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RESEARCH ARTICLE

Tandem 41-bp repeats in chicken and Japanese quail genomes: FISH mapping and transcription analysis on lampbrush chromosomes

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Abstract The chromosomal distribution of 41-bp repeats, known as CNM and PO41 repeats in the chicken genome and *Bgl*II repeats in the Japanese quail, was analyzed precisely using giant lampbrush chromosomes (LBC) from chicken, Japanese quail, and turkey growing oocytes. The PO41 repeat is conserved in all galliform species, whereas the other repeats are species specific. In chicken and quail, the centromere and subtelomere regions share homologous satellite sequences. RNA polymerase II transcribes the 41-bp repeats in both centromere and subtelomere regions. Ongoing transcription of these repeats was demonstrated by incorporation of BrUTP injected into oocytes at the lampbrush stage. RNA complementary to both strands of CNM and PO41 repeats is present on chicken LBC loops, whereas strand-specific G-rich transcripts are characteristic of *Bgl*II repeats in the Japanese quail. The RNA from 41-bp repeats does not undergo cotranscriptional U snRNP-dependent splicing. At the same time, the ribonucleoprotein matrix of transcription units with C-rich RNA of CNM and PO41 repeats was enriched with hnRNP protein K. Potential promoters for satellite transcription are discussed.

Introduction

Tandem repeats typical of centromeric regions of microchromosomes have been isolated from genomes of different representatives of the order Galliformes: CNM from the chicken *Gallus gallus domesticus* (Matzke et al. 1990), TM from the turkey *Meleagris gallopavo* (Matzke et al. 1992), *Bgl*II repeat from the Japanese quail *Coturnix coturnix* (Tanaka et al. 2000), and 578-bp *Eco*RI repeat from the blue-breasted quail *Coturnix chinensis* (Yamada et al. 2002). These repeats are species specific. At the same time, they have a basic 20-bp internal repeat that must have originated from a common ancestral sequence. Moreover, in chicken, turkey, and Japanese quail, the repeats are all 41 bp in length. Recently, another 41-bp repeat, the so-called PO41, has been isolated from the chicken genome (Wang et al. 2002; Wicker et al. 2005). The PO41 sequences have been assigned only to virtual chicken chromosomes (ChrUn). Additionally, PO41 and CNM arrays were next to each other in two clones (Wicker et al. 2005). These data allow us to predict that PO41 has a microchromosomal localization. However, the precise chromosomal distribution of the PO41 repeat has not yet been analyzed. Because the majority of microchromosomes are minute and difficult to identify, precise fluorescence in situ hybridization (FISH) mapping on the microchromosomes is not easy.

Giant lampbrush chromosomes (LBCs), which occur at the diplotene stage of prophase I during oogenesis, represent an excellent system for high-resolution cytogenetic analysis, especially for microchromosomes. Avian LBCs are more than 30 times longer than the corresponding mitotic metaphase chromosomes. They have distinctive chromomere-loop patterns that make it possible to recognize each LBC. Use of LBCs has already allowed us to define the gene order in chicken and quail genomes more precisely (Galkina et al. 2006). FISH on chicken LBCs has recently revealed numerous q-terminal arrays of CNM repeat in addition to the centromeric clusters (Krasikova et al. 2006). It is interesting to note that both centromere and q terminus associated CNM clusters are transcribed at

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the lampbrush stage (Krasikova et al. 2006). However, the transcription of related repeats in other galliform species has not been studied. Transcripts of other tandem repeats, including $(TTAGGG)_n$, the Z-chromosome macrosatellite in the chicken, and the centromeric satellite PR1 in pigeons, have been demonstrated on LBCs (Solovei et al. 1994; Hori et al. 1996; Solovei et al. 1996).

Recent studies on RNA interference have totally changed our view of the role of noncoding RNAs. Nowadays, their participation in regulation of gene expression, chromatin organization, and genome functioning is well established (Almeida and Allshire 2005; Kim 2005; Matzke and Birchler 2005; Prasanth and Spector 2007). Thus, studies on the location and transcription of highly repeated, noncoding sequences may well provide insight into fundamental issues of chromosome organization and function.

We focus in this study on the comparative analysis of the CNM repeat in the chicken and the CNM-like *Bgl*II repeat in the Japanese quail, as well as on their colocalization with the PO41 repeat. Using the advantages of the LBC system, we analyzed the intrachromosomal distribution of the 41-bp repeats in chicken and Japanese quail at high resolution. The highly conserved PO41 repeat is associated with the species-specific CNM and *Bgl*II repeats on certain microchromosomes in the chicken and Japanese quail, respectively. All three repeats are transcribed in long transcription units on lateral loops. Because individual transcription units on LBCs can be visualized by light microscopy, it is possible to determine the composition of the nascent ribonucleoprotein (RNP) matrix of transcriptionally active sequences. The tandem 41-bp repeats in centromeric and subtelomeric regions are transcribed by RNA polymerase II (Pol-II). At the same time, the RNP matrix of these loops lacks splicing factors, suggesting that snRNP-dependent cotranscriptional splicing does not occur there.

Materials and methods

Chromosome preparation

Chicken (*G. gallus domesticus*, GGA), Japanese quail (*C. coturnix japonica*, CCO), and turkey (*M. gallopavo*, MGA) LBCs were isolated manually from oocytes of 1.0–1.5 mm diameter according to the standard technique (Solovei et al. 1994). Adult females were bought from commercial stocks. After overnight fixation in 70% ethanol, preparations were dehydrated in 96% ethanol and were air-dried before using for FISH. Preparations for immunostaining were kept in 70% ethanol and never dried. Full sets of chicken and Japanese quail LBCs were analyzed.

Mitotic metaphase chromosomes were prepared from chicken and Japanese quail embryonic fibroblasts using conventional techniques.

Immunofluorescent staining

Immunostaining of chicken and quail LBCs was carried out as previously described (Krasikova et al. 2004, 2005) with the following mouse monoclonal antibodies: H14 (BAbCO) and V22 (kindly donated by U. Scheer and R. Hock) against the phosphorylated C-terminal domain (CTD) of RNA polymerase II, K121 against the trimethylguanosine cap existing on most splicing snRNAs (Oncogene Research Products), Y12 against the SmB/SmB′ core proteins found on most splicing snRNPs (Lerner et al. 1981), 3C2 against hnRNP K/J (Matunis et al. 1992), and rabbit polyclonal antibody K828 against STAG2 (Prieto et al. 2004), a centromere marker on LBCs (Krasikova et al. 2006). The specificity of these antibodies to avian proteins was confirmed by Western blot analysis using standard techniques (Krasikova et al. 2004, 2005). To avoid RNA degradation during immunostaining, PBS containing 1% blocking reagent (Roche) was autoclaved and kept at −20°C before using. After immunostaining and image acquisition, LBC preparations were used for FISH. Slides were washed in 2× SSC at 42°C, were dehydrated in a 70–80–96% ethanol series, and were air-dried before applying FISH probes.

