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On the positions of centromeres in chicken lampbrush chromosomes

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Abstract

Using immunostaining with antibodies against cohesin subunits, we show here that cohesin-enriched structures analogous to the so-called centromere protein bodies (PB) are the characteristic of galliform lampbrush chromosomes. Their centromeric location was verified by FISH with certain DNA probes. PB-like structures were used as markers for centromere localization in chicken lampbrush chromosomes. The gap predicted to be centromeric in current chicken chromosome 3 sequence assembly was found to correspond to the non-centromeric cluster of CNM repeat on the q-arm of chromosome 3; the centromere is proposed to be placed at another position. The majority of chicken microchromosomes were found to be acrocentric, in contrast to Japanese quail microchromosomes which are biarmed. Centromere cohesin-enriched structures on chicken and quail lampbrush microchromosomes co-localize with pericentromeric CNM and *Bgl*II− repeats respectively. FISH to the nascent transcripts on chicken lampbrush chromosomes revealed numerous non-centromeric CNM clusters in addition to pericentromeric arrays. Complementary CNM transcripts from both C- and G-rich DNA strands were revealed during the lampbrush stage.

Introduction

The domestic chicken (*Gallus gallus domesticus*) is widely used as a model organism for biomedical research. Because of its importance the chicken genome is now one of the best-investigated bird genomes. Available resources include a consensus genetic map with more than 2000 markers (http://acedb.asg.wur.nl/), BAC libraries representing several genome-equivalents organized in physical maps (http://www.bioinformatics.nl/gbrowse/cgi-bin/gbrowse/, http://www.animalsciences.nl/cmap/), the first draft of the chicken genome sequence (Schmid *et al.* 2000, 2005, ICGSC 2004), and the complete description of the chicken karyotype (Masabanda *et al.* 2004). The chicken karyotype (2n = 78) consists of 11 pairs of macrochromosomes, including autosomes 1–10 and gonosomes ZZ/ZW, and 28 pairs of chromosomes called microchromosomes (Masabanda *et al.* 2004). The smallest chromosome has a size of about 3.4 Mb (Pichugin *et al.* 2001).

Notwithstanding the progress of the research, the chicken genome sequence still contains numerous gaps. The DNA sequences in centromeric regions are unknown and are therefore represented by gaps in the current chicken chromosome sequence assembly (accessible at http://www.ensembl.org, http://www.ncbi.nih.gov, http://genome.ucsc.edu/cgi-bin/hgGateway). Since any chicken chromosome sequence contains a number of gaps at the boundaries of the sequence forming supercontigs, the positions of centromeric and non-centromeric satellite arrays are not clear. For a number of microchromosomes (chromosomes 25, 29–31, 33–38) no sequence information is yet available.

Moreover, on metaphase preparations, even in the case of the largest microchromosomes, the position of primary constriction is undetectable. Integration of physical mapping and sequence analyses represents a powerful cytogenomic approach (Romanov *et al.* 2005). Cytogenetic analysis at higher resolution level would provide essential information about the regions absent from the sequence maps of chicken chromosomes including centromeres, telomeres and other heterochromatic regions enriched with tandem repeats.

Avian chromosomes in the lampbrush form, which develop during the diplotene stage of the meiotic prophase I in females, represent an excellent system for high-resolution cytogenetic analysis. Active transcription in avian pre-vitellogenic oocytes leads to the decondensation of the chromatin and formation of the greatly elongated lampbrush chromosomes (LBC), so called because of their fuzzy appearance produced by hundreds of laterally projecting loops. The chicken chromosome length increases more than 30-fold between the mitotic metaphase and lampbrush stages and between pachytene chromosomes and LBC – up to 10-fold (Chelysheva *et al.* 1990). Each LBC represents a succession of chromomeres (condensed chromatin) with one or more pairs of lateral loops (transcriptionally active chromatin) extended from each chromomere (reviewed in Callan 1986, Macgregor 1986, 2002, Morgan 2002).

Chicken lampbrush chromosomes are well characterized (Hutchison 1987, Chelysheva *et al.* 1990, Solovei *et al.* 1993, 1994, 1998, Galkina *et al.* 2005, Schmid *et al.* 2005). In particular, the correspondence of chicken LBC 1–6, Z and W and the corresponding metaphase chromosomes has been confirmed (Solovei *et al.* 1993, Derjusheva *et al.* 2003, Galkina *et al.* 2006). Cytogenetic maps for chicken LBC 1–6, Z and W are published (Chelysheva *et al.* 1990, Galkina *et al.* 2006). In contrast to mitotic metaphase preparations, on LBC spreads individual microbivalents can be recognized by unique chromomere and loop patterns. Notwithstanding the detailed descriptions of chicken LBC, centromeric regions have not been unequivocally located on them.

In many urodele species (Amphibia), centromeres on LBC are marked by elongated and loopless chromomeres called centromere or axial bars by Callan (1986). Until now certain bar-like regions lacking lateral loops were considered as possible indicators of centromere regions on chicken LBC (Chelysheva *et al.* 1990, Schmid *et al.* 2005). However, some chicken LBC do not have prominent axial bars while others have several loopless bars. For example a prominent loopless region on LBC3, predicted as centromeric, has recently been shown by FISH mapping of BAC clones to represent the distal region of 3q (Galkina *et al.* 2006). So centromere positions on most chicken LBC remain unknown.

Information on chicken centromere-specific sequences is sparse. Only two repeats from chicken genome are known to be centromeric satellites. One is the partially inverted satellite repeat (PIR) that localizes at the centromere region of chromosome 8 (Wang *et al.* 2002). The other tandem repeat – CNM – is proposed to have centromeric localization on a number of microchromosomes and two intermediate chromosomes (Matzke *et al.* 1990, Wang *et al.* 2002, Romanov *et al.* 2005). Many recent efforts, including Cot-based sequencing did not reveal any new satellites of centromere localization within the repetitive landscape of the chicken genome (Wicker *et al.* 2005). The aim of the present work was to localize the centromeres on chicken LBC using molecular markers other than DNA sequences.

