SC35 protein that are typical of Pol-II loops. For this reason, it is unlikely that Pol-III occurs on these loops. Instead, the antibody RPC39 probably crossreacts with an unrelated protein in the loop matrix. Similar behaviour is known, for instance, for certain loops on *Triturus* LBCs that show intense matrix staining with mAb 8WG16 against the non-phosphorylated Pol-II CTD, presumably because a matrix component shares an epitope with the Pol-II CTD (Morgan 2002). The fact that transcriptionally active special loops on chicken LBC 1 stain intensively with two polyclonal sera against ADAR1 merits attention. It is tempting to assume that they are involved in ADAR catalysed deamination of adenosines to inosines, which is well known to be important for RNA processing *in vivo* and presumably required during early embryogenesis (Gerber & Keller 2001).

Interestingly, with the exception of the special loops on LBC 1, other remarkable structures on chicken LBCs, such as lumpy loops, telomere giant

Figure 3. Chicken chromosome 1 in the mitotic metaphase (insert on **C**) and in the lampbrush phase (**A–D**). (**A**) FISH with chicken chromosome 1 paint on LBC 1 without RNase A treatment: hybridization signal in the Cy3 channel (top image) and DAPI staining (bottom image). The single pair of lateral loops brightly stains with chromosome 1 paint even after increasing the Cot-1 DNA and the pre-annealing time. (**C**) FISH with chicken chromosome 1 paint on LBC 1 treated with RNase A: hybridization signal in the Cy3 channel (top image) and DAPI staining (bottom image). All chromomeres on LBC 1 are painted with chromosome 1 paint. Two chromomeres in the predicted centromeric region and the 'special loop' chromomere look unexpectedly bright. (**B, D**) Phase contrast images of the same lampbrush chromosomes before FISH. Black arrow shows PBL11 marker loop; white arrows, 'special loop' or 'special loop' chromomere; bracket on **D** shows the region of the 'special loop'; arrowheads, centromeric regions. Metaphase chromosomes are counterstained with DAPI. Scale bar = 10 μm.

loops, and spaghetti marker (Solovei *et al.* 1992), did not reveal any unusual characteristics after FISH with chromosome painting probes (Figures 1 & 2).

In conclusion, chicken LBCs are a promising system for investigating principles of genomic structure and function. The gene map of the chicken is now well developed. Transferring this map to the LBCs will provide an opportunity to explore gene function and to better understand lampbrush loops themselves.

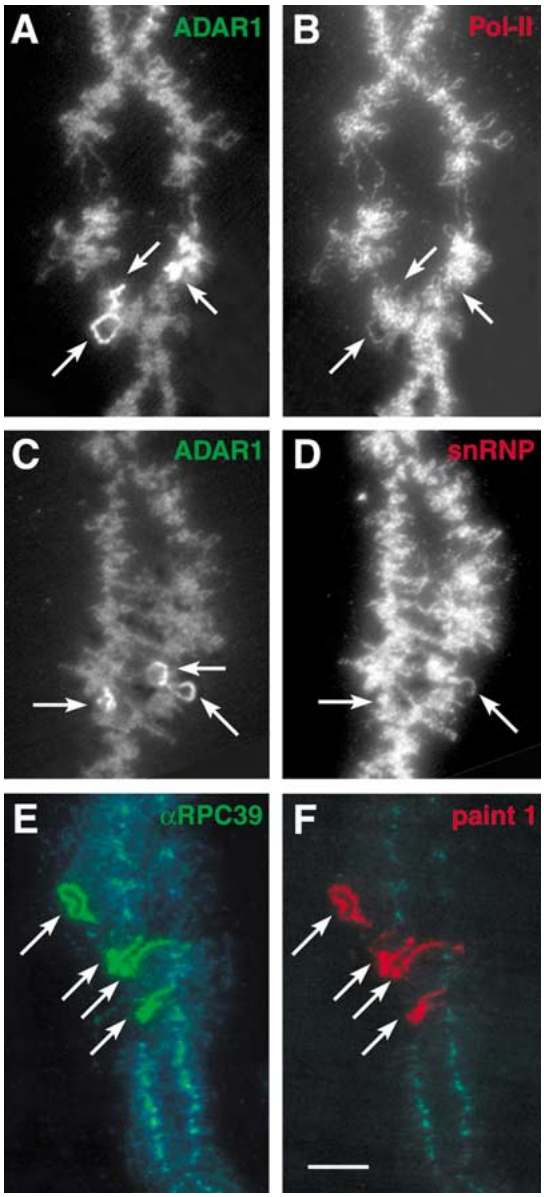


Figure 4. The 'special loop' on chicken lampbrush chromosome 1. (**A, B**) Double staining with serum Sat3 against ADAR1 in the Cy2 channel (**A**) and mAb V22 against the phosphorylated CTD of Pol-II in the Cy3 channel (**B**). (**C, D**) Double staining with serum Sat3 in the Cy2 channel (**C**) and with mAb Y12 against the Sm epitope of snRNPs in the Cy3 channel (**D**). Double staining of the 'special loop' with serum Sat3 and either mAb K121 against the trimethylguanosine cap or anti-SC35 against SR protein SC35 are similar. ADAR1 is localized to lateral loops and is enriched on the special loops (**A, C**). Staining with mAb V22 shows the presence of RNA Pol-II on all regular loops including the special loops (**B**). The Pol-II transcriptionally active loops are decorated with splicing components, in particular staining with mAb Y12 indicates the presence of Sm epitope on splicing snRNPs (**D**). (**E, F**) Immunostaining with a polyclonal serum against RPC39 (**E**) followed by FISH with chromosome 1 paint without RNase A treatment (**F**); LBC 1 is counterstained with DAPI. Arrows indicate the 'special loops'. Scale bar = 10 μm.

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