Fluorescence in situ hybridization

LBCs and mitotic metaphase chromosomes were probed with oligonucleotides that were designed according to the consensus sequences of 41-bp tandem repeats: CNM (Matzke et al. 1990; NCBI accession number X51431), *Bgl*II repeat (Tanaka et al. 2000; NCBI accession numbers AB035968–AB035970) and PO41 (Wang et al. 2002; Wicker et al. 2005; NCBI accession number AF124926).

Oligonucleotides were as follows:

- CNMpos, 5′-TGTTTTCTCTTCGAAAATCCCCCATTT-3′
- CNMneg, 5′-AAATGGGGGATTTTCGAAGAGAAAACA-3′
- CCOpos, 5′-ATGGGGCAGGAGCTGCTGTGGGGCAGATGT-3′
- CCOneg, 5′-ACATCTGCCCCACAGCAGCTCCTGCCCCAT-3′
- PO41pos, 5′-TATGGGGCTCTATGGGGCTCTATGGGGCGGC-3′
- PO41neg, 5′-GCCGCCCCATAGAGCCCCATAGAGCCCCATA-3′

A telomere-specific oligonucleotide $(CCCTAA)_5$ was also applied to Japanese quail LBCs along with 41-bp repeat probes. The oligonucleotides were biotinylated or labeled with either Cy3 or Cy5.

The labeled oligonucleotides were dissolved to a final concentration of 5 ng/μl in hybridization buffer (40% formamide, 2× SSC, 10% dextran sulfate). A 50-fold excess of salmon sperm DNA was used with 41-bp repeat probes, and the same excess of tRNA was utilized with the telomeric oligonucleotide. They were either used separately or mixed in such a way as to detect colocalization of different repeats and/or to analyze strand-specific hybridization using multicolor FISH.

In the case of FISH to metaphase chromosomes, preparations were pretreated 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 standard procedures. Chromosomal DNA and probes were denatured together on the slide under a coverslip at 82°C for 5 min. Then slides were incubated overnight at room temperature.

Three variants of FISH were carried out on LBC preparations: (1) For DNA/DNA hybridization, LBCs were pretreated 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, LBCs were denatured as described above; in the third one, chromosomal DNA was not denatured. As a negative control for DNA/RNA hybridization, we used LBCs pretreated with RNase A.

After hybridization, the slides were washed in four changes of 2× SSC at 37°C. Avidin-FITC (Vector Laboratories) or Avidin-Cy3 (Jackson ImmunoResearch Laboratories) was used to detect biotin-labeled probes. All preparations after FISH were mounted in antifade solution containing 1 μg/ml DAPI.

Microinjection of BrUTP

BrUTP (Sigma) was dissolved in sterile PBS to a concentration of 20 mM. BrUTP solution of 23 nl was injected into the cytoplasm of oocytes using a Drummond Nanoject II automatic nanoliter injector (Drummond Scientific Company, Broomall, PA, USA). After microinjection, oocytes were incubated for 1–4 h. For immunofluorescent detection of BrUTP incorporation, LBCs were isolated from the injected oocytes and were probed with anti-BrdU mouse monoclonal antibody (produced and kindly donated by M. V. Filatov).

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 multicolor images.

Results

Comparative FISH mapping of PO41 and CNM-like repeats

Oligonucleotides specific for the PO41 repeat were applied to chicken and Japanese quail mitotic and lampbrush chromosomes, along with either CNM or *Bgl*II-repeat-specific oligonucleotides. PO41 probes effectively hybridized to both chicken and Japanese quail chromosomes, suggesting that the PO41 repeat from the chicken genome is present in the quail. As expected, the majority of PO41 sites were found on microchromosomes (Fig. 1). Apart from the microchromosomes, clusters of PO41 repeat were revealed at terminal regions of GGA1p, GGA1q, GGA2q, GGAZp, and GGAWp (Figs. 1a, 2a, d, and 5b) and CCO1p, CCO1q, CCO2p, CCOZp, and CCOWp (Figs. 1b, 2b, g, and 5d). These same regions on chromosomes 1 and 2 are known to share strong homology between chicken and Japanese quail due to a pericentric inversion CCO2p being homologous to the distal part of GGA2q (Schmid et al. 2005; Galkina et al. 2006); PO41 sites on chromosomes Z and W belong to pseudoautosomal regions, which are very close to the chiasma on the ZW lampbrush bivalent (Fig. 2d, g). In contrast to CNM-like repeats (CNM, TM, *Bgl*II repeat, and 578-bp *Eco*RI repeat), which never hybridize across species (Matzke et al. 1990; Matzke et al. 1992; Tanaka et al. 2000; Yamada et al. 2002; our data), the PO41 repeat seems to be highly conserved among galliform species both in its sequence and chromosomal distribution. In fact, in turkey, the PO41 probe also hybridized to a number of smaller microchromosomes, to pseudoautosomal regions of Z and W chromosomes, to both termini of chromosome 1, and to the end of the long arm of chromosome 3 (Fig. 2c), which is homologous to GGA2q (Schmid et al. 2005).

FISH on LBCs allowed us to identify all sites of the *Bgl*II repeat in Japanese quail, both macro- (CCO4p, shown in the study of Galkina et al. (2006), and CCOWcen, shown in Fig. 2g) and microchromosomal ones. The *Bgl*II repeat was revealed on 27 quail microbivalents; because one of the largest microchromosomes is polymorphic, it can lack the *Bgl*II repeat on one (Fig. 3a) or even both homologues, giving 26 microbivalents that are *Bgl*II repeat positive. Recently, we have confirmed a preferential centromeric localization of the *Bgl*II repeat (Krasikova et al. 2006), though additional noncentromeric clusters are detectable on a number of quail microchromosomes at the prominent terminal heterochromatic chromomeres (Fig. 3d, i). Using FISH on chicken LBCs, the chromosomal distribution of the chicken CNM repeat was described in detail earlier (Krasikova et al. 2006). Along with centromeric clusters on chromosomes 6, 9, and 11–38, numerous CNM arrays were found on terminal