Our approach is based on information about LBC centromeric regions of other avian species. The centromeric regions of pigeon and chaffinch LBC were shown to be marked with prominent spherical structures called protein bodies – PB (Gaginskaya 1972, Solovei *et al.* 1996, Saifitdinova *et al.* 2003). In Galliformes – in particular in chicken, quail and turkey – similar prominent spherical bodies on LBC have not been described at light microscopic level (Kropotova & Gaginskaya 1984, Hutchison 1987, Chelysheva *et al.* 1990, Myakoshina & Rodionov 1994, Rodionov & Chechik 2002, Schmid *et al.* 2005). Recently we have shown that centromere PB in non-galliform species comprise certain chromosome structural proteins, including cohesin subunits SMC3, SMC1α, Rad21, STAG1 and STAG2 (Krasikova *et al.* 2005). Keeping in mind that in mammals cohesin complex proteins are enriched at chromosome centromeric regions and proposed to be involved in joining of sister kinetochores during late meiotic prophase I (Parra *et al.* 2004, Prieto *et al.* 2004), we applied antibodies against cohesin subunits to galliform LBC. This approach allowed us to detect PB-like agglomerates of cohesin subunits on lampbrush bivalents of chicken and Japanese quail (*Coturnix coturnix japonica*). We argue that these sites are strong candidates for universal markers for centromere positions on lampbrush chromosomes.

Materials and methods

Chromosome preparation

Chicken (*Gallus gallus domesticus*, GGA) and Japanese quail (*Coturnix coturnix japonica*, CCO) LBC were isolated manually from oocyte nuclei according to the standard technique (Solovei *et al.* 1993, 1994, Macgregor 2002, Saifitdinova *et al.* 2003). Adult females were bought from commercial stocks. Preparations were dehydrated in 96% ethanol and air-dried before using for FISH. Preparations for immunostaining were never dried. Full sets of chicken LBC were analysed. Mitotic metaphase chromosomes were obtained from chicken embryonic fibroblasts using conventional techniques.

Immunofluorescent staining

Immunostaining of chicken and quail LBC was carried out as previously described (Krasikova *et al.* 2005) with the following rabbit polyclonal antibodies: K853 and K854 against Rad21, and K828 against STAG2 (Prieto *et al.* 2004). After immunostaining and image acquiring some LBC preparations were used for FISH. In this case slides were washed in 2 × SSC at 42°C, dehydrated in ethanol and air-dried before applying FISH probes.

DNA probes

For identification of chicken LBC and certain LBC regions, the following probes were used: (1) whole chromosome paints specific for individual chromosomes 7–10 and the two largest microchromosomes (chromosomes 11 and 12) (Griffin *et al.* 1999); (2) chicken BAC clones bW043G06 (1p11), bW025G16 (1q11), bW026B13 (2p12), bW014J06 (2q11), bW029L12 (3pter) and bW125P16 (4p11) from the Wageningen chicken BAC library (Crooijmans *et al.* 2000, http://www.zod.wau.nl/vf/); their chromosome position and microsatellite markers are described in Galkina *et al.* (2006). BAC and paints were labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche). The labelled probes were dissolved to a final concentration of 10–50 ng/μl in hybridization buffer (50% formamide, 2 × SSC, 10% dextran sulphate) with 10–50-fold excess of chicken Cot-1 DNA and/or salmon sperm DNA.

Full-length repeated unit of centromeric satellite PIR specific for chicken chromosome 8 (Wang *et al.* 2002) was amplified from chicken genome DNA by a PCR reaction with a primer (5′-GTTTGTTGTTTTGGTTGC CGTTT-3′). Chicken W specific *Ssp*I repeat was amplified from chicken genome DNA by a PCR reaction with the primers SSPF1 and SSPR1 described by Itoh & Mizuno (2002). Then the repeats were labelled with biotin-16-dUTP (Roche) in PCR reactions with the same primers. The labelled probes were dissolved to a final concentration of 5 ng/μl in the hybridization buffer with 50-fold excess of salmon sperm DNA.

Chicken LBC were also probed with biotinylated oligonucleotides: $(CCCTAA)_5$ specific for telomeric repeat, and CNMpos and CNMneg specific for microchromosomal repeat CNM – a ~41 bp tandem repeat isolated from the chicken genome and described by Matzke *et al.* (1990). The CNMpos and CNMneg oligonucleotides were designed according to the consensus sequence to produce strand-specific hybridization. Oligonucleotides were as follows:

