The Use of Primed *in situ* Synthesis (PRINS) to Analyze Nucleolar Organizer Regions (NORs) and Telomeric DNA Sequences in the Domestic Chicken Genome

Monika BUGNO-PONIEWIERSKA, Leszek POTOCKI, Beata BŁĄDEK, Klaudia PAWLINA, Maciej WNUK, Mariusz PIETRAS, and Ewa SŁOTA

Accepted May 15, 2013

BUGNO-PONIEWIERSKA M., POTOCKIL., BŁĄDEK B., PAWLINA K., WNUK M., PIETRAS M., SŁOTA E. 2013. The use of primed *in situ* synthesis (PRINS) to analyze nucleolar organizer regions (NORs) and telomeric DNA sequences in the domestic chicken genome. Folia Biologica (Kraków) **61**: 149-153.

One of the most often analyzed avian genomes is the domestic chicken genome (*Gallus domesticus*) whose diploid number is 2n=78. In the chicken karyotype, similarly to other birds, there is a group of microchromosomes for which the determination of morphology and banding pattern is impossible using classic cytogenetics methods. The aim of this study was to evaluate telomeric and rDNA repetitive sequences in the chicken genome by the PRINS technique as an alternative method to fluorescence *in situ* hybridization. This is the first report on the application of the PRINS method to locate these repetitive sequences in the chicken nuclei and metaphase chromosomes.

Key words: Chicken, chromosomes, PRINS technique, rDNA, telomeric DNA.

Monika BUGNO-PONIEWIERSKA, Klaudia PAWLINA, Laboratory of Genomics, National Research Institute of Animal Production, Krakowska 1, 32-083 Balice, Poland. Monika BUGNO-PONIEWIERSKA, Leszek POTOCKI, Beata BŁĄDEK, Maciej WNUK, Ewa SŁOTA, Department of Genetics, University of Rzeszow, 35-959 Rzeszów, Poland; Centre of Applied Biotechnology and Basic Sciences, University of Rzeszów, 36-100 Kolbuszowa, Poland. E-mail: monika.bugno@izoo.krakow.pl

Mariusz PIETRAS, Department of Animal Nutrition and Feed Science, National Research Institute of Animal Production, 32-083 Balice, Poland.

The chicken chromosomes (*Gallus domesticus*) are relatively difficult material to study because of their large diploid number and the presence of microchromosomes, the identification of which is impossible using classical cytogenetic methods. Therefore precise tools providing reliable results are necessary to study the chicken karyotype. These tools are offered by molecular cytogenetics and one of them is the PRINS technique - primed in situ synthesis (KOCH 1996), which combines elements of the PCR reaction and cytogenetic methods. This technique allows for the detection of small amounts of transcripts or single copies of genes at the cellular level; it also creates the possibility of detection of repetitive sequences (satellite sequences, rDNA sequences, telomeric sequences, dispersed Alu sequences) (GU & HINDKJAER 1996; COULLIN et al. 2002; WNUK et al. 2008; BUGNO--PONIEWIERSKA et al. 2009; WNUK et al. 2010). It also allows for the rapid identification of chromosomes by the detection of specific centromeric sequences (PELLESTOR *et al.* 1996). Furthermore, the PRINS technique in some aspects has advantages over the FISH technique. It is characterized by greater sensitivity and specificity, is less timeconsuming and laborious and applied primers are for specific sequences, reducing the risk of nonspecific and background signals. The application of short oligonucleotide primers allows for the localization of even short DNA sequences (PELL-ESTOR 2006) and moreover, there is no risk of the lack of cell structure penetration, which may occur when the FISH method with large probes is used.

Recent analysis of the chicken genome has shown that microchromosomes, despite their small size, are richer in coding sequences than macrochromosomes (ICGSC 2004). In spite of the fact that they constitute only 23% of the total DNA, they comprise 50% of all genes (NANDA *et al.* 2002). Therefore it is important to examine them while studying the avian karyotype. The NOR regions are responsible for nucleolus synthesis, a structure managing the transcription and synthesis of 18S, 5.8S and 28S ribosomal subunits (HADJIOLOV 1985). In the chicken genome, they have been widely studied at the cellular, genetic and molecular levels, in order to understand the role of rDNA genes during the development and growth of an organism (SCHMID *et al.* 2005). The rDNA genes in the chicken karyotype are located on the 16th microchromosome (MASA-BANDA *et al.* 2004).

