A HIGHLY REPEATED FCP CENTROMERIC SEQUENCE FROM CHAFFINCH (*FRINGILLA COELEBS: AVES*) GENOME IS REVEALED WITHIN INTERCHROMOSOMAL CONNECTIVES DURING MITOSIS

© *A. F. Saifitdinova*,[1] *L. P. Timofejeva*,[2] *V. G. Zhurov*,[1] *E. R. Gaginskaya*[1]

[1] *Biological Institute of St. Petersburg University,*
[2] *Oranienbaumskoye sch., Stary Peterhof, St. Petersburg, egag@chromo.lgu.spb.su &*
[2] *Institute of Experimental Biology of Estonian Agricultural University, Harku*

A highly repeated FCP (*Fringilla coelebs PstI* element) sequence was localized by FISH in centromeric regions of all chromosomes of the chaffinch. Besides, FISH signal was found also in interchromosomal connectives linking centromeres of non-homologous chromosomes in mitotic cells. The presence of DNA in the connectives was confirmed by immunostaining with anti-dsDNA antibodies as well as in experiments on nick-translation and random primed labeling *in situ*. Non-denaturing FISH with FCP probe and random primed labeling of non-denatured chromosomes resulted in fluorescence signal on both centromeres and intercentromeric connectives, thus providing evidence for the availability of single-strand DNA tracts in FCP sequence. It is suggested that the highly repeated FCP centromeric sequence may be respondible for interconnection of mitotic chromosomes and may by involved in nuclear architecture maintenance in the chaffinch.

Key words: centromeres, *Fringilla coelebs* (*Aves*), mitotic chromosomes, interchromosomal connectives, repeated DNA sequences.

The problem of spatial chromosome arrangement in the nucleus has been attracting attention since the famous works by Rabl (1885) and Boveri (1888). Non-random distribution and arrangement of chromosomes was shown both during interphase and in dividing cells (Navashin, 1947; Takayama, 1975, 1976; Chiarelli et al., 1977; Avivi, Feldman, 1980; Hilliker, Appels, 1989; Nagele et al., 1995, 1998; Klein et al., 1998; Visser et al., 1998; Zalensky, 1998). In the 1970s, end-to-end side-by-side associations between non-homologous metaphase chromosomes were repeatedly observed by light and electron microscopy. Interchromosomal connectives were found by Chiarelli et al. (1977) and hypothesized to result from end-to-end contacts between chromosomes. Other authors watched connections between centromeres, and speculated that a centromeric ring might be maintained in the eukaryotic nucleus throughout the cell cycle (Mosolov, 1973). Using living metaphase cells from mouse fibroblasts, HeLa, rat kangaroo kidney, and Chinese hamster lung, Takayama (1975, 1976) demonstrated that the interchromosomal connectives were neither artefacts of colchicine or hypotonic treatments, no something attrbutable to spindle elements. Twenty years later, a method of fluorescence *in situ* hybridization (FISH), using centromere- and telomere-specific probes, and chromosome painting techniques were introduced to further explore the nuclear architecture. The centromeres of mitotic chromosomes were shown to form a ring with chromosome arms extending outwards in a rosette pattern (Ferguson, Ward, 1992; Nagele et al., 1995; Slijepcevic et al., 1997). More recently, elegant studies by Nagele et al. (1998) provided evidence that chromosomes within rosettes were segregated into haploid sets, with each chromosome occupying a preferred position within a set. Moreover, these authors were able to demonstrate that the spatial organization of chromosomes could be maintained from gestation to adulthood.

It is now clear that centromeric associations between non-homologous chromosomes do exist in mitosis and may serve for maintaining non-random spatial chromosome arrangement within the cell. However, the structural and mechanical bases of mitotic interchromosomal associations remain unknown. Nevertheless, some evidence exists that satellite DNA is involved in interchromosomal interconnections of other types. For example, ectopic pairing is thought to result from interchromosome conjugation between homologous DNA sequences of heterochromatic regions, and ectopic threads, binding non-homologous polytene chromosomes in *Diptera*, were shown by *in situ* hybridization to contain satDNA (Hankeln et al., 1994). In *Drosophila* mitotic cells, dodecasatellite DNA was revealed by FISH in a string-like structure linking sister chromatids at the region of chromosomal localization (Carmena et al., 1993). H. Macgregor has kindly informed us about his unpublished observation about apparent Feulgen-positive connectives between chromosomes in salamander meiotic cells (personal communication). As to the maintenance of chromosomal associations within mitotic cells, Ikemura and coauthors (1998) suggest that interactions between repeated centromeric DNA may constitute a general mechanism of interchromosome binding via formaton of intermolecular non-B DNA structures.