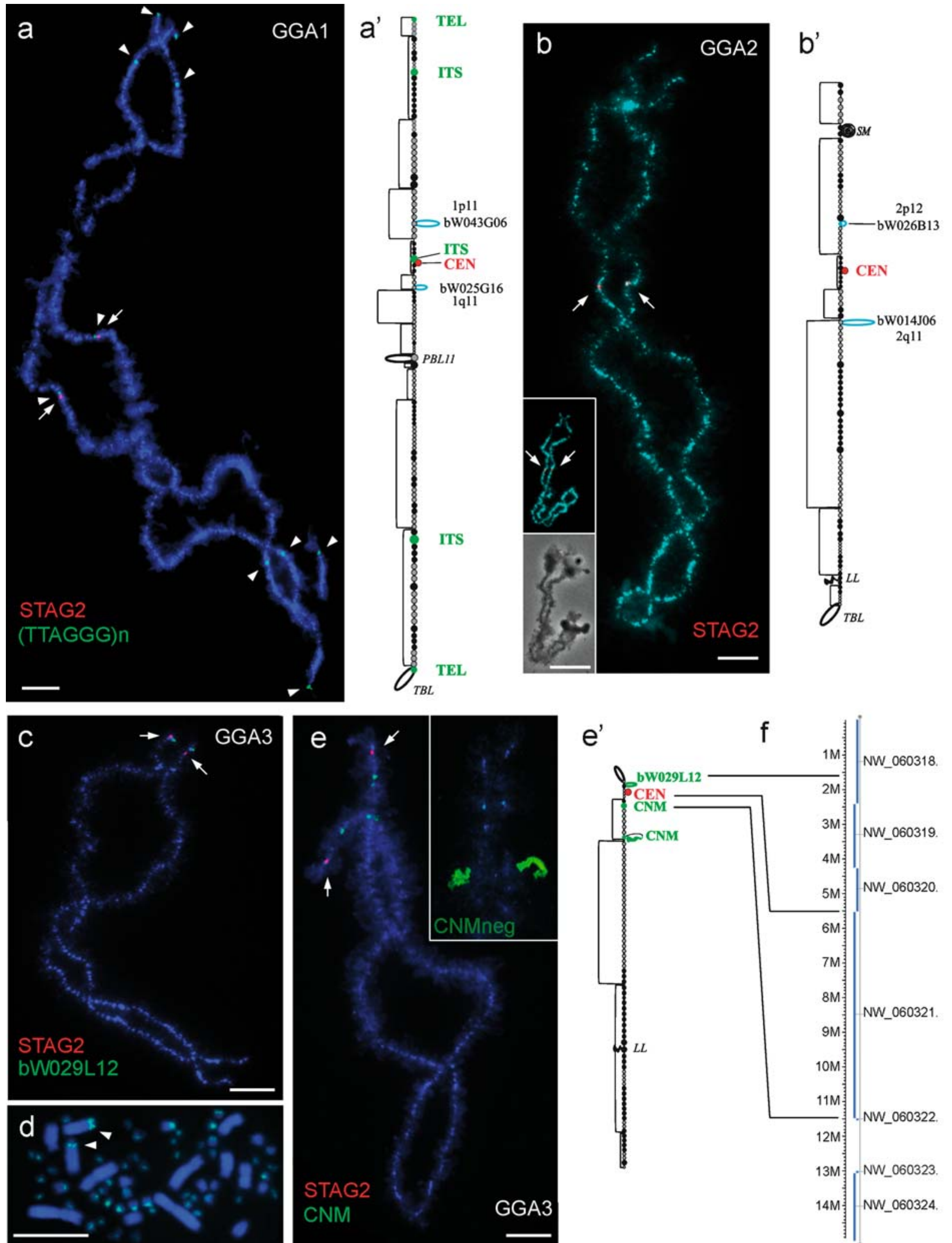
CNMpos, 5′-biotin-TGTTTTCTCTTCGAAAAT CCCCCATTT-3′
CNMneg, 5′-biotin-AAATGGGGGATTTTCGAA GAGAAAACA-3′.

Japanese quail LBC were probed with biotinylated CCOpos and CCOneg oligonucleotides (see Galkina *et al.* 2006) designed according to the consensus sequence of a *Bgl*II- 41 bp tandem repeat isolated from the Japanese quail genome by Tanaka *et al.* (2000). The biotinylated oligonucleotides were dissolved to a final concentration of 5 ng/μl in 40% formamide hybridization buffer. The 50-fold excess of salmon sperm DNA was used in the case of CNM or CCO oligonucleotides, and the same excess of tRNA – in the case of telomeric oligonucleotide. The CNMpos and CNMneg oligonucleotides were either mixed, or used separately in the case of strand-specific hybridization.

Fluorescence in-situ *hybridization (FISH)*

Chromosome painting of chicken LBC was performed according to Derjusheva *et al.* (2003). In some experiments the pre-annealing of painting probes was omitted, to allow the preferential hybridization to repetitive sequences.

LBC preparations were also probed with chromosome-specific PIR or *Ssp*I-repeat probes and with


a
GGA1
STAG2
(TTAGGG)n
a'
TEL
ITS
1p11
bW043G06
ITS
CEN
bW025G16
1q11
PBL11
ITS
TEL
TBL
b
GGA2
STAG2
b'
SM
2p12
bW026B13
CEN
bW014J06
2q11
LL
TBL
c
GGA3
STAG2
bW029L12
d
e
CNMneg
STAG2
CNM
GGA3
e'
bW029L12
CEN
CNM
CNM
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f
1M
2M
3M
4M
5M
6M
7M
8M
9M
10M
11M
12M
13M
14M
NW_060318.
NW_060319.
NW_060320.
NW_060321.
NW_060322.
NW_060323.
NW_060324.

BAC. LBC and probes were denatured together on the slide under a coverslip at 82°C for 5 min. Hybridization was performed according to Galkina *et al.* (2006).

When labelled oligonucleotides were used as probes, three variants of FISH were carried out: (1) for DNA/DNA hybridization, LBC were pre-treated with RNase A; (2) for DNA/(DNA+RNA) hybridization and (3) for DNA/RNA hybridization, RNase A treatment was omitted, allowing us to reveal RNA transcripts. In the first two variants LBC were denatured as described above; in the third one chromosomal DNA was not denatured. Then slides were incubated overnight at RT. After hybridization the slides were washed in four changes of 2 × SSC at 37°C.

In the case of FISH to metaphase chromosomes, chromosome preparations were pre-treated with RNase A (100–200 μg/ml), pepsin (0.01% in 0.01 N HCl) and formaldehyde (1% in PBS, 50 mM $MgCl_2$) according to the standard procedures. Hybridization was performed as described above.

Avidin-FITC (Vector Laboratories) or Avidin-Cy3 (Jackson ImmunoResearch Laboratories) was used to detect biotin-labelled probes. Antibody against digoxigenin conjugated with Cy3 (Jackson ImmunoResearch Laboratories) was used to detect digoxigenin-labelled probes. All preparations after FISH were mounted in antifade solution containing 1 μg/ml DAPI.

Microscopy

Preparations were examined using a Leica fluorescence microscope DM4000 equipped with a monochrome digital camera DFC350 FX and appropriate filter cubes. Leica CW 4000 FISH software was used to acquire and process multicolour images.