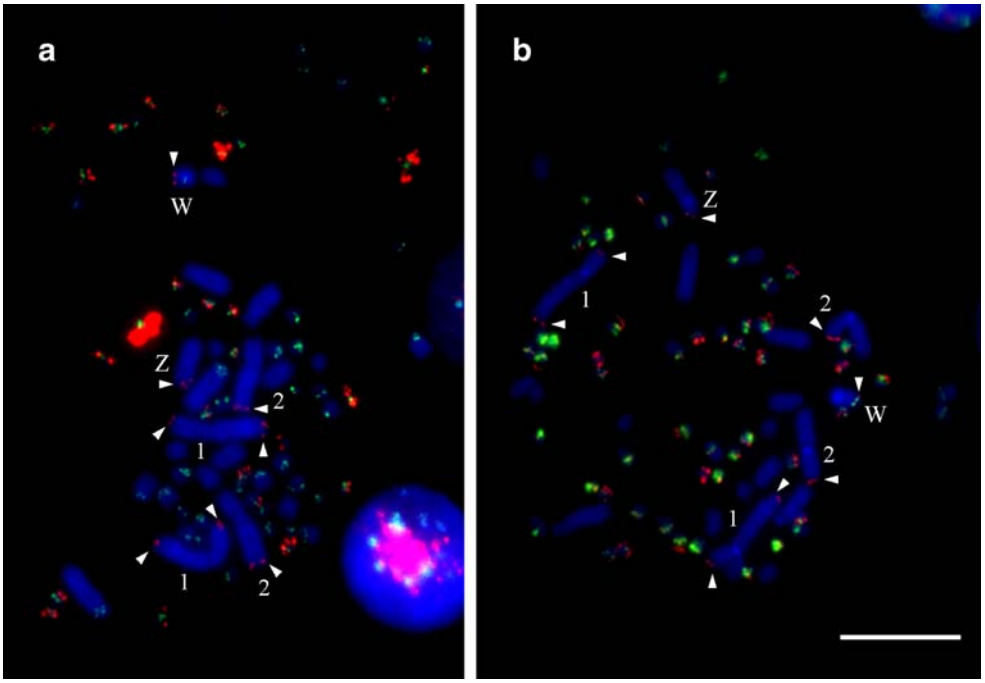


Fig. 1 Two-color FISH with CNM (*green signal*) and PO41 (*red signal*) probes on chicken metaphase chromosomes (**a**), and with *Bgl*II repeat (*green signal*) and PO41 (*red signal*) probes on Japanese quail metaphase chromosomes (**b**). *Arrowheads* indicate PO41 arrays on macrochromosomes. Chromosomes are counterstained with DAPI. *Scale bar* 10 μm

lateral loops on the long arms of certain microchromosomes and in two clusters on the proximal region of GGA3q (Krasikova et al. 2006). Some microchromosomes with noncentromeric CNM clusters can be seen in this study in Figs. 2f, 4g, g″, and i, and 5c. Thus, in both species, the microchromosomal repeats that were proposed to be centromeric were also found in noncentromeric sites.

Turning back to the PO41 repeat, it is important to note that PO41 was originally presumed to be a subtelomeric repeat (Wicker et al. 2005). Indeed, it was found in subtelomeric sites on chromosomes 1, 2, ZW, and a number of microchromosomes both in chicken and Japanese quail (Figs. 2, 3b–d, and 5d). However, this localization is not invariable. In Japanese quail, this sequence is preferentially contiguous with pericentromeric arrays of *Bgl*II repeat (Figs. 3 and 5e–g). The centromere chromatin of the smallest quail microchromosome lacking the *Bgl*II repeat consists of PO41 repeat (Fig. 3f, g). In chicken, a number of PO41-repeat arrays were mapped to the middle of microchromosomes (Fig. 2e, f). In addition to GGAUn sites of the PO41 repeat, sequences with 83% identity to PO41 can be recognized by an NCBI BLAST search in nonterminal sites of GGA1 and GGA5 sequence assembly, though these clusters are undetectable by FISH with the PO41 probe we used.

FISH on LBCs made it possible to analyze colocalization of the arrays of PO41 and CNM-like repeats more precisely than on mitotic metaphase chromosomes. We found several types of microchromosomes that differ in the FISH patterns of the repeats (Figs. 2f and 3). The most striking FISH patterns occur when two sequences hybridized to one lateral loop (Figs. 3h′, j, and k and 5f, g). This clearly shows that some arrays of CNM-like (both CNM in chicken and the *Bgl*II repeat in Japanese quail) and PO41 repeats are immediately adjacent to one another.

Transcribing strand

It should be mentioned in this study again that all three 41-bp repeats hybridized to lateral loops on LBCs from chicken and quail oocytes. FISH signals on the lateral loops were highly sensitive to RNase treatment; their visualization did not require chromosome denaturing. These features are characteristic of FISH to RNA transcripts. Thus, we can be sure that certain lateral loops contain nascent transcripts complementary to satellite DNAs with 41-bp repeating units—PO41, CNM, and *Bgl*II repeats. Transcription of the repeats is not obligatory. In fact, there is a quail microchromosome whose centromere region consists of both PO41 and *Bgl*II-repeat DNA (Fig. 3e′) but never shows fluorescent signals when FISH is performed according to the DNA/RNA hybridization protocol (Fig. 3e). The centromere cluster of the *Bgl*II repeat on the W chromosome is nontranscribing. Additionally, the *Bgl*II-repeat clusters detected at terminal heterochromatic chromomeres (Fig. 3d, i) were shown to be nontranscribing, whereas transcription of PO41 may occur on lateral loops arising from the terminal heterochromatic chromomeres (Fig. 3a, b). In chicken, we showed nontranscribing clusters of CNM repeat on GGA3 and on the centromeric regions of a number of microchromosomes (Krasikova et al. 2006).

In our previous study, we demonstrated that the CNM repeat transcribes from both strands in the chicken; C-rich