In this work we investigated a chromosomal localization of GC-enriched tandem repeat FCP (*Fringilla coelebs PstI* element) previously cloned from the genomic DNA of chaffinch (*Fringilla coelebs* L.) which is available under GenBank accession number AF160980. Corresponding FISH signals were observed in centromeric regions of all the mitotic chromosomes and, which is most important, in threads connecting centromeres of non-homologous chromosomes. We have demonstrated that the threads contain DNA, and concluded that the FCP centromere repeat may be responsible for interchromosome interactions during mitosis in the chaffinch.

Materials and methods

Chromosome preparation. Primary fibroblast cultures were isolated from 4—5 day chaffinch embryos (*Fringilla coelebs* L.) using standard methods. To prepare mitotic chromosomes, cells were treated with 0.1 % colchicine for 35 min followed by a 20 min soaking in hypotonic solution (1 % sodium citrate) and fixation overnight in methanol—acetic acid (3 : 1). Fixed cells were dropped onto glass slides, rinsed in 96 % ethanol, dried, and stored at –20 °C until use (the length of fixation is critical for DNA preservation within the interchromosomal connectives, since in preparations after a prolonged fixation they were not revealed). In some experiments, slides were stained with AT-specific fluorochrome DAPI in the presence of actinomycin D (Schweizer, 1981).

FCP probe. Highly repetitive sequences were obtained by digesting the chaffinch genomic DNA with *PstI* and cloning into the vector pTZ19U (Gaginskaya et al., in preparation). As previously found, the FCP repetitive unit is 505/506 bp in length, and contains 57 % G + C (GenBank accession number AF160980). The FCP insert was labeled with biotin-16-dUTP by PCR, using M13 forward and reverse primers. After lyophilization, the probe was dissolved in hybridization buffer (50 % formamide, 2 × SSC, 10 % dextran sulphate) to a concentration of 5 ng/μl.

Fluorescence *in situ* hybridization. Chromosome spreads were pre-treated with RNAse A and pepsin, and were used either without further treatment (non-denatured, native chromosomes), or were denatured in 70 % formamide in 2 × SSC at 70 °C for 2 min. The FCP probe was denatured for 5 min at 100 °C, and 20 μl of probe solution was added to each slide, which was then covered with a glass coverslip and sealed with rubber cement. After 12—18 hr hybridization at 37 °C in a moist chamber, preparations were washed in 50 % formamide (in 2 × SSC) at 42 °C, followed by washes in 2 × SSC. Hybridization reaction was detected using avidin DN conjugated with FITC (Vector Laboratories) and amplified using biotinilated anti-avidin D (Vector Laboratories) followed by another incubation with avidin-FITC. Chromosomes were counterstained with propidium iodide.

Immunocytochemistry. Chromosome preparations were pre-incubated in PBS containing 10 % normal donkey serum (Jackson ImmunoResearch) for 30 min, followed by overnight incubation at 4 °C in anti-dsDNA (Chemicon International; diluted 1 : 200 in PBS + 5 % donkey serum). Slides were then washed 3 times in PBS containing 0.02 % Tween-20, followed by 40 min incubation with FITC-conjugated goat anti-mouse IgG (ICN; 1 : 85 in PBS + 5 % donkey serum). After washing, slides were mounted into an anti-bleaching solution. Control slides were processed accordingly with the same protocol except anti-dsDNA treatment.

Nick-translation *in situ*. Chromosomal preparations were incubated for 1 hr at 16 °C in 50 μl nick-translation-solution (10 μM 2-mercaptoethanol, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 μg/ml BSA, 50 μM dNTP-mix) containing 20 μM biotin-16-dUTP and 10 units DNA polymerase I (Boehringer Mannheim), followed by incubating for 60 min at 16 °C, 30 min at 18 °C, and 30 min at 20 °C. The reaction was stopped by addition of 200 μl stop buffer (500 mM NaCl, 50 mM EDTA) per slide. After washing in 2 × SSC, newly-synthesized DNA was detected with avidin-FITC treatment as described above, and chromosomes were counterstained with propidium iodide.