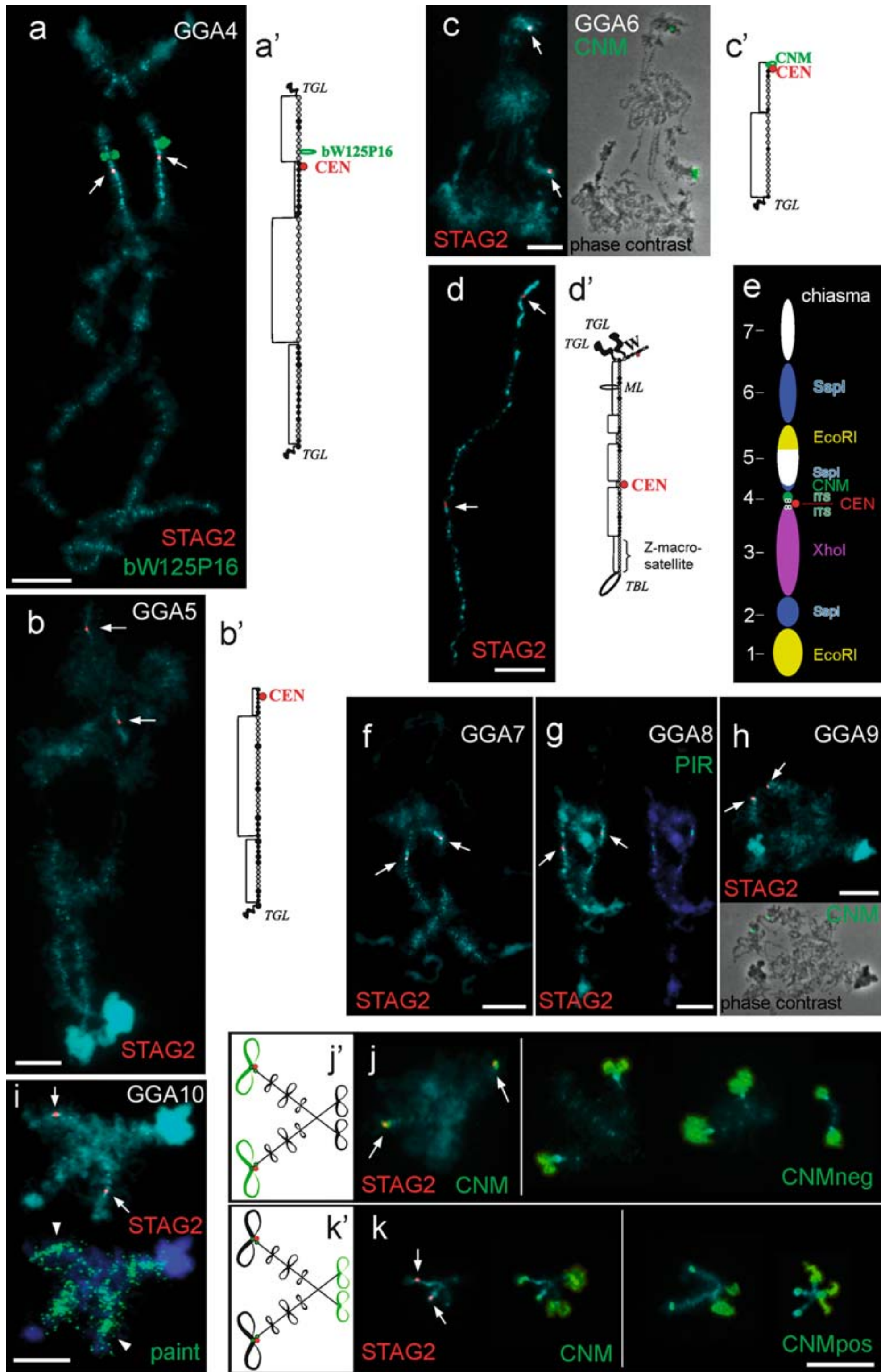
Results

PB-like cohesin-enriched structures on galliform lampbrush chromosomes

Immunofluorescent staining of chicken LBC spreads with either anti-STAG2 or anti-Rad21 antibodies revealed predominantly one prominent focus on each LBC homologue (Figures 1 and 2). The diameter of these cohesin-enriched structures does not exceed 1 μm, a size several times smaller than the average centromere PB of chaffinch and pigeon. These cohesin-enriched structures were found at the constant positions characteristic of each lampbrush bivalent. During chromosome contraction after the lampbrush stage these PB-like structures persist at the same sites on chromosomes (Figure 1b). Similar results of labelling with antibodies against cohesin subunits were observed in Japanese quail: both macro- and micro-LBC bore one major labelling site for each homologue (Figure 3).

In chicken the cohesin-enriched structures were plotted on the cytogenetic maps available for lampbrush chromosomes 1–6, Z and W (Figure 1a′, b′, e′ and Figure 2a′–d′, e). Macrochromosomes 7–10 and the two largest microchromosomes (chromosomes 11 and 12) were identified using chromosome-specific paints. On these LBC PB-like structures were mapped at chromosome regions that could be identified by marker chromomeres (Figure 2f–i). On the rest of chicken LBC, micros 13–38, PB-like

◀ *Figure 1.* Centromere localization on chicken lampbrush chromosomes 1–3. (**a, b, c** and **e**): Immunofluorescent detection of STAG2 cohesin subunit (red). Centromere PB-like structures (red) are marked by arrows. (**a**) Lampbrush chromosome 1. FISH localization of TTAGGG-repeat (green, arrowheads) performed after immunostaining. Cohesin-enriched structure (red) co-localizes with one of the interstitial (TTAGGG)n sites (ITSs). (**b**) Bivalent 2 at the middle and at the end of lampbrush stage (insert) shown at the same magnification. (**c**) Lampbrush chromosome 3. FISH mapping of BAC clone bW029L12 (green) after immunostaining. (**d**) FISH with CNM probe (green) to chicken metaphase chromosomes. Arrowheads indicate CNM signals on chromosomes 3. (**e**) Lampbrush chromosome 3. FISH mapping of CNM repeat according to the DNA/DNA hybridization protocol after immunostaining. Two CNM sites (green) are visible on the long arm of each homologue. FISH with CNMneg oligonucleotide to nascent RNA (insert) reveals polarized transcription units containing CNM transcripts (green). (**a**–**e**) Chromosomes are counterstained with DAPI (blue). Scale bars = 10 μm. (**a**′, **b**′ and **e**′) Cytological maps of chromomere-loop pattern of chicken LBC 1–3 respectively. DAPI-positive chromomeres are represented by black or grey axial dots. Step line reflects the average loop length over a single region. Red circles indicate centromere cohesin-enriched structures (CEN). The positions of certain BAC clones are depicted in blue. Chromomeres comprising TTAGGG-repeat in LBC1 (**a**′), as well as loops and chromomeres comprising CNM or bW029L12 in LBC3 (**e**′), are shown in green. Landmark structures are depicted: LL, lumpy loop; PBL11, marker loop on LBC1; SM, spaghetti marker; TBL, telomere bow-like loop. (**f**) Scheme of contigs constituting first 15 Mb of the draft chicken chromosome 3 sequence assembly (from http://www.ncbi.nih.gov). The positions of centromere and distal CNM site are transferred from the cytological map with corresponding black lines.