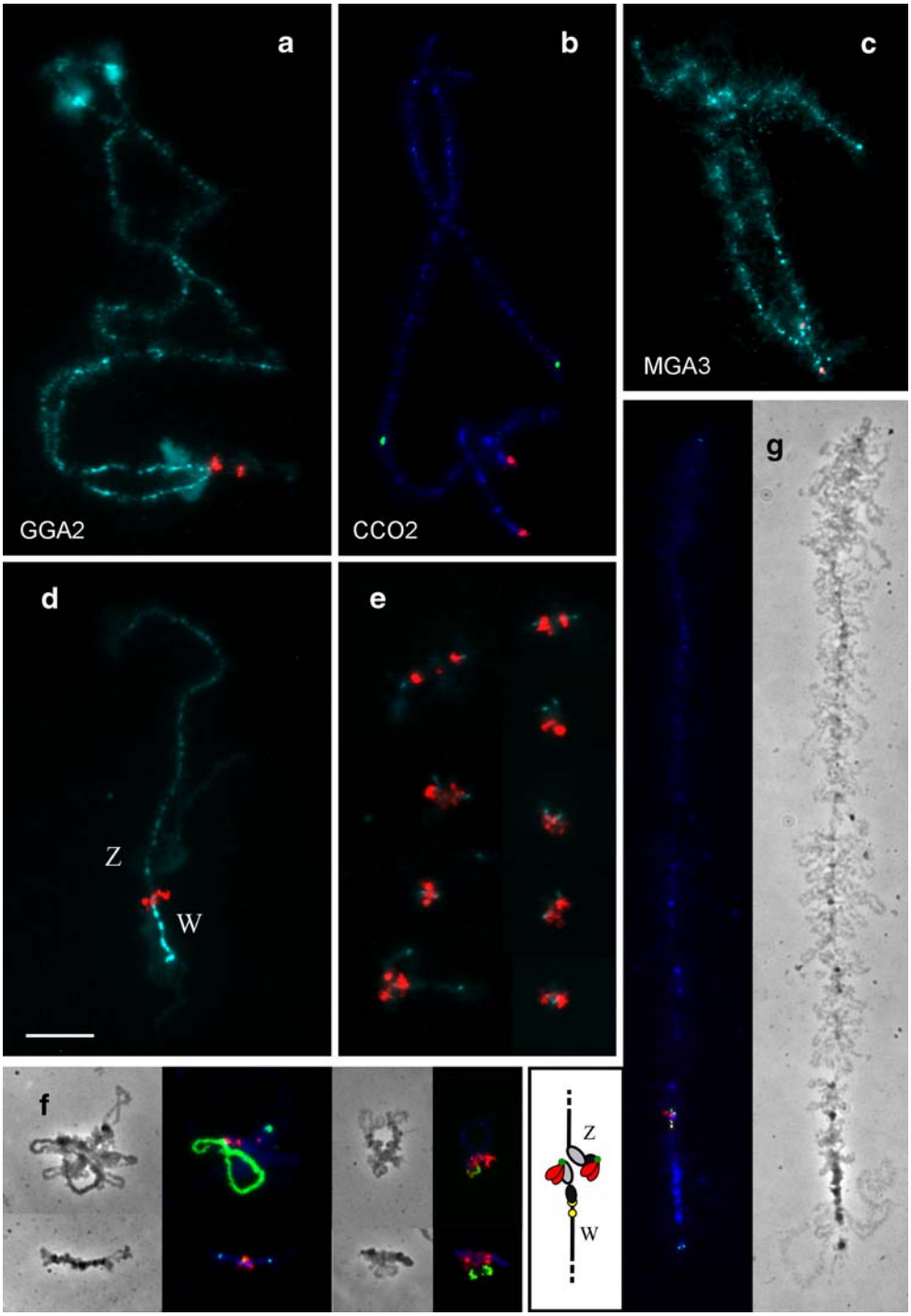


Fig. 2 FISH with PO41 probe (*red signal*) on lampbrush chromosomes: **a** chicken LBC2 (GGA2); **b** Japanese quail LBC2 (CCO2), centromere position detected by immunostaining with an antibody against STAG2 (*green signal*); **c** turkey LBC3 (MGA3); **d** chicken LBC ZW; **e**, **f** representative chicken microchromosomes probed with PO41 (*red signal*) (**e**) or PO41 (*red signal*) along with CNM (*green signal*) (**f**). **g** Quail LBC ZW, probed with PO41 (*red signal*), telomeric TTAGGG repeat (*green signal*) and centromeric *Bgl*II repeat (*yellow signal*). Schematic drawing of the ZW chiasma region shows the distribution of FISH signals (colored *green*, *red*, and *yellow* as on the corresponding image). FISH was performed according to DNA/(DNA+RNA) hybridization protocol. Chromosomes are counterstained with DAPI. *Left panels* on **f** and *right panel* on **g** are phase contrast images. *Scale bar* 10 μm

CNM transcripts were detected on loops extending from centromere-associated chromomeres of certain microchromosomes and on lateral loops extending from the distal CNM site on LBC3, whereas G-rich transcripts occurred on the loops extending from the q-terminal chromomeres of microchromosomes (Krasikova et al. 2006; Fig. 5c). The behavior of other 41-bp repeats—PO41 and *Bgl*II repeats—were different from CNM. In Japanese quail, transcription of the CNM-like *Bgl*II repeat occurs on long pericentromeric loops; only G-rich *Bgl*II-repeat transcripts were revealed on these loops (Fig. 3h, h′, j, and k). An oligonucleotide complementary to the C-rich strand of the repeat hybridized to quail LBCs only when chromosomal DNA was denatured (Fig. 3i). PO41-repeat RNA hybridized with oligonucleotides complementary to either the C-rich or G-rich strand of the PO41 repeating unit. In