Research on the occurrence of telomere sequences in different avian species showed that these sequences are conservative and microchromosomes are richer in telomere sequences than macrochromosomes (NANDA et al. 2002), and, in extreme cases, are almost entirely covered by them (DELANY et al. 2000). Such numerous repetitions of telomere sequences in chicken microchromosomes may be caused by the amplification of a telomere sequence or may indicate high recombination frequency (NANDA et al. 2002). Despite the fact that the avian genome constitutes only 1/3 the size of an average mammalian genome, telomere sequences constitute 4% of the avian genome (0.3-0.4% in mammals). The abundance of telomere sequences in the avian genome, the activity of telomerase in embryonal cells and the relatively long life span of birds compared to mammals, make them a good model for research on the dynamics of telomeres during in vivo and in vitro ageing (TAYLOR & DELANY 2000).

Material and Methods

Embryo metaphase preparation

Cells obtained from blastodiscs of domestic chicken eggs incubated for 16 to 18 h at 37°C were incubated in RPMI medium with 0.05% colchicine at 37°C for 3 h. After centrifugation (5 min, 900 rpm/min) and disposal of supernatant, 0.8% sodium citrate was added up to 5 ml and the specimens were incubated for 5 min at room (21°C) temperature. After another centrifugation (the same parameters) and disposal of supernatant, 4 ml of Carnoy's fixative (methanol:acetic acid 3:1) cooled at 5°C was added. The mixture was incubated for 20 min in a refigerator at 4°C; then the centrifugation and disposal of supernatant were repeated and a new portion of the fixative was added. The suspension of the fixed metaphase cells was used for preparations.

PRINS technique with the use of primers specific for repeated sequences (telomeric DNA, rDNA) of the domestic chicken

Fixed metaphase chromosomes obtained after standard procedure were treated with 100 μ g/ml RNase in 2 x saline sodium citrate (SSC) buffer in a humidified chamber at 37°C for 1 h for better results. After RNase treatment, slides were washed three times with 2xSSC and then were treated with 0.01% pepsin in 10 mM HCl at 37°C for 30 min. The slides were then washed twice with PBS (pH 7.4) and once with PBS with 50 mM MgCl₂ and passed through a set of ethanol solutions (70%, 80% and 95%).

PRINS with oligonucleotides complementary to rRNA

Primers specific for the mammalian 18S ribosomal RNA gene were used (Genomed, Poland):

-Forward: 5'CCACGCTCTCGCCAACGTTAAT3'

-Reverse: 5'CGCTGTCCCACCGTCTCCTA3'

The PRINS reaction mixture in a total volume of 25 μ l per slide contained: 0.2 mM dATP, dCTP and dGTP; 0.025 mM dTTP; 1 nmol biotin-16dUTP; 0,2 U Ampli *Taq* GOLD polymerase (Invitrogen, Germany); 1x *Taq* polymerase buffer with MgCl₂, 0.2 μ M Fwd primer and 0.2 μ M Rev primer, and sterile distilled water (milliQ).

The PRINS reaction mixture was applied on a slide, covered with a coverslip and sealed with fixogum to prevent evaporation. Reaction conditions were as follows: denaturation at 95°C for 5 min; annealing at 57°C for 15 min and extension at 68°C for 15 min. The PRINS reaction was stopped by transferring the slides to stop buffer containing 50 mM NaCl and 50 mM EDTA (pH 8.0) at 58°C for 5 min and next to the same buffer (50 mM NaCl and 50 mM EDTA, pH 8.0) at room temperature for 7 min.

PRINS with oligonucleotides complementary to telomeric DNA

Primers specific to both forward and reverse telomeric DNA strands were used ((TTTAGGG)₇ and (CCCTAA)₇ respectively).