a
GGA4
STAG2
bW125P16
a'
TGL
bW125P16
CEN
TGL
b
GGA5
STAG2
b'
CEN
TGL
c
GGA6
CNM
STAG2
phase contrast
c'
CNM
CEN
TGL
d
STAG2
d'
TGL
TGL
W
ML
CEN
Z-macro-
satellite
TBL
e
chiasma
7–
6–
SspI
EcoRI
5–
SspI
CNM
4–
ITS
ITS
CEN
3–
XhoI
2–
SspI
1–
EcoRI
f
GGA7
STAG2
g
GGA8
PIR
STAG2
h
GGA9
STAG2
CNM
phase contrast
i
GGA10
STAG2
paint
j'
j
STAG2
CNM
CNMneg
k'
k
STAG2
CNM
CNMpos

structures were found near the most prominent chromomere at one of the LBC ends (Figure 2j, k); this is similar to LBC 6, 9, 11 and 12.

Verification of centromeric position of PB-like structures

To confirm the centromeric location of the PB-like structures on galliform LBC, FISH with available probes specific for the centromeres was performed after the immunostaining.

In lampbrush bivalent 1, PB-like structures associate with two chromomeres in a prominent loopless bar (Figure 1a). This axial bar is surrounded with BAC bW043G06 and bW025G16, assigned to 1p11 and 1q11, respectively (Galkina *et al.* 2006). These chromomeres hybridize with the chromosome 1 paint more intensively than expected (Derjusheva *et al.* 2003), leading to the presumption that they contain repetitive DNA typical of centromeres. Although centromeric repeats specific for chromosome 1 are not yet available, one of the interstitial telomeric sites (ITS) is known to mark the centromeric region of this chicken chromosome in mitotic metaphase preparations (Nanda & Schmid 1994). FISH with biotinylated $(CCCTAA)_5$ oligonucleotide as a probe after immunostaining with antibody against STAG2 showed the PB-like structure on LBC1 to nearly co-localize with the ITS signal (Figure 1a, a′).

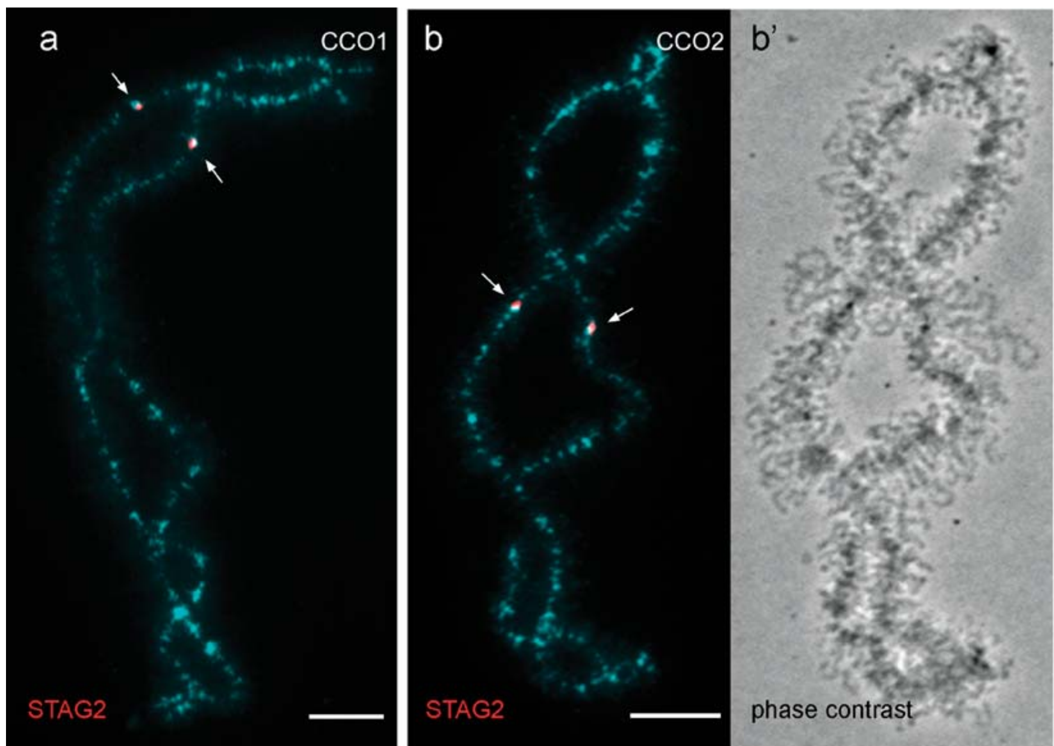
Keeping in mind the average amount of DNA per chromomere in chicken LBC (1.5–2.0 Mb) and the position of the putative centromeric gap (18.4–19.9 Mb) in the GGA4 sequence assembly, we predicted the centromere localization on LBC4 within the two-chromomere distance from the BAC clone bW125P16, assigned to the 16 Mb position (Galkina *et al.* 2006). FISH with bW125P16 probe performed after immunostaining showed close localization of the PB-like structure and bW125P16-bearing loop on LBC4; the signals were separated by two chromomeres as expected (Figure 2a, a′).