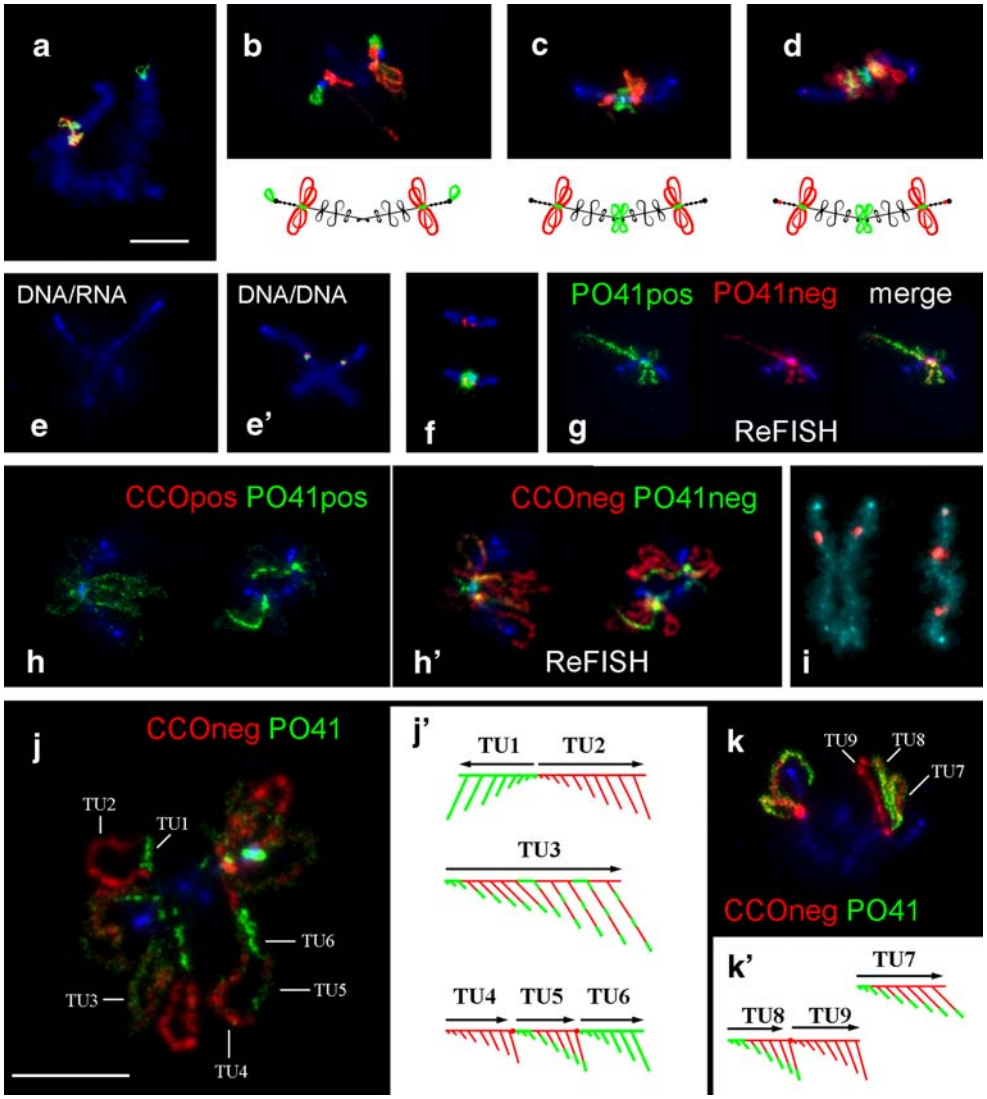


Fig. 3 High-resolution analysis of the distribution of PO41 and *Bgl*II repeats on Japanese quail microchromosomes. (**a**–**d**, **k**) Representative microchromosomes differing in their PO41 (*green*) and *Bgl*II repeat (*red*) FISH patterns. **e**, **e′** A microchromosome with nontranscribing centromeric PO41 repeats and *Bgl*II-repeat clusters (**e** DNA/RNA hybridization, FISH signals are absent; **e′**, DNA/DNA hybridization, both repeats are detected). **f** The smallest microchromosome contains PO41 repeat (*green signal*) in its centromere region, the centromere position being detected by immunostaining with an antibody against STAG2 (*red signal*). **g** Transcription units bearing PO41 RNA hybridize with both C-rich and G-rich oligonucleotide probes, DNA/RNA hybridization. **h**, **h′** G-rich transcripts of the *Bgl*II repeat are revealed on pericentromeric lateral loops using FISH with oligonucleotide CCOneg complementary to the G-rich strand of the repeating unit (**h′**, DNA/RNA hybridization). Oligonucleotide CCOpos complementary to C-rich strand hybridizes to chromomeric DNA only (**h**, DNA/RNA hybridization, *red signal* is absent) (**i** DNA/DNA hybridization, *red signal*). **j** A microchromosome after FISH with PO41 (*green*) and *Bgl*II-repeat (*red*) probes shown at higher magnification than chromosomes on panels **a**–**i** and **k**. **j′**, **k′** Diagrams to illustrate how the labeling patterns arise on transcription units (TU) indicated on panels **j** and **k**. Sister loops on panel **k** display different transcription patterns: one long transcription unit (TU7) in the first loop and two TUs (TU8, TU9), which differ in the sequences they contain, in the other loop. PO41 repeat colored *green*; *Bgl*II repeat colored *red*. *Arrows* indicate directions of transcription. Chromosomes are counterstained with DAPI. *Scale bars* 10 μm

contrast to the CNM repeat, the majority of PO41 transcription units bind both PO41pos and PO41neg oligonucleotide probes (Fig. 3g, h and h′). As can be seen in Fig. 3, PO41 and *Bgl*II repeats are transcribed either independently in different transcription units (Fig. 3a–d and j) or continuously without truncation in one unit (Fig. 3h, h′, j, and k). In the latter case, PO41 transcripts are C-rich, whereas *Bgl*II-repeat transcripts are G-rich (Figs. 3h, h′ and 5f, g).

FISH with single-stranded oligonucleotide probes not only permits determination of the transcribed strand but also shows the organization of the repeats within clusters: CNM

in chicken and the *Bgl*II repeat in quail are preferentially organized in long tandem arrays with repeating units oriented head to tail, whereas within long transcribing clusters of the PO41 tandem repeat, there are short tracks of inverted repeats.

Composition of the transcription units bearing 41-bp tandem repeats

To confirm the ongoing transcription on loops bearing 41-bp tandem repeats, we analyzed dynamics of BrUTP incorporation into RNA of these loops. After a 1-h pulse of BrUTP, newly synthesized RNA was restricted to the axis of loops; pericentromeric loops did not differ from the majority of normal lateral loops. The polymerase seems to be more or less uniformly distributed along the axis, so that during a short labeling period, each polymerase will move the same distance and hence incorporate the same amount of label. At 3–4 h after BrUTP injection, transit of the polymerase around the loop makes it possible to see completely labeled matrix on most of the loops, including satellite DNA bearing loops (Fig. 4a–c). These results imply that transcription of noncoding tandem repeats is very rapid in oocytes at the lampbrush stage.

Immunostaining with antibodies against the hyperphosphorylated elongating form of RNA polymerase II (mAbs H14 and V22), following by FISH to RNA transcripts, showed that Pol-II-dependent transcription occurs on loops bearing 41-bp repeats—CNM, PO41, and *Bgl*II repeats (Fig. 4d, d′ and d″). At the same time, we did not see splicing snRNPs in the matrix of these loops, as is typical of most Pol-II loops (Fig. 4e–i).