The PRINS reaction mixture in a total volume of 10 μ l per slide contained: 0.2 mM dATP, dCTP and dGTP; 0.025 mM dTTP; 1 nmol biotin-16dUTP; 0.2 U Ampli *Taq* GOLD polymerase (Invitrogen, Germany); 1x *Taq* polymerase buffer with MgCl₂, 0.4 μ M Fwd primer and 0.4 μ M Rev primer, and sterile distilled water (milliQ). Reaction conditions were: denaturation at 94°C for 2 min; annealing at 58°C for 30 min. The PRINS reaction was stopped by transferring the slides to stop buffer

containing 50 mM NaCl and 50 mM EDTA (pH 8.0) at 58°C for 5 min and next to the same buffer (50 mM NaCl and 50 mM EDTA, pH 8.0) at room temperature for 7 min.

The detection of biotin labeled sequences

Biotin Painting Kit (3 Step) with FITC (Cambio UK) were used to visualize biotin-labeled PRINS products. Chromosome staining was performed with DAPI. Photos were captured in a fluorescence microscope Olympus BX61 equipped with a DP72 CCD camera and Olympus CellF software. The mean telomeric DNA area in an interphase nuclei of the chicken was measured with the TFL-TELO (Telomere Measurements and Analysis) Software. Telomere length was expressed as the mean telomere area per cell which is the equivalent of the fluorescence area (number of pixels) occupied by a single spot.

Results and Discussion

Using primed *in situ* synthesis (PRINS) with two specific sets of primers and 16-d-UTP nucleotide, we studied the chromosomal distribution of the highly conserved telomere repeats as well as 18S rDNA sequences of *Gallus domesticus* (Figs 1-3).

The PRINS reaction successfully labeled telomeric sequences in metaphase spreads and interphase nuclei of the chicken embryo (Fig. 1 A and B). Moreover, the PRINS profile of telomeric sequences in chicken embryos showed terminal fluorescence signals (FITC) in most chromosomes including macrochromosomes and microchromosomes as well as interstitial telomeric sequences on some of the largest macrochromosomes (Fig. 1B). Additionally, Quantitative – PRINS (Q-PRINS) analysis performed in 50 embryo nuclei revealed 86.25 ± 24.18 of the mean telomere area per cell (Fig. 1A).

Summarizing, PRINS telomere labeling showed three major features of the chicken karyotype a) telomeric sequences on all macrochromosomes and microchromosomes b) the existence of interstitial telomeric DNA sequences on the macrochromosomes c) no fluorescence telomeric signals in the centromere regions. These observations are consistent with studies carried out previously by the FISH technique (DELANY *et al.* 2000; NANDA *et al.* 2002; SWANBERG *et al.* 2010).

DELANY *et al.* (2000), NANDA *et al.* (2002) and SWANBERG *et al.* (2010) concluded that avian telomeric repeat arrays are generally bigger than in mammals and are organized into at least three classes. Each of these classes is characterized by a specific size and/or location. Class I arrays display



Fig. 1. The PRINS technique with the use of primers specific for telomeric sequences in interphase nuclei (A) and metaphase chromosomes (B) of the domestic chicken.

a distinctive profile of interstitial telomeric DNA arrays ranging from 0.5 to 10 kb in length on several macrochromosomes of the chicken (SWAN-BERG *et al.* 2010). The ends of chromosomes are occupied by class II and class III arrays ranging from 10 to 40 kb and from 200 kb to 4 Mb respectively (DELANY *et al.* 2000; DELANY *et al.* 2007; O'HARE & DELANY 2009; RODRIGUE *et al.* 2005).

Our results also confirmed the earlier observation that fluorescence signals localized on microchromosomes were generally stronger than on macrochromosomes (DELANY *et al.* 2000). This phenomenon can be explained by a higher frequency of recombination or the amplification of telomeric sequences in/on the microchromosomes which are richer in these sequences than the macrochromosomes (NANDA *et al.* 2002).



Fig. 2. Chicken metaphases with rDNA sequences labeled with PRINS. Two microchromosomes with green fluorescence signals indicated by arrows are shown in magnification in the upper and lower corner of the microphotograph.