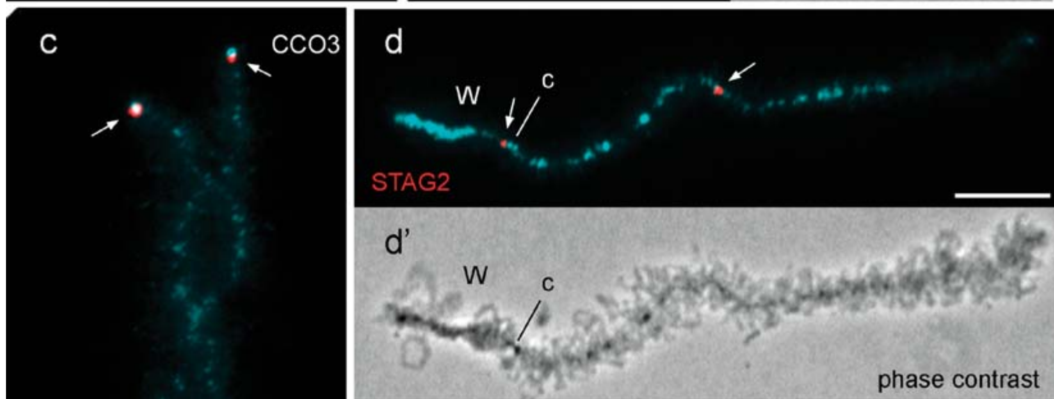
In chicken LBC8 the PB-like structure associates with the pair of chromomeres hybridizing with the satellite repeat PIR (Figure 2g) specific for the centromere region of GGA8.

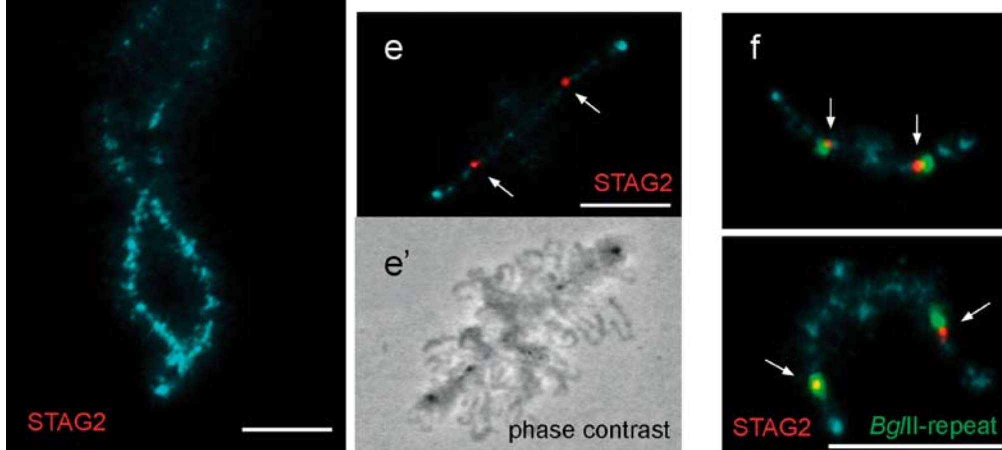
Since chicken repetitive sequence CNM was presumed to have pericentromeric localization on chromosomes 6, 9 and microchromosomes (Matzke *et al.* 1990, Wang *et al.* 2002, Romanov *et al.* 2005), we analysed CNM distribution on LBC. CNM-bearing LBC were identified as chromosomes 3, 6, 9, W and micros 11–38. In LBC 6, 9, 11–38 CNM signals were found in the terminal chromomeres adjacent to PB-like structures (Figure 2c, c′, h, j, k). CNM was not detected in eight LBC (chromosomes 1, 2, 4, 5, 7, 8, 10 and Z) that correspond to the data of CNM mapping on metaphase chromosomes (Figure 1d).

In chicken female-specific W-chromosome the kinetochore position was earlier identified on pachytene ZW bivalents (Solari 1992); the kinetochore localizes between the major cluster of *Xho*I repeat and the minor cluster of *Eco*RI repeat (Solari & Dresser 1995). On chromosome W in the lampbrush form these pericentric blocks of *Xho*I and *Eco*RI sequences were mapped in chromomeres 3 and 5 correspondingly (Solovei *et al.* 1998). We show here

Figure 2. Centromere localization on chicken lampbrush chromosomes 4–10, Z, W and micro-LBC. (**a**–**d**, **f**–**k**) Immunofluorescent detection of STAG2 cohesin subunit (red). Lampbrush bivalents 4 (**a**), 5 (**b**), 6 (**c**), ZW (**d**), 7 (**f**), 8 (**g**), 9 (**h**), 10 (**i**) and microchromosomes (**j**, **k**) Centromere PB-like structures (red) are marked by arrows. (**a**) Lampbrush chromosome 4. FISH mapping of BAC clone bW125P16 (green) after immunostaining. (**c**, **h**) Lampbrush chromosomes 6 and 9. FISH mapping of CNM repeat after immunostaining, hybridization signals (green) are shown on the corresponding phase contrast images. (**a′**, **b′**, **c′**, **d′**) Cytological maps of chromomere-loop pattern of chicken LBC 4–6 and Z. Red circles indicate centromere cohesin-enriched structures (CEN). Curly brace indicates the region of LBCZ containing the Z-Ma-satellite; TGL, terminal giant loop; ML, marker loop; the rest of indications are the same as in Figure 1. (**e**) Chromomere map of chicken LBCW according to Ogawa *et al.* (1997) with modifications. Red circle indicates centromere cohesin-enriched structure (CEN). CNM-positive chromomere is shown in green. Sites of XhoI and EcoRI repeats (Solovei *et al.* 1998), major site of SspI repeat (chromomere 6, Itoh & Mizuno 2002 and our data), minor sites of SspI repeat (chromomeres 2 and 5, our data), and interstitial (TTAGGG)n sites (ITS) (Solovei *et al.* 1993 and our data) are indicated. (**g**) Lampbrush chromosome 8. FISH mapping of PIR repeat after immunostaining. PB-like structure (red) localizes between two PIR-comprising chromomeres (green). (**i**) Lampbrush chromosome 10. FISH with chicken chromosome 10 paint after immunostaining. In conditions favouring the hybridization of repetitive sequences painting probe (green) preferentially hybridizes to the centromeric chromomeres of LBC10 (arrowheads), whereas no signal was detected at the centromere chromomeres of other LBC. (**j**, **j′**, **k**, **k′**) Two types of chicken microchromosomes grouped according to the CNM hybridization pattern. (**j′**, **k′**) – schematic representation, red circles indicate centromere cohesin-enriched structures, CNM-bearing chromomeres and loops are shown in green. (**j**, **k**) FISH with CNM probe (green) after immunostaining (red) (left panels). FISH with CNMpos or CNMneg oligonucleotide (green) allows determination of transcribing strand (right panels). (**a**–**d**, **f**–**k**) Chromosomes are counterstained with DAPI (blue). Scale bars = 10 μm.


a
CCO1
STAG2
b
CCO2
STAG2
b'
phase contrast


c
CCO3
d
w
c
STAG2
d'
w
c
phase contrast


STAG2
e
STAG2
e'
phase contrast
f
STAG2
BglII-repeat

that the PB-like structure locates between the chromomere 3 and the tiny chromomere 4 of LBCW (Figure 2d, e). It is worth noting that the chromomere 4, which lacks other known W-specific repetitive sequences (*Eco*RI, *Xho*I, and *Ssp*I), hybridizes with a probe homologous to the CNM repeat (Figure 2e). Similarly, CNM was detected at the primary constriction of GGAW in DT40 cells (Wang *et al.* 2002).