Random-primed labeling *in situ*. Slides were random-primed with digoxigenin-11-dUTP using Random Primed DNA Labeling Kit (Boehringer Mannheim) overnight at 37 °C, according to the manufacturer's instructions. In control experiments, labeling mixture contained no primers. As indicated below, some samples were denatured for 2 min at 70 °C in 70 % formamide/2 × SSC, while other preparations were not denatured. The labeling reaction was stopped by addition of 500 mM NaCl + 50 mM EDTA, and slides were washed in 2 × SSC prior to staining with Cy-3-conjugated monoclonal mouse anti-digoxin IgG (Jackson ImmunoResearch). Chromosomes were counterstained with DAPI.

Microscopic analysis. Microscopy was carried out using a DMRXA fluorescence microscope (Leica Microsystems Wetzlar GmbH) equipped with a FLUOTAR × 100/1.30 objective, a 1.6 × tube lens, a CCD camera and appropriate filter cubes. QFISH software (Leica Imaging Systems Cambridge Ltd.) was used for analysis of microscopic images.

Results

The karyotype of *Fringilla coelebs* is typical of passerine birds, as previously described (Piccinni, Stella, 1970). Chromosomes may be precisely divided into three groups (Fig. 1). The first group contains seven pairs of macrochromosomes, including sex chromosomes Z and W. The second group consists of three pairs of larger microchromosomes, or medium chromosomes (Rodionov, 1996). And the third group includes 30 pairs of smaller microchromosomes. Z and W are of almost similar size being the only metacentric macrochromosomes in the chaffinch karyotype. Staining with DAPI/actinomycin D (Fig. 1) has revealed DAPI-positive blocks along the arms on macro- and medium chromosomes. Centromeric heterochromatin of chromosomes was readily recognized by this technique as DAPI-negative (GC-enriched) regions (Fig. 1). It was rather difficult to identify centromeric regions on microchromosomes due to their small size. However, it is reasonable to suggest that centromeric regions of all chaffinch chromosomes contain GC-enriched heterochromatin blocks.

DNA/DNA FISH, applied to the chaffinch mitotic chromosomes using FCP repeat as a probe, has revealed centromeric location of this sequence (Fig. 2, *A*). Hybridization signals were visible on each chromosome, although fluorescence intensities varied between individual chromoso-

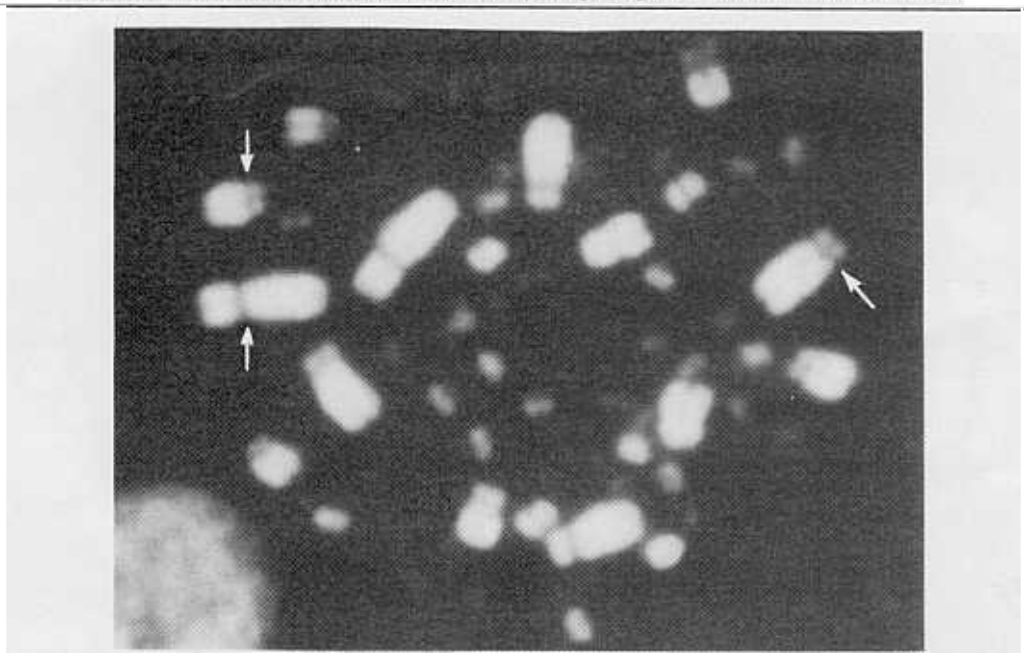

Fig. 1. The full metaphase plate of the chaffinch chromosomes stained with A/T-specific fluorochrome DAPI and counterstained with Actinomycin D. Note that centromeres are DAPI-negative (arrowheads).

mes, and was not correlated with chromosome size. The pattern of variation was the same on all chromosome plates investigated, which enabled us to conclude that the number of FCP repeats may vary considerably in different chromosomes.