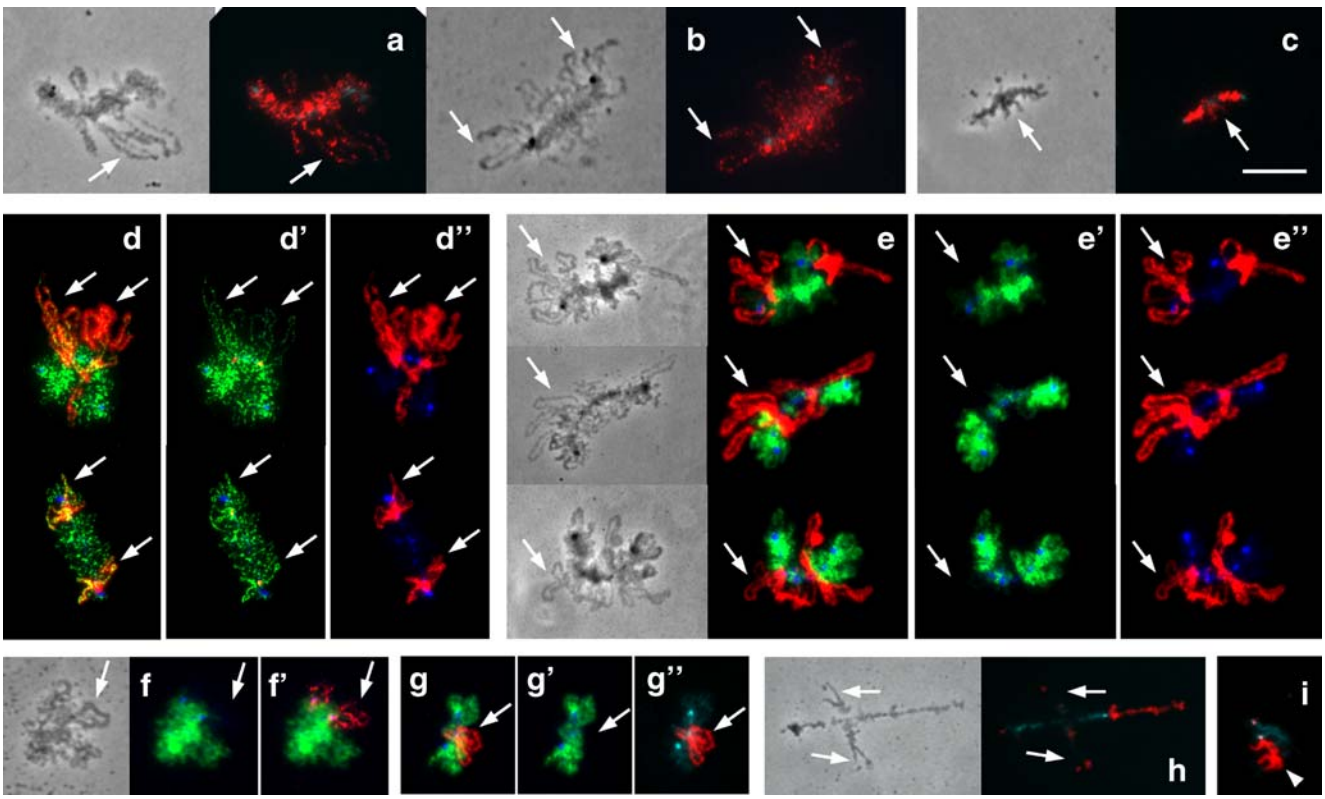
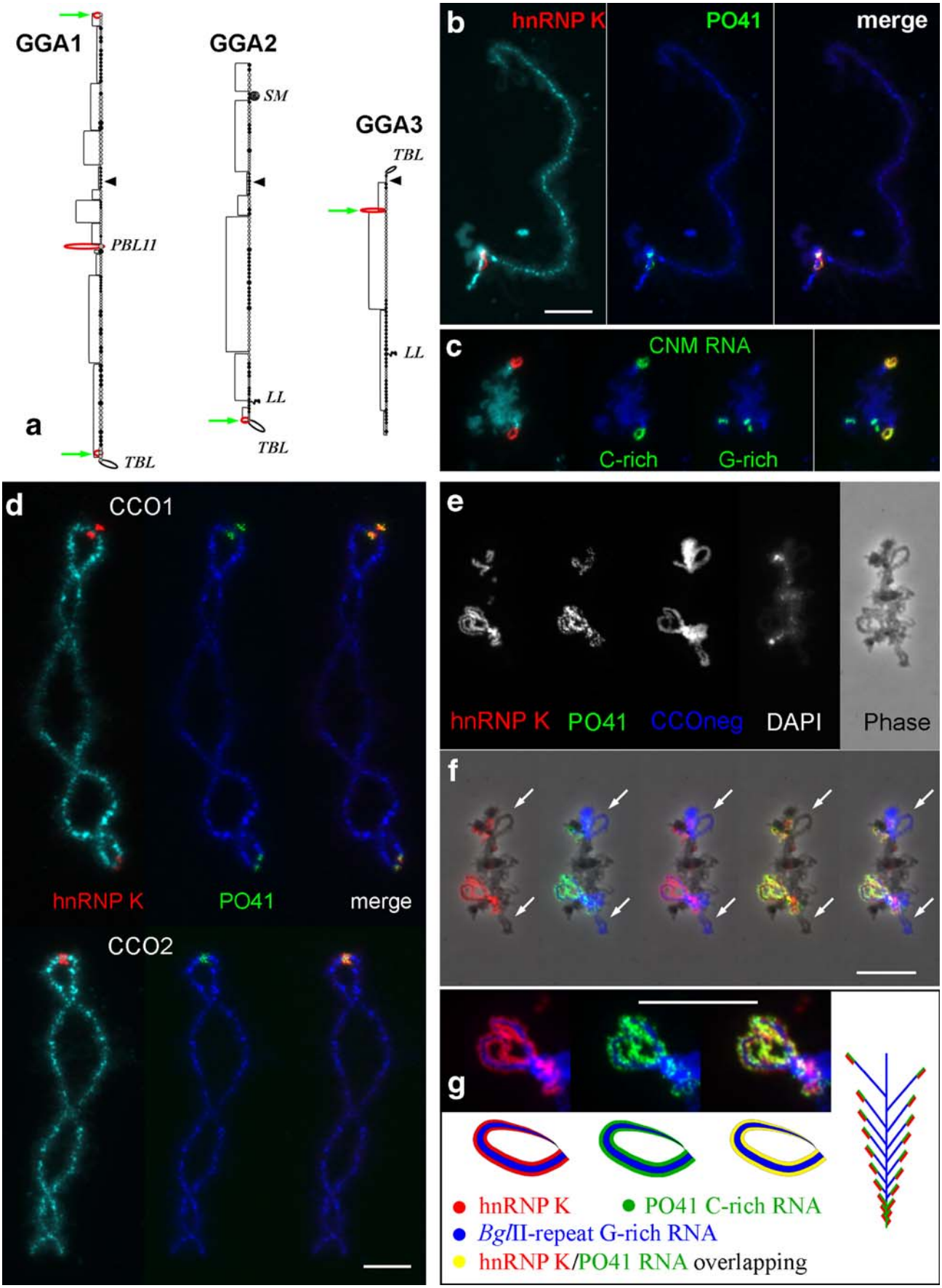


Fig. 4 Ongoing transcription of satellite 41-bp repeats in chicken and Japanese quail oocytes during the lampbrush phase. Quail (**a**, **b**) and chicken lampbrush chromosomes (**c**) from oocytes injected with BrUTP and detected with anti-BrdU antibody (*red signal*). **d**, **d′** Immunodetection of hyperphosphorylated elongating RNA polymerase II with mAb V22 (*green signal*) in the axes of loops containing *Bgl*II-repeat RNA (*red signal*) (**d**, **d″**), centromere position detected by immunostaining with an antibody against STAG2 (*red signal* on panel **d′**). **e**, **e′** Immunostaining with mAb Y12 against the Sm epitope of snRNPs (*green signal*) following by FISH with oligonucleotide CCOneg to *Bgl*II-repeat RNA (*red signal*) on quail lampbrush chromosomes (**e**, **e″**). **f**, **f′** Immunostaining with mAb Y12 (*green signal*) following by FISH with CNM probe (*red signal*) on a chicken lampbrush chromosome, CNM transcribing on centromere loops. **g**, **g′** Immunostaining with mAb Y12 (*green signal*) following by FISH with CNM probe (*red signal*) on a chicken lampbrush chromosome (**g**, **g″**), CNM transcribing on q-terminal loops. **h** Immunostaining of a chicken lampbrush chromosome with mAb K121 against the trimethylguanosine cap (*red signal*); splicing snRNAs are undetectable in the matrix of q-terminal lateral loops, on which transcripts of CNM repeat are detected by FISH (**i**). *Left panels* on **a**–**c**, **e**, **f**, and **h** are phase contrast images. *Arrows* indicate lateral loops with satellite RNA. Chromosomes are counterstained with DAPI. *Scale bar* 10 μm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GGA1
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G-rich
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hnRNP K
PO41 C-rich RNA
BglII-repeat G-rich RNA
hnRNP K/PO41 RNA overlapping