Comparative alignment analysis performed by SCAN2 software by Softberry (<u>http://linux1.softberry.com/berry.phtml</u>) for aligning two multimegabyte-size nucleotide sequences revealed that our set of primers is partially complementary to 18S and 28S rDNA sequences (SHAO *et al.* 2009). SCAN2 analysis showed at least one significant alignment between our primers and chicken 18S rRNA and 28S rRNA genes.

The PRINS technique with the use of rDNAspecific oligonucleotides gave fluorescence signals on two microchromosomes (Fig. 2). These results are consistent with previous observations (DELANY *et al.* 2009).

Additionally, the fluorescence labeling of rDNA sequences was analyzed in interphase nuclei of the chicken embryo (Fig. 3). A total of 100 chicken embryo cells were analyzed. The rDNA regions appeared variable in relative size and intensity of fluorescent signals. Cells consistently showed an extensive single rDNA focus (31% of cells) (Fig. 3. A-F), double rDNA foci (35% of cells) (Fig. 3. G-I) and a compact single rDNA focus



Fig. 3 Intercellular variation of rDNA foci in chicken interphase nuclei. Representative microphotography of different types of rDNA signals in chicken embryonic cells with extensive single rDNA focus (A-F), double rDNA foci (G-I) and compact single rDNA focus (J-L), ×100.

(34% of cells) (Fig. 3. J-L). Most fluorescence signals were located peripherally in cells (Fig. 3). We further characterized a set of subpopulations on the basis of the size and shape of rDNA labeled foci. Cells with a single signal most often demonstrated a conspicuous ovoid rDNA region which was diffuse in appearance and had a generally equatorial location (Fig. 3. A-F). Changes of fluorescence signal foci detected in interphase nuclei (number and size, intensity) may reflect dynamic (probably epigenetic) processes that occur during the growth and differentiation of a cell (DELANY et al. 1995; DELANY et al. 2009; JACOB 1995). The correct number and structure of repetitive sequences are very important for normal development of chicken embryos (DELANY et al. 1995). A reduced number of repetitions of rDNA genes can induce lethality (DELANY et al. 1995); while an increased number of microchromosomes with NOR regions in aneuploid lines of chickens did not cause visible effects, however, it poses questions concerning the regulation of additional rDNA genes. This is due to the fact that rRNA synthesis is closely related to the cellular ability to produce proteins determining the size of the nucleolus organizing region (gene copy number) and its expression which have a major impact on the growth and differentiation of a cell, and on all levels of growth and development (DELANY et al. 1995; DELANY et al. 2009; JACOB 1995).

The application of the PRINS technique showed that it is an attractive method to locate repetitive sequences in the domestic chicken. The PRINS method can be used to compare the domestic chicken genome with genomes of other economically important poultry species.

References

- BUGNO-PONIEWIERSKA M., WNUK M., WITARSKI W., SLOTA E. 2009. The fluorescence *in situ* study of highly repeated DNA sequences in domestic horse (*Equus caballus*) and domestic donkey (*Equus asinus*) – Advantages and limits of usefulness in phylogenetic analyses. J. Anim. Feed Sci. **18**: 723-732.
- COULLIN P., ROY L., PELLESTOR F., CANDELIER J.J., BED-HOM B., GUILLIER-GENCIK Z., BERNHEIM A. 2002. PRINS, the other *in situ* DNA labeling method useful in cellular biology. Am. J. Med. Genet. **107**: 127-135.
- DELANY M.E., KRUPKIN A.B., MILLER M.M. 2000. Organization of telomere sequences in birds: evidence for arrays of extreme length and for in vivo shortening. Cytogenet. Cell Genet. **90**: 139-145.
- DELANY M.E., ROBINSON C.M., GOTO R.M., MILLER M.M. 2009. Architecture and organization of chicken microchromosome 16: order of the NOR, MHC-Y, and MHC-B subregions. J. Hered. **100**: 507-514.