It seems most important, that the ECP probe also hybridized with thin thread-like strands extending from the centromeres, in some cases linking non-homologous chromosomes (Fig. 2, *A*). Short «threads» extending from individual chromosomes are likely to be damaged interchromo-

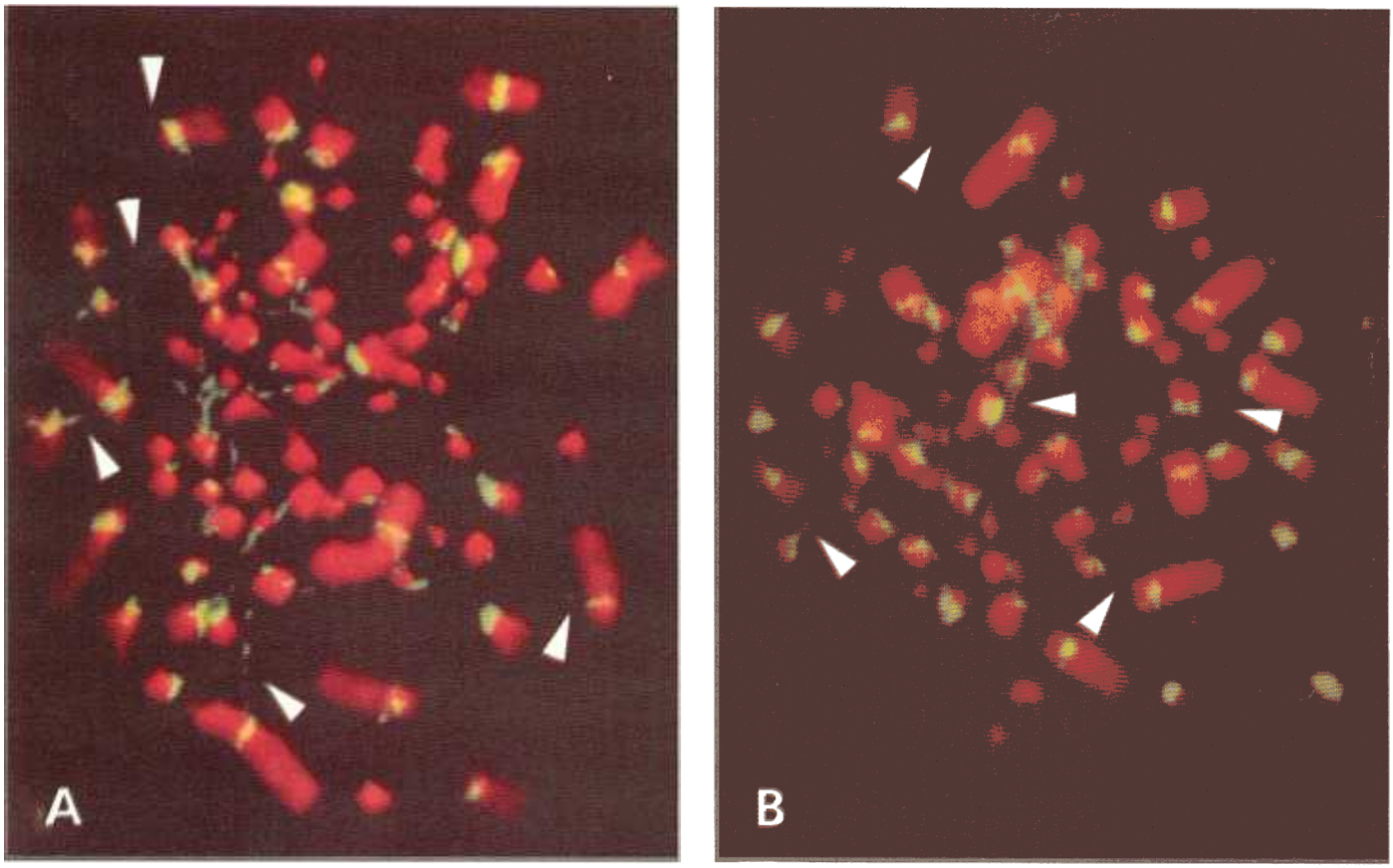

Fig. 2. FISH applied to the chaffinch metaphase chromosomes with biotinilated FCP probe (followed by FITC-labeled avidin) reveals staining of centromeric regions and of intercentromeric connectives (arrowheads) in denaturing (*A*) and non-denaturing (*B*) conditions. Chromosomes are counterstained with PI.

