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Localization of single-copy sequences on chicken synaptonemal complex spreads using fluorescence in situ hybridization (FISH)

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Abstract. Synaptonemal complex (SC) spreads from bird oocytes and spermatocytes show the complete chromosome complement and can be observed at the light microscope using immunostaining of the proteins that compose the lateral elements. To investigate the use of avian SC spreads as substrates for fluorescent in situ hybridization (FISH) in combination with immunostaining, we applied two single-copy sequences to chicken oocyte spreads. Signals for both target sequences were consistently observed on the short arm of bivalent 1 in a large number of nuclei. Based on previous data about the size of chromosome 1 and from measurements on probed SC spreads, an estimate of the physical distance in Mb between each sequence and the telomere was calculated. The crossover frequencies along SC 1 obtained by immunolocalization of MLH1 foci during pachytene were used to calculate the distances in cM to the target sequences and to compare this cytogenetic SC map with the consensus linkage map for GGA1. The combination of SC-FISH and immunostaining could be generally applied to obtain high-resolution mapping of single-copy sequences in birds and, coupled with MLH1 crossover maps, it could be a reliable approach to obtain genetic distances between markers to test the genetic linkage maps generated from molecular markers.

Determining the physical location of a gene on a chromosome is of interest since its expression often depends on its location in a genome through its interaction with other genes and DNA sequences. One approach to locate the relative positions of genes or molecular markers on a linkage group is by producing multi-hybrid crosses and determining the frequency of recombination between them to obtain a genetic map. A drawback associated with this methodology is that the physical location cannot be overlaid directly on chromosomes since map distances are not proportional to physical distances (Sturtevant and Beadle, 1939). As an alternative, cytogenetic maps show the position of markers on chromosomes relative to cytological landmarks such as centromeres or telomeres. Cytogenetic maps are also valuable to integrate genetic, molecular and cytological information as well as to understand better the organization of the genome in the context of chromosomes. One strategy to locate sequences on the chromosomes is to visualize them directly using fluorescence in situ hybridization (FISH). This technique has been widely used for localization of genes and repetitive sequences on mitotic chromosomes in a large variety of species, helping to integrate genetic and physical maps.

Among birds, most efforts in genome mapping have concentrated on chicken *(Gallus gallus)* and recently this species has become the first agricultural animal to have its genome sequenced (Hillier et al., 2004). The haploid chicken genome has 1,250 Mb of DNA distributed in 39 chromo-

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somes, compared to the 3,000 Mb of mice and humans that, at the same time, have fewer chromosomes. A consequence of this reduced amount of DNA is that the average macrochromosome in the chicken and in most birds is considerably smaller than the average mammalian chromosome. Therefore, achieving a high resolution in the localization of sequences on mitotic chromosomes in birds represents a challenge when compared to mammals, especially on the smaller macrochromosomes and the numerous dot-like microchromosomes. An approach to obtain better resolution in cytogenetic maps is to use meiotic chromosomes that are considerably longer than their mitotic counterparts. Birds, like other vertebrates with yolk-rich eggs, develop lampbrush chromosomes during the diplotene stage of the first meiotic prophase (Hutchison, 1987). Lampbrush chromosomes can certainly provide excellent resolution to locate sequences on avian chromosomes and the long lampbrush bivalents of the chicken have been used to localize repetitive sequences and single-copy sequences (Hori et al., 1996; Solovei et al., 1998; Derjusheva et al., 2003; Galkina et al., 2005, 2006). In spite of the benefits of using lampbrush bivalents to map sequences on bird chromosomes, it is difficult to obtain good numbers of preparations from ovarian follicles of a sexually mature hen; a single spread rarely shows all the bivalents (Hutchison, 1987) and the largest microbivalents are poorly preserved (Rodionov et al., 1992).

In certain organisms the use of FISH on pachytene chromosomes has become usual practice as an alternative to mitotic chromosomes to map single- or low-copy sequences. An appealing feature of pachytene chromosomes as substrates for localization of single-copy sequences is that they are several times longer than their mitotic counterparts, thus offering better resolution for closely linked markers. Especially among plants this method has served to integrate genetic and cytological maps in several species, either using acid-fixed pachytene chromosomes or synaptonemal complex (SC) spreads fixed with paraformaldehyde (Peterson et al., 1999; Zhong et al., 1999; Islam-Faridi et al., 2002; Wang et al., 2006). Single-copy sequences have also been localized on mouse pachytene bivalents using SC spreads and their positions compared with those obtained by genetic analyses (Froenicke et al., 2002). Among birds, the use of FISH on pachytene bivalents is restricted to a single report that showed the distribution of the repetitive *Eco*R1 sequences on the W chromosome of the chicken in silver-stained SC spreads (Solari and Dresser, 1995), but no single-copy sequences have been localized yet.