In Japanese quail, a CNM-like ~41 bp repeat concentrating in the centromeric heterochromatin of microchromosomes was described (Tanaka *et al.* 2000). It is worth noting that, in quail micro-LBC, this repeat was revealed within chromomeres, adjacent to the PB-like structure (Figure 3f).

Taken together, these results confirm the centromeric localization of cohesin-enriched structures not only on chaffinch and pigeon LBC, but also on galliform LBC.

Centromere localization in chicken lampbrush chromosomes

Using antibodies against cohesin complex subunits we defined the centromere position for each chicken LBC. Immunofluorescence experiments demonstrated that chicken LBC 1, 2, 4, 7, 8, 10, Z and W are submetacentric, whereas chromosomes 3, 5, 6, 9 and 11–38 are acrocentric (Figures 1 and 2). The characterization of chicken microchromosomes as acrocentric is in agreement with the synaptonemal complex analysis (Solari 1980, Kaelbling & Fechheimer 1983). In contrast to chicken microchromosomes (Figure 2j, k), the majority of quail microchromosomes are biarmed, as demonstrated by immunofluorescent staining of Japanese quail LBC spreads. The PB-like structures on quail micro-LBC do not co-localize with the most prominent chromomeres, which have terminal locations (Figure 3e). FISH with pericentromeric *Bgl*II-repeat confirmed these data (Figure 3f). Interestingly, the centromere in quail W chromosome localizes in close vicinity to the pseudoautosomal region so that the terminal chiasma in the sex ZW lampbrush bivalent (Figure 3d) always has a pericentric position in CCOW.

Centromere positions in chicken macro-LBC determined by PB-like structures apparently correspond to the centromeric regions in the draft chromosome sequence assembly, proposed to be 1.5 Mb lengths and symbolized by gaps. The only exception is chromosome 3. In chicken LBC3, PB-like structure presents within two chromomere distance (3.0–4.0 Mb) from the loop, bearing BAC clone bW029L12 (Figure 1c). This BAC localizes at 3pter, the position of linked microsatellite marker in the GGA3 sequence assembly – 1.6 Mb (Galkina *et al.* 2006). Thus the position of centromere is calculated to be at 4.6–5.6 Mb in the GGA3 sequence assembly (Figure 1f), whereas the gap for the proposed region of pericentromeric DNA exists at the 11.5–13.0 Mb position. In GGA3 sequence map the 1.5 Mb gap, which refers to the centromere, is bordered with several CNM repeats. CNM repeats in the chromosome sequence assembly having 80–100% identity to $(\text{CNM unit})_{10}$ (CNM unit sequence was identical to X51431, Matzke *et al.* 1990) were recognized by NCBI BLAST search. Interestingly, on metaphase chromosomes, the CNM signal seems to localize exactly at the primary constriction of chromosome 3 (Figure 1d). At the same time, FISH on LBC3 revealed two clusters of CNM repeat (Figure 1e); these clusters are separated by five chromomeres and are located on the long arm of chromosome 3, the proximal CNM block being separated from the centromeric PB-like structure by three chromomeres (Figure 1e, e′).

CNM transcription on chicken lampbrush chromosomes

When FISH with CNM was performed on chicken LBC according to the hybridization protocol, which reveals both DNA and nascent RNA transcripts, new clusters of transcribing CNM repeat on lateral loops were observed in addition to the chromomere hybridization (Figures 1e and 2j, k). On LBC3 the

Figure 3. Positions of PB-like structures on lampbrush chromosomes in Japanese quail. (**a**–**f**) Lampbrush bivalents 1 (**a**), 2 (**b**), 3 (**c**), ZW (**d**) and micro-LBCs (**e**, **f**). Immunofluorescent detection of STAG2 cohesin subunit (red). Centromere PB-like structures are marked by arrows. W chromosome is indicated: c, chiasma position in ZW lampbrush bivalent (**d**). (**b′**, **d′**, **e′**) Corresponding phase contrast images. (**f**) FISH with *Bgl*II-repeat probe to quail micro-LBC according to the DNA/DNA hybridization protocol after immunostaining. Cohesin-enriched structures (red) co-localize with pericentromeric *Bgl*II-repeat sites (green). Chromosomes are counterstained with DAPI (blue). Scale bars = 10 μm.

CNM signal was revealed within the polarized RNP-matrix on the pair of lateral loops arising from the distal CNM site (Figure 1e, e′). CNM-bearing loops were found on all micro-LBC (Figure 2j, k). Chicken micro-LBC can be classified into two types according to the CNM distribution pattern (Figure 2j′, k′). In one group of microchromosomes the CNM occupies one or two terminal chromomeres at the centromere region and the certain pairs of lateral loops with long transcription units extending from these chromomeres (Figure 2j, j′). In the other group of microchromosomes CNM repeats were found at both termini: in centromeric chromomeres and on a pair of loops with long transcription units at the terminal region of the long arm (Figure 2k, k′).