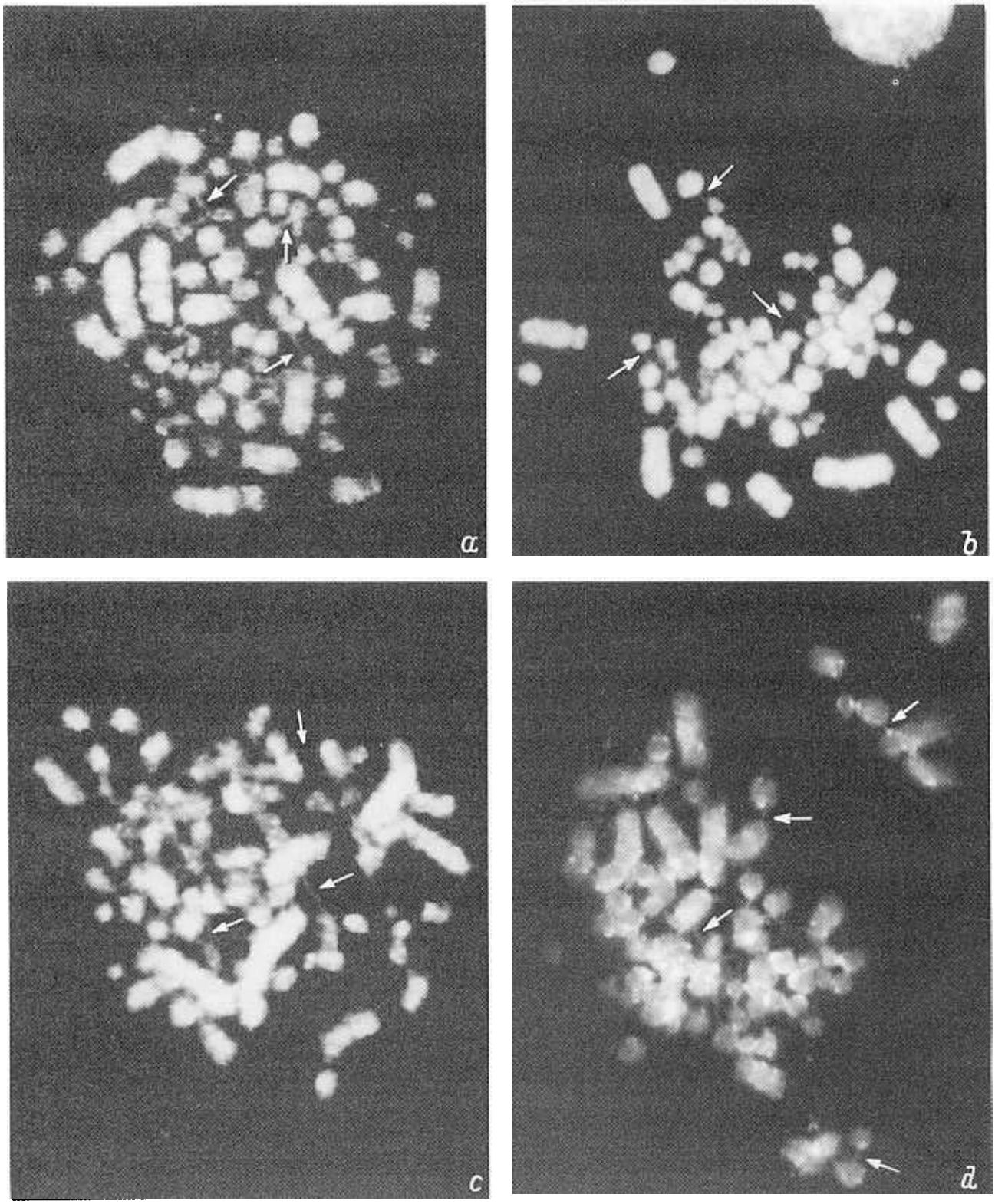

Fig. 3. DNA visualization in the chaffinch metaphase chromosomes.