The aim of this paper was to investigate the use of pachytene SC spreads as substrates to localize single-copy sequences in the chicken and to test this method as a means to anchor the genetic and physical maps in this species. Two single-copy sequences located on the short arm of chromosome 1 were employed: one of them contains a microsatellite genetically mapped on the short arm of chromosome 1 and the other sequence is close to the end of the same arm. The recombination frequencies along bivalent 1 of the chicken are available from genetic mapping (reviewed in Schmid et al., 2005) and also from MLH1-focus data in oocytes (Pigozzi, 2001; this report). Therefore it should be possible to compare the distances in cM obtained for the target sequences by the genetic method and the SC-FISH method.

Materials and methods

Target sequences and probe preparation

The BACs identified as CH135N20 and CH174B20 (referred to as N20 and B20 from now on) were obtained from the BACPAC Resources Center at the Children's Hospital Oakland Research Center (http://bacpac.chori.org). The first BAC was assigned to a position around 3.2 Mb from the end of GGA1 in the assembly of May 2006 (http://genome.ucsc.edu) and it has not been linked to a genetically mapped marker yet. It was selected due to its proximity to the end of the chromosome in order to compare directly its physical distance to the telomere in pachytene bivalents and mitotic chromosomes. The second BAC contains the microsatellite MCW0111 that maps at 118 cM from GGA1pter (Groenen et al., 1998). This marker and the centromere serve to compare the distances in cM in the consensus linkage map and the cytogenetic SC-FISH map generated in combination with MLH1 data (see below). The BAC DNA was extracted using the Phase Prep kit from Sigma, quantified using a spectrophotometer and labeled by nick translation with biotin-16-dUTP (Roche Applied Science) according to the manufacturer's instructions.

Synaptonemal complex spreads

SC spreads were prepared from oocytes one day after hatching. Briefly, the single (left) functional ovary was dissected, placed into Hanks balanced salt solution and cut in three or four small pieces. The small pieces were treated with a hypotonic extraction buffer (Peters et al., 1997) for 30 min and then gently disaggregated in 100 mM sucrose at pH 8.5 to give a homogenous cell suspension. About 30 μl of this suspension were dropped onto glass slides covered with a thin layer of 1% PFA, 0.1% Triton X-100. The slides dried for about 1 h in a humid chamber and then were washed in 0.4% Photoflo. The slides were used immediately for FISH and immunostaining or alternatively they were kept at –70°C for later use.

Fluorescence in situ hybridization and immunostaining

Slides with SC spreads were immersed in 70% formamide, 2× SSC at 72°C for 5 min to denature the DNA and then rapidly dehydrated in a cold ethanol series. The probes were resuspended in hybridization buffer containing 50% formamide, 10% dextran sulfate and salmon sperm DNA in 2× SSC, and denatured in a thermal cycler at 85°C for 5 min. Twenty microliters of probe (about 100 ng of labeled DNA) were applied to each slide, covered with a 22-mm coverslip and sealed with rubber cement. Slides were incubated overnight at 37°C in a humid chamber. Stringent washes were performed in 50% formamide, 2× SSC and 0.1× SSC for 5 min at 60–62°C. After washes in 2× SSC and PBS, an antibody against the cohesin subunit SMC3, that recognizes the lateral elements of the synaptonemal complexes, was applied to each slide diluted 1:1000 in PBT (3% BSA, 0.05% Triton X-100 in PBS) and incubated at 37°C for 1 h. Slides were subsequently washed in 1× PBS, 0.1% Tween 20 before the simultaneous incubation with streptavidin-FITC and goat anti-rabbit rhodamine to detect the probe and the anti-SC antibody, respectively. After 1 h of incubation in a humid chamber slides were washed in PBS/Tween and then mounted in Vectashield (Vector laboratories) with DAPI as counterstain. Slides were examined under a Leica fluorescence microscope and images of well spread pachytene nuclei with clear FISH signals and SC labeling were captured using a CCD camera. Two separate images were taken: one with the FITC filter for the probe signal and the other with the rhodamine filter to obtain the image of the SCs, and then merged using Adobe Photoshop. MLH1 focus data were obtained from SC spreads immunostained with antibodies against SMC3, CREST serum that labels the centromeres and an anti-MLH1 antibody (Pharmingen) that recognizes a protein present at the sites of crossovers in a variety of organisms (Baker et al., 1996).