◀ **Fig. 5** Immunostaining with mAb 3C2 against hnRNP protein K following by FISH with satellite DNA probes on chicken (**a**–**c**) and quail lampbrush chromosomes (**d**–**g**). **a** Schemes of chromomeric-loop patterns of the first three chicken lampbrush chromosomes (GGA1, GGA2, GGA3) showing the set of loops that bind mAb 3C2 (colored *red*) and hybridize with PO41 (GGA1, GGA2) or CNM (GGA3) probes (indicated by *green arrows*). *Axial dots* represent DAPI-stained chromomeres. *Long brackets* show the average loop lengths. *Arrowheads* indicate centromeric regions. *LL* Nontranscribing lumpy loops; *PBL11* marker loop, which is known to bind C-rich single-stranded nucleic acids and mAb 3C2 (Solovei et al. 1995), but neither CNM nor PO41 probes; *SM* spaghetti marker; *TBL* telomere bow-like loops, which are known to contain Z chromosome macrosatellite DNA intensely transcribed during the lampbrush stage (Hori et al. 1996). **b** Chicken lampbrush bivalent ZW after immunostaining with mAb 3C2 (*red signal*) and FISH with PO41 probe (*green signal*). The hnRNP K enriched sites on the chicken ZW bivalent are PO41 transcribing loops. **c** FISH on a chicken lampbrush microbivalent with CNM oligonucleotide probes complementary to either C-rich strand (CNMneg, *green signal*) or G-rich strand (CNMpos, *green signal*) after immunostaining with mAb 3C2 (*red signal*). Hn RNP K colocalizes with C-rich CNM transcripts but not with G-rich ones. **d** Quail lampbrush chromosomes 1 (CCO 1) and 2 (CCO 2) after immunostaining with mAb 3C2 (*red signal*) and FISH with oligonucleotide PO41pos complementary to the C-rich strand of PO41 repeat (*green signal*). **e**–**g** A quail lampbrush microbivalent after immunostaining with mAb 3C2 against hnRNP K (*red signal*) followed by FISH with the oligonucleotide PO41pos against C-rich PO41 RNA transcripts (*green signal*) and oligonucleotide CCOneg complementary to *Bgl*II-repeat transcripts (*blue signal*). **e** Corresponding fluorescent signals and phase contrast image in *black and white*. **f** Colored hnRNP K immunostaining (*red*) and FISH signals (PO41—*green*, *Bgl*II repeat—*blue*) accompanied by three combinations of overlapping signals are shown on *top* of the phase contrast image. *Arrows* indicate loops with *Bgl*II-repeat transcripts only. **g** Enlarged representative loops. Schematic drawing shows the labeling pattern: hnRNP K is detected in RNP matrix that contains C-rich PO41 satellite RNA but not in RNP matrix that contains G-rich *Bgl*II-repeat RNA. Chromosomes are counterstained with DAPI. *Scale bar* 10 μm

The pattern of loops containing C-rich CNM and PO41 transcripts on chicken LBCs reminded us of the C-rich RNA binding loops that are enriched for the heterogeneous nuclear ribonucleoprotein complex K protein (hnRNP K) (Solovei et al. 1995). FISH with CNM and PO41 probes after immunodetection of hnRNP K revealed C-rich transcripts of these repeats in the hnRNP-K-enriched matrix (Fig. 5a–c). In Japanese quail, mAb 3C2 against hnRNP K stained the transcription units that contain C-rich RNA transcripts of the PO41 repeat but did not stain those with G-rich RNA of the *Bgl*II repeat only (Fig. 5d–g). These patterns were predictable because preferential binding of protein K to C-rich RNA is known (Matunis et al. 1992). In contrast to the binding of the single-stranded, C-rich homopolymeric probe, described by Solovei et al. (1995), FISH with CNM and PO41 probes is RNase sensitive. Nevertheless, RNase treatment of fixed LBCs does not eliminate hnRNP K staining. These observations confirm that the hybridization of oligonucleotides is to complementary RNA transcripts.

Discussion

Using three 41-bp tandem repeats in the chicken and the Japanese quail—PO41, CNM, and the *Bgl*II repeat—we have demonstrated that pericentromeric and subtelomeric regions share homology in satellite DNA. Both the pericentromeric and subtelomeric clusters of the repeats are transcribed intensely in oocytes at the lampbrush stage during diplotene of meiotic prophase I. These data imply that the same machinery might be involved in the process of centromeric and subtelomeric chromatin formation.

The transcripts of 41-bp repeats in chicken and quail oocytes are just a few of many examples of satellite transcription during the lampbrush phase of oogenesis. Satellite RNAs on LBCs were revealed in newts *Triturus cristatus* (Varley et al. 1980, Baldwin and Macgregor 1985), *Triturus vulgaris* (Barsacchi-Pilone et al. 1986), and *Notophthalmus viridescens* (Diaz et al. 1981; Epstein et al. 1986), in the frog *Xenopus laevis* (Jamrich et al. 1983), and in the birds *G. gallus domesticus* (Solovei et al. 1994; Hori et al. 1996), *Columba palumbus*, and *Columba livia* (Solovei et al. 1996). These clustered repeats may be centromeric (Diaz et al. 1981; Baldwin and Macgregor 1985; Barsacchi-Pilone et al. 1986; Solovei et al. 1996), subtelomeric (Jamrich et al. 1983; Solovei et al. 1994; Hori et al. 1996), or dispersed (Varley et al. 1980; Jamrich et al. 1983; Epstein et al. 1986). Their transcription was shown to be species specific and to occur in some instances from both strands (Diaz et al. 1981; Jamrich et al. 1983). We have shown by the use of single-stranded oligonucleotide probes that sequences from both strands of CNM and PO41 repeats are present in transcripts on loops in the chicken and the Japanese quail. The transcription of both strands of satellite DNA could result in double-stranded RNA (dsRNA). It is tempting to assume that long dsRNAs might be processed into small interfering RNA (siRNA), which is known to be involved in heterochromatin formation (Almedia and Allshire 2005; Kim 2005; Matzke and Birchler 2005; Prasanth and Spector 2007). However, it is still unknown if noncoding RNA products transcribed from different strands in far-away loci can hybridize to form long dsRNA. If the formation of long dsRNA is required, the following question emerges. What mechanisms are triggered if nascent RNA complementary to one strand of a repeat is detected, as in the case of the *Bgl*II repeat in quail (our data) or satellite 2 in *N. viridescens* (Epstein et al. 1986)? On the other hand, the absolute necessity of repetitive RNA processing with formation of repeat-associated siRNA (rasiRNA) is not obvious. In fact, centromere-encoded RNAs in maize, both sense and antisense strands, but *not* small interfering RNAs, were tightly bound to centromeric histone H3 (Topp et al. 2004).