a — immunostaining with anti-dsDNA antibodies (FITC fluorescence); *b* — *in situ* nick-translation in the presence of DNase I and biotin-16-dUTP (FITC fluorescence); *c* — *in situ* random-primed labeling of denatured chromosomes using digoxigenin-11-dUTP (Cy3 fluorescence); *d* — *in situ* random-primed labeling of non-denatured chromosomes using digoxigenin-11-dUTP (Cy3 fluorescence). Arrowheads — interchromosomal connectives.

somal connectives occasionally disrupted during chromosome preparation. The staining intensity of centromeres and interchromosomal «threads» was insensitive to RNase A treatment, indicating that these connections were not attributable to RNA.

Surprisingly, FISH experiments using non-denatured, RNase-treated chromosomes, which served as a control, yielded the same staining pattern as FISH on denatured slides (Fig. 2, *B*). This result seemed to equally testify that FCP repeat contains some single-strand DNA able to bind

single-strand nucleic acid probes, or alternatively, that interchromosomal connectives may consist only of proteins specifically adsorbing the FCP probe. To solve this problem, additional experiments were carried out for identification of both double-strand and single-strand DNAs in the intercentromere «threads».

Staining of the chaffinch metaphase plates with anti-dsDNA antibodies shown in Fig. 3, *a* reveals the irregular fluorescence-banding pattern, presumably reflecting differences in DNA compaction along the chromosome arms. Faint interconnections between centromeres are visible in these images, similar to those observed after FISH with the FCP probe. This was definitely a specific staining, because on the control slides with the omitted step of treatment with primary antibody no staining was seen at all.

Images comparable with anti-dsDNA staining were also obtained when DNA was labeled by nick-translation (Fig. 3, *b*) or annealing and extension of random hexamers on denatured slides (Fig. 3, *c*), conforming DNA presence in the thin interchromosomal connectives.

Because FISH with the specific FCP probe labeled intercentromeric threads even under non-denaturing conditions, we also attempted *in situ* labeling of native (non-denatured) chromosomal DNA using random primers. As shown in Fig. 3, *d*, the fluorescence was observed on throughout the whole chromosomes, the brightest signal coinciding with centromeric regions, and fluorescent labeled threads were seen to connect the number of centromeres. On the control slides (labeling procedure without hexanucleotides) no fluorescence signal was registered. The results seem to indicate that DNA of centromeres and interchromosomal connectives on the slides contains single-stranded tracts which can bind single-stranded DNA probes and hexanucleotide primers.

It is interesting to note once more that random-primed labeling of non-denatured chromosomes (Fig. 3, *d*) produced irregular fluorescence signals throughout the whole chromosomes, with the brightest fluorescence being observed in centromeric regions, and the pattern of centromere fluorescence signal directly correlated with that in FISH experiments (Fig. 2, *A*, *B*). Different staining of individual chromosomes was consistently observed in all chromosome plates on the same slides providing an evidence that single-strand DNA is preferentially exposed in specific chromosomal regions.

Discussion

Using high-resolution light microscopy and variety of molecular-cytogenetic approaches, we have localized repetitive FCP DNA elements to the centromeres of the chaffinch chromosomes. In addition, these DNA sequences were revealed in fine threads connecting centromeres of non-homologous chromosomes on slides. The results indicate that centromeric regions of non-homologous chromosomes in the chaffinch are interlinked, similarly as it was previously suggested for other organisms (Takayama, 1975, 1976; Avivi, Feldman, 1980; Hilliker, Appels, 1989), and the highly repeated FCP sequence appears to be responsible for these mitotic chromosome associations.