To determine the transcribing strand of CNM satellite, either CNMpos or CNMneg oligonucleotide complementary to the G- or C-rich strand of CNM consensus sequence respectively was applied to native LBC preparations. As a negative control we used LBC preparations treated with RNase A. Both the C- and the G-rich complementary CNM transcripts were revealed (Figure 2j, k). On each pair of the labelled loops CNM is transcribed only from one of the strands; the transcription occurs in long transcription units. Interestingly, tandem arrays of CNM transcribed from the G-rich strand localize on loops extended from centromere-associated chromomeres of certain micro-LBC, whereas those transcribed from the C-rich strands localize on the loops extended from the qter chromomeres of other micro-LBC (Figure 2j, k), with one exception: a microchromosome with CNM transcribed from the C-rich strand on centromere-associated loops (not shown). On the long arm of LBC3 the C-rich CNM transcripts were revealed on lateral loops extended from the distal CNM site (Figure 1e).

Discussion

Centromere PB were earlier described as spherical objects, which occur on LBC and, in some avian species, reach 12 μm in diameter (Gaginskaya & Gruzova 1969, Gaginskaya 1972). PB may represent a special type of oocyte-specific intranuclear structures. They contain a set of chromosome structural proteins but lack fibrillarin, pre-mRNA splicing factors, snRNA and phosphorylated RNA polymerase II (Krasikova *et al.* 2004). This composition differs from the composition of other known nuclear bodies that have been examined so far (Matera 1999, Handwerger & Gall 2006). Centromere PB form at the time of chromosomes becoming lampbrush, and in many birds participate in the karyosphere formation in more mature oocytes (Gaginskaya 1972, Saifitdinova *et al.* 2003), possibly retaining condensed bivalents within a confined space of large oocyte nucleus. Morphologically similar round granules at the LBC centromeric regions flanked by heterochromatin were described in some amphibian species (reviewed in Callan 1986, Macgregor 1986, Gall 1992). Here we show that the presence of PB-like cohesin-enriched structures specifically at centromeric sites is a universal feature of LBC in all birds studied, including those belonging to the Galliformes. Centromere PB-like structures represent a promising marker for the kinetochore domain of giant LBC, allowing cytological centromere mapping with high resolution.

The centromeric indexes of chicken LBC 1–8 determined in the present work correspond to those of the same chromosomes at the metaphase stage. However, the centromeric indexes of GGAZ in the metaphase ($p/L = 0.48$) and in the lampbrush form ($p/L = 0.55$) differ significantly, perhaps due to unequal decondensation in p and q arms of LBCZ. In spite of the active transcription of Z-macrosatellite on the long arm of LBCZ (Hori *et al.* 1996) the heterochromatin region containing this repeat is less elongated than euchromatin regions of the LBCZ. The centromeric index of hypothetical chromosome Z without Z-macrosatellite was found to be almost identical at the lampbrush and metaphase chromosomes. The degree of chromatin condensation/decondansation along LBC axes seems to be different in euchromatin and C-band-positive heterochromatin regions. This regularity is obvious in the case of highly heterochromatic avian chromosome W that is strongly condensed as compared to other chromosomes in the lampbrush form (Solovei *et al.* 1993, Saifitdinova *et al.* 2003). Another instance is the C-positive short arm of Japanese quail chromosome 4, that at the lampbrush stage is as condensed as at the metaphase stage (Galkina *et al.* 2006).

The striking example of employment of cohesin-enriched body as a marker for centromeric region is detailed analysis of the GGA3 centromere position. In the draft GGA3 sequence assembly the 1.5 Mb gap at the 11.5–13.0 Mb position is proposed to be pericentromeric DNA (ICGSC 2004). However, the

centromere determined by mapping of the PB-like structure in chicken LBC3 is predicted to be between 4.6 Mb and 5.6 Mb. In fact, in the GGA3 sequence map, there is a gap between the supercontigs at the position of ~5.5 Mb (Figure 1f). This very gap is supposed to be centromeric. This gap thus could be extended up to 1.5 Mb to compensate for the pericentromeric sequences.

With regard to the gap at the position of 11.5–13.0 Mb, it should be said that supercontig belonging to GGA3 sequence assembly contains a few repeated units of CNM at the ~11.5 Mb position. FISH with CNM probe to the chicken LBC3 after immunodetection of PB-like structures (Figure 1e) makes it possible to distinguish CNM sites and the centromere. We demonstrate that proximal array of CNM repeat on GGA3 localizes at the 5 chromomere distance (~10 Mb) from the BAC clone bW029L12 with genomic position 1.6 Mb (Figure 1e′). These results indicate that the currently known pericentromere DNA gap at the 11.5–13.0 Mb position in the GGA3 sequence assembly corresponds to the proximal non-centromeric array of CNM repeat (Figure 1e′, f). Notwithstanding moving of the centromere gap to another position – close to 5.5 Mb – the gap (11.5–13.0 Mb) should be preserved, since the cluster of CNM repeat occupies two chromomeres in LBC3 (Figure 1e).

CNM repetitive DNA sequence is practically absent from the current draft of the chicken genome sequence map (http://www.ensembl.org). Only for chromosomes 3, 16, 23, 24 and 28 is it represented in several copies at one of the ends of a particular chromosome. Detailing of the CNM repeat chromosomal distribution by hybridization to the nascent transcripts on the lateral loops of giant LBC allowed us to reveal numerous non-centromeric sites of CNM-repeat in addition to the pericentromeric arrays.

On LBC the CNM repeat is transcribed on many long lateral loops of microchromosomes and also on the pair of loops of LBC3. Various highly repeated sequences including centromere satellites were shown to transcribe in amphibian and avian oocyte nuclei (reviewed in Callan 1986, Macgregor 1986, Morgan 2002). It was found that in many cases amphibian LBC transcribe both strands of centromere repeats, though non-proportionally. Transcription of both strands of satellite DNA in metazoan centromeres resulting in the production of long double-stranded (ds) precursor RNA and their subsequent procession into small interfering RNA (siRNA) are required for the centromere heterochromatin formation and centromere cohesion (reviewed in Almedia & Allshire 2005). It is unknown whether non-coding RNA products of CNM transcription on the LBC lateral loops both from the C- and G-rich strands can hybridize to form long dsRNA. If CNM dsRNA formation occurs, we can hypothesize that such long dsRNA could be processed into siRNA for participation in the compact chromomere formation at the pericentric regions of LBC, or/and could be stored within the oocyte for the early stages of embryogenesis to provide a pool of long pericentromeric CNM dsRNA for the production of siRNA in the absence of own synthetic activity of chromosomes in early embryos.

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