- DELANY M., TAYLOR R., BLOOM S. 1995. Teratogenic development in chicken embryos associated with a major deletion in the rRNA gene cluster. Develop. Growth Differ. **37**: 403-421.
- GU F., HINDKJAER J. 1996. Primed *in situ* labeling (PRINS) detection of the telomeric (CCCTAA)n sequences in chromosomes of domestic animals. Mammalian Genome 7: 231-232.
- HADJIOL OV A.A. 1985. The nucleolus and ribosome biogenesis. (In: Cell Biology Monographs. A.M. Beerman, L. Goldstein, K.R. Portrer, P. Sitte eds. NewYork): 1-263.
- ICGSC 2004. Sequence and Comparative Analysis of the Chicken Genome Provide Unique Perspectives on Vertebrate Evolution. Nature **432**: 695-716.
- JACOB S.T. 1995. Regulation of ribosomal gene transcription. Biochem. J. **306**: 617-626.
- KOCH J. 1996. Primed *in situ* labeling as a fast and sensitive method for the detection of specific DNA sequences in chromosomes and nuclei. Methods 9: 122-8.
- MASABANDA J.S., BURT D.W., O'BRIEN P.C., VIGNAL A., FILLON V., WALSH P.S., COX H., TEMPEST H.G., SMITH J., HABERMANN F., SCHMID M., MATSUDA Y., FERGU-SON-SMITH M.A., CROOIJMANS R.P.M.A., GROENEN M.A.M., GRIFFIN D.K. 2004. Molecular cytogenetic definition of the chicken genome: The first complete avian karyotype. Genetics **166**: 1367-1373.
- NANDA I., SCHRAMA D., FEICHTINGER W., HAAF T., SCHARTL M., SCHMID M. 2002. Distribution of telomeric (TTAGGG)n sequences in avian chromosomes. Chromosoma 111: 215-227.
- O'HARE T.H., DELANY T.H. 2009. Genetic variation exists for telomeric array organization within and among the genomes of normal, immortalized, and transformed chicken systems. Chromosome Res. 17: 947-964
- PELLESTOR F. 2006. *In situ* aneuploidy assessment in human sperm: the use of primed *in situ* and peptide nucleic acid-fluorescence *in situ* hybridization techniques. Asian J. Androl. **8**: 387-392.
- PELLESTOR F., GIRARDET A., COIGNET L., ANDRÉO B., CHARLIEU J.P. 1996. Assessment of an euploidy for chromosomes 8, 9, 13, 16, and 21 in human sperm by using primed *in situ* labeling technique. Hum. Genet. **58**: 797-802.
- RODRIGUE K.L., MAY B.P., FAMULA T.R., DELANY M.E. 2005. Meiotic instability of chicken ultra-long telomeres and mapping of a 2.8 megabase array to the W-sex chromosome. Chromosome Res. **13**: 581-591
- SHAO P., YANG J., ZHOU H., GUAN D., QU L. 2009. Genome-wide analysis of chicken snoRNAs provides unique implications for the evolution of vertebrate snoR-NAs. BMC Genomics **10**: 86.
- SWANBERG S.E., O'HARE T.H., ROBB E.A., ROBINSON C.M., CHANG H., DELANY M.E. 2010. Telomere biology of the chicken: a model for aging research. Exp. Gerontol. **45**: 647-654.
- TAYLOR H.A., DELANY M.E. 2000. Ontogeny of telomerase in chicken: impact of down regulation on pre- and postnatal telomere length in vivo. Dev. Growth Differ. **42**: 613-621.
- WNUK M., BUGNO M., SLOTA E. 2008. Application of primed *in situ* DNA synthesis (PRINS) with telomere human commercial kit in molecular cytogenetics of *Equus caballus* and *Sus scrofa scrofa*. Folia Histochem. Cytobiol. **46**: 85-88.
- WNUK M., OKLEJEWICZ B., LEWINSKA A., ZABEK T., BAR-TOSZ G., SLOTA E., BUGNO-PONIEWIERSKA M. 2010. PRINS detection of 18S rDNA in pig, red fox and Chinese raccoon dog, and centromere DNA in horse. Hereditas 147: 320-324.