The discovery of centromere repeated DNA within intercentomeric connections raises several questions, particularly, how these connections arise, and what molecular mechanisms may be responsible for the maintenance of chromosome (intercentromere) associations. It seems most likely that chromosome rosettes, as they were desctibed by Nagele et al. (1995, 1998) and Klein et al. (1998), represent a native spatial organization of chromosomes during mitosis to be maintained by interaction between chromatin domains of adjacent centromeres. Interchromosome connectives seen on chromosome spreads may result from extension of interacting domains from the chromosomes during preparation procedure. As regards mechanisms of tight interchromatin associations, Ikemura et al. (1998) have suggested that non-B DNA structures (such as triplex DNA) may be important for DNA-DNA interchromosomal associations determining the spatial organization in the nucleus. However, analysis of the FCP sequence (GenBank accession number AF160980) has shown that it does not contain potential sites for triplex DNA formation (Gaginskaya et al., in preparation). Our ability to label the centromeres and intercentromere connectives of non-denatured chromosomes by FISH indicates the presence of the single-strand DNA in the chaffinch centromeric heterochromatin. The labeling of non-denatured chromosomes with random primers preferentially stained the centromeres, supporting that this region may be rich in single-stranded DNAs. The single-stranded DNA tracts may be involved in DNA-DNA interaction, making intercentromere associations, but without special investigations it is impossible to say with a confidence if their occurrence in the chaffinch DNA is a characteristics of native chromosomes or a result of acetic acid fixation. Interestingly, that analysis of the FCP sequence has revealed the number of target sites for transcriptional factors (Gaginskaya et al., in preparation). In particular, the FCP was found to contain at least four target sites for Sp1 protein. Some transcription factors, such as SpGCF1 in sea urchins and Sp1 in other animals, were shown to form stable multimeric complexes when bound *in vitro* to DNA specific probe at target sites remote from each other (Zeller et al., 1995). These data might serve an indirect evidence to say confidently that the formation of stable complexes between Sp1 molecules bound to FCP sequences in different chromosomes may become a molecular mechanism responsible for the maintenance of intercentromere associations in the chaffinch mitotic cells.

Acknowledgements

We thank Drs. Tatiana Kislyakova and Tatiana Sukhih for their help with cell cultivation. We are especially grateful to Prof. Herbert C. Macgregor, Prof. David Roos, Dr. Andrei Zalensky and Dr. Alexander Rodionov for critical reading of the manuscript and useful discussions.

The reseacrch was supported by Russian Basic Research Foundation (grant #99-04-49592), Russian Universities State Programme (grant #2259), Russian Ministry of Education (grant #97-10-6 for Basic Science Programme), and by Russian Ministry of Sciences for COMUS-1 unique instruments (registration number 06-03).

Referenc

Avivi L., Feldman M. 1980. Arrangement of chromosomes in the interphase nucleus of plants. Hum. Genet. 55 : 281—295.

Boveri K. T. 1888. Die Befruchtung und Teilung des Eies von *Ascaris megalocephala.* In: Zellen-Studies. Jena: G. Fischer. 1—189

Carmena M., Abad J. P., Villasante A., Gonzalez C. 1993. The *Drosophila melanogaster dodecasatellite* sequence is closely linked to the centromere and can form connections between sister chromatids during mitosis. J. Cell Sci. 105 : 41—50.

Chiarelli B., Ardito G., Brogger A. 1977. The non-random distribution of human chromosomes at metaphase. II. Chromosomal interconnections. The nucleus. 20 (3) : 249—251.

Ferguson M., Ward D. C. 1992. Cell-cycle dependent chromosome movement in pre-mitotic human T-lymphocyte nuclei. Chromosoma. 101 : 557—565.

Hankeln T., Fillippova M. A., Kiknadze I. I., Aimanova K. G., Schmidt E. R. 1994. Centromeric heterohromatin and satellite DNA in the *Chironomus plumosus* species group. Genome. 37 : 925—934.

Hilliker A. J., Appels R. 1989. The arrangement of interphase chromosomes: structural and functional aspects. Exp. Cell Res. 185 : 297—318.

Ikemura T., Ohno M., Uemura T., Sasaki H., Tenzen T. 1998. Genome and subnuclear organization of non-B forming DNA in mammalian chromosomes. Cytogenet. Cell Genet. 81 (2) : 101—102.

Klein C., Cheutin T., O'Donohue M.-F., Rothlum L., Kaplan H., Beorchia A., Lucas L., Heliot L., Ploton D. 1998. The three-dimensional study of chromosomes and upstream binding factor-immunolabeled nucleolar organizer regions demonstrates their non-random spatial arrangement during mitosis. Mol. Biol. Cell. 9 : 3147—3159.

Mosolov A. N. 1973. Hypothesis of centromeric ring unifying eukaryote chromosomes at all stages of the cell cycle. (In Russian). Dokl. Akad. Nauk. SSSR. 212 (2) : 483—484.