The comparative analysis of the transcription patterns of a variety of amphibian and avian satellites (Varley et al. 1980;

Diaz et al. 1981; Jamrich et al. 1983; Baldwin and Macgregor 1985; Barsacchi-Pilone et al. 1986; Epstein et al. 1986; Solovei et al. 1996; our data) suggests that satellite transcription does not depend on the repeating sequences themselves but rather on other sequences with which the transcribing satellites are associated. The most striking example is the transcription of satellite 1 of *Notophthalmus*, which occurs coordinately with histone gene transcription, the satellite transcribing by read-through from histone gene promoters (Diaz et al. 1981). The read-through hypothesis postulates that transcription of satellite sequences begins at a promoter of a structural gene but proceeds through termination signals into the downstream satellite region (Varley et al. 1980; Diaz et al. 1981; Diaz and Gall 1985). The failure of termination signals seems to be general in transcripts of LBCs. The read-through transcripts produced during the lampbrush stage might be required for events after oocyte maturation and the start of embryo development. These transcripts, which are polyadenylated and accumulate in the oocyte, can be utilized in the absence of RNA synthesis in the period from oocyte maturation to activation of zygotic gene expression. In fact, in *Xenopus* oocytes, replication-dependent histone genes were found to transcribe through the stem-loop sequence, the cleavage site, and the U7 snRNA-binding site, continuing downstream to include an antisense transcript of adjacent histone genes. These read-through poly(A)+ transcripts are known to degrade by the mid-blastula transition, when expression from the zygotic genome commences, whereas when they are injected into the germinal vesicle, they can be converted to a precisely processed form (Masi and Johnson 2003). Thus, in *Notophthalmus*, the satellite 1 sequences in oocyte-specific "read-through" histone transcripts appear to be incidental due to satellite insertion into the read-through transcribing histone gene cluster.

Keeping in mind the read-through model of satellite transcription from upstream coding gene promoters, we screened the Chicken Genome databases available online (http://www.ncbi.nih.gov, http://www.girinst.org) to analyze sequences bordering the CNM and PO41 arrays; 107 contigs containing CNM and/or PO41 repeat sequences were found and analyzed. The results of this search are summarized in Fig. 6. Besides contigs containing open reading frames (ORF) for the endogenous avian retrovirus (ERV) pol gene, no other ORFs were found. Also, no 5S rRNA or U snRNA genes were found. However, long terminal repeat (LTR) sequences of the ERVs were present immediately adjacent to either CNM or PO41 arrays in a number of analyzed contigs (Fig. 6). Exactly the same LTR sequences were shown to contain fully functional promoters and to exist both in chicken and Japanese quail genomes (Boyce-Jacino et al. 1989). These findings allow us to predict that satellite transcription begins at LTR promoters rather than at more distant structural gene promoters. Alternative transcription of

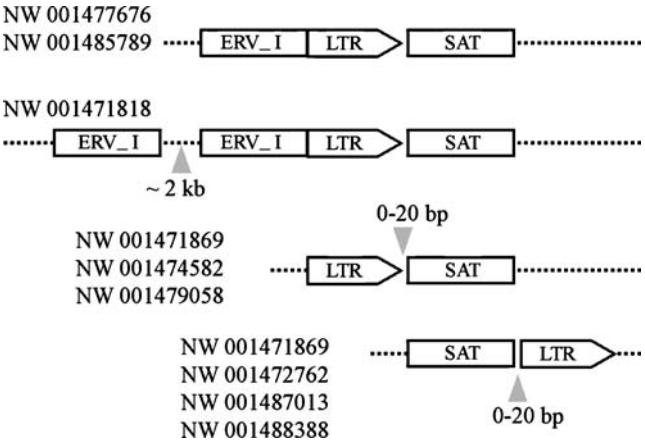

Fig. 6 Diagram of CNM and/or PO41 repeats with adjacent sequences based on Chicken build 2 genome database (Gallus_gallus-2.1) analysis. NCBI accession numbers for contigs containing CNM/PO41 repeats along with endogenous avian retrovirus sequences are listed. "SAT" indicates CNM/PO41 arrays. "ERV_I" indicates sequences of the 3′-part of endogenous avian retrovirus pol gene—3′ terminus of integrase open reading frame, the promoter region being absent. "LTR" indicates the sequence of endogenous avian retrovirus long terminal repeat, which is extremely short (229 or 243 bp) but is known to be an active promoter (Boyce-Jacino et al. 1989)

endogenous coding genes from LTR promoters is well known (Dunn et al. 2006). It is important to mention that LTRs are known to possess promoter activity in both directions (Christy and Huang 1988; Dunn et al. 2006) and that the contrary orientation of the LTR would not prevent its functioning as a promoter for adjacent satellite repeat transcription (Fig. 6). We believe that satellite transcription is more likely to begin normally at retrotransposon promoters, moreover at LTR promoters, rather than at normal promoters next to protein-coding regions. Our model is consistent with the facts that (1) retrotransposons are embedded in centromeric satellite DNA in plants (Topp et al. 2004; Ma and Jackson 2006), *Drosophila* (Sun et al. 2003), and mammals (Prades et al. 1996; Laurent et al. 1999) and that (2) retrotransposon promoters regulate only Pol-II-dependent transcription, as is the case for satellite transcription on LBCs (Fig. 4d).

Although it is clear that Pol-II transcription occurs on pericentromeric and subtelomeric satellite repeats in avian LBCs, it is intriguing that splicing snRNPs do not accompany the nascent RNA. A similar phenomenon was found in the newt *N. viridescens* whose pericentromeric giant loops on LBC2 were shown not to stain with mAb Y12 against the Sm-epitope of snRNPs (Pinol-Roma et al. 1989). These data suggest that satellite RNAs are not subject to cotranscriptional snRNP-dependent splicing. In fact, the machinery involved in typical mRNA splicing (Dreyfuss et al. 2002) might be quite different from that which is involved in cotranscriptional processing of satellite RNA.

Along with the lack of snRNPs, the presence of hnRNP K protein in loops that transcribe 41-bp repeats merits

special attention. HnRNP K is a multifunctional protein involved in chromatin remodeling, transcription, pre-mRNA splicing, and RNA export and translation (Dreyfuss et al. 2002; Bomsztyk et al. 2004). Noncoding satellite DNA is well known to transcribe from both strands in normal (Rudert et al. 1995; Klimek-Tomczak et al. 2006; Prasanth and Spector 2007) and stressed somatic cells (Jolly and Lakhotia 2006; Prasanth and Spector 2007). Satellite RNA–hnRNP K complexes were found in rat hepatoma cells, where transcripts originating from satellite I DNA were the most abundant RNAs that copurified with hnRNP K protein (Klimek-Tomczak et al. 2006). HnRNPs K and M were associated with noncoding RNAs in two cases: with the heat shock RNA omega in omega speckles in *Drosophila*, and with satellite III transcripts in nuclear stress bodies in human cells (Jolly and Lakhotia 2006). In bird oocytes, hnRNP K was found to associate with C-rich transcripts of satellite DNA. The giant loops in *N. viridescens* are similar to bird pericentromeric loops in their snRNP patterns and are enriched in hnRNP L (Pinol-Roma et al. 1989) that is known to interact with hnRNP K (Kim et al. 2000). Taken together, these data allow us to postulate a key role of hnRNP K protein in regulation of satellite DNA transcription and processing.

In conclusion, many questions remain open about satellite RNA transcription initiation and processing, as well as the fate of these RNAs in the oocyte. These aspects deserve further analysis. It is worth noting that chromatin-associated RNP complexes are difficult to analyze using biochemical approaches (Dreyfuss et al. 2002), whereas oocytes at the lampbrush stage provide a promising system in which to study the interactions between transcribing DNA, nascent RNA, and proteins in situ.

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