Nagele R. C., Freeman T., Fazekas J., Lee K.-M., Thomson Z., Lee H.-Y. 1998. Chromosome spatial order in human cells: evidence for early origin and faithful propagation. Chromosoma. 107 : 330—338.

Nagele R., Freeman T., McMorrow L., Lee H. 1996. Precise spatial positioning of chromosomes during prometaphase: evidence for chromosomal order. Science. 270 : 1831—1834.

Navashin M. S. 1947. Metaphase chromosome situation and nuclear dynamics. (In Russian). Dokl. Akad. Nauk SSSR. 57 (6) : 613—616.

Piccinni E., Stella M. 1970. Some avian karyograms. Caryologia. 23 : 189—202.

Rabl C. 1885. Über Zellteilung. Morphologisches Jahrbuch. 10 : 214—330.

Rodionov A. V. 1996. Micro versus macro: a review of structure and functions of avian micro- and macrochromosomes. Russian J. Genetics. 32 (5) : 517—527.

Schweizer D. 1981. Counterstain-enhanced chromosome banding. Hum. Genet. 57 : 1—14.

Slijepcevic P., Hande M. P., Bouffler S. D., Landsdorp P., Bryant P. E. 1997. Telomere length, chromatin structure and chromosome fusigenic potential. Chromosoma. 106 : 413—421.

Takayama S. 1975. Interchromosomal connectives in squash preparations of L cells. Exp. Cell Res. 91 : 408—412.

Takayama S. 1976. Interchromosomal connectives in air-dried metaphase chromosomes of some mammalian cells. Ann. Zool. Jap. 49 (1) : 38—47.

Visser A. E., Eils R., Jauch A., Little G., Bakker P. J. M., Cremer T., Tten J. A. 1998. Spatial distributions of early and late replicating chromatin in interphase chromosome territories. Exp. Cell Res. 243 : 398—407.

Zalensky A. O. 1998. Genome architecture. Adv. Genome Biol. 5 (A) : 179—210.

Zeller R. W., Griffith J. D., Moore J. G., Kirshhamer C. V., Britten R. J., Davidson E. H. 1995. A multimerizing transcription factor of sea urchin embryos capable of looping DNA. Proc. Nat. Acad. Sci. USA. 92 : 2989—2993.

Recieved 6 X 1999

ЛОКАЛИЗАЦИЯ ВЫСОКОПОВТОРЯЮЩЕЙСЯ ЦЕНТРОМЕРНОЙ ПОСЛЕДОВАТЕЛЬНОСТИ FCP ИЗ ГЕНОМА ЗЯБЛИКА (FRINGILLA COELEBS: AVES) В МИТОТИЧЕСКИХ ХРОМОСОМАХ И ИНТЕРХРОМОСОМНЫХ НИТЯХ

А. Ф. Сайфитдинова,[1] *Л. П. Тимофеева,*[2] *В. Г. Журов,*[1] *Е. Р. Гагинская*[1]

[1] Биологический Научно-исследовательский институт С.-Петербургского государственного университета, электронный адрес: egag@chromo.lgu.spb.su, и

[2] Институт экспериментальной биологии Эстонского сельскохозяйственного университета, Харку

Выделенная из геномной ДНК зяблика (Fringilla coelebs. L.) и клонированная в бактериальной плазмиде высокоповторяющаяся последовательность FCP (Fringilla coelebs PstI-элемент) методом флуоресцентной гибридизации (FISH) локализована в центромерных районах всех хромосом исследуемого вида. Гибридизационный сигнал обнаружен также на тонких нитях, связывающих центромерные районы негомологических хромосом. Присутствие ДНК в межцентромерных нитях подтверждено иммуноцитохимической реакцией с антителами против двунитчатой ДНК, а также в экспериментах на денатурированных хромосомах по ник-трансляции in situ и праймерному мечению in situ с использованием случайных праймеров. Более слабая флуоресценция центромеров и межцентромерных нитей после FISH неденатурированных хромосом с зондом FCP и после праймерного мечения неденатурированных хромосом свидетельствует о присутствии участков однонитевой ДНК в исследуемом повторе. Предполагается, что высокоповторяющаяся центромерная последовательность FCP ответственна за осуществление межхромосомных связей в так называемых митотических розетках и таким образом вовлечена в поддержание пространственной организации клеточного ядра у зяблика.