Mitotic chromosomes were obtained from whole embryos treated with colchicine and used for FISH with the same probes employing a procedure similar to that described for SC spreads.

SC measurements and the cytogenetic SC-FISH map

Linear distances between each FISH signal and the nearest telomere were measured using the program Micromeasure (Reeves, 2001). Relative positions along a given SC are equivalent in spite of variations in the absolute SC length between nuclei (Sherman and Stack, 1995; Pigozzi and Solari, 1999). Thus, the program was used to obtain the relative distance between the probe signal and the nearest telomere expressed as a fraction of the whole SC which is calculated by dividing the absolute distance from the telomere to the probe by the total SC length in each particular nucleus. Both sequences are clearly closer to one of the telomeres that can be identified as the telomere of the short arm of SC 1 based on the physical location of the BACs in the chicken genome assembly and by FISH on mitotic chromosomes (see Results). The number of MLH1 foci between this telomere and the average relative position of each FISH signal was scored using the same measuring device on immunostained SC spreads. Since one MLH1 focus corresponds to one crossover event, the SC segment that has on average one focus would be 50 map units long, which is the map distance between two loci that show on average one crossover per meiosis. This is the basis to calculate the genetic distances on SCs using MLH1 focus data and, in combination to FISH, generate a cytogenetic SC map to compare with the consensus genetic linkage map.

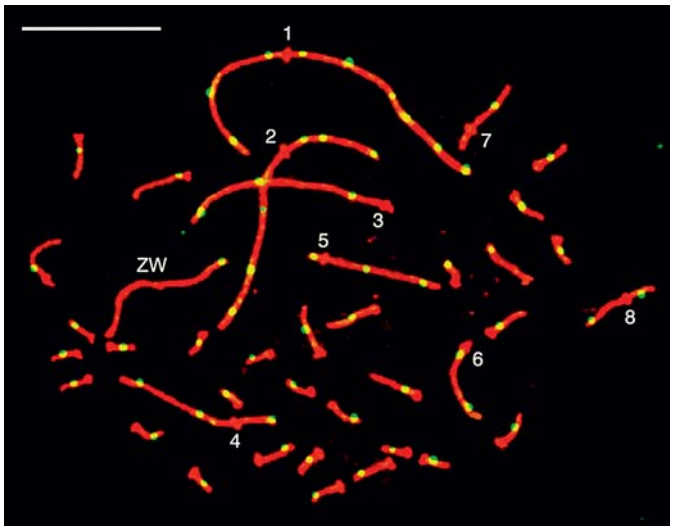

Fig. 1. Complete SC set immunostained with anti-SMC3 antibody and CREST serum (red) and anti-MLH1 antibody (yellow). The numbers next to the kinetochores label the SCs of the largest macrobivalents. The SC corresponding to chromosome 1 has three foci on the short arm and six on the long arm. The ZW pair has a single terminal focus. Bar: 10 μm.

Results

The distribution of MLH1 foci on SC 1

The number and distribution of MLH1 foci on SC 1 were scored on well spread oocytes showing identifiable macrobivalents (Fig. 1). A sample of 62 oocytes was analyzed to count the MLH1 foci on SC 1 and to determine their positions along each arm. In the chicken, the largest SC with a centromeric index of 0.4 corresponds to bivalent 1 and its relative length and kinetochore position in SC spreads match very well those of the mitotic chromosome pair (Kaelbling and Fechheimer, 1983; this report). The average length of SC 1 was 32.9 ± 3.1 μm and it showed a mean number of 9 foci (SD = 1.4), with 3.5 foci being present on the short arm. From these data, the total genetic map for chromosome 1 is therefore 450 cM, with 175 cM (39%) corresponding to the short arm. The frequency distribution of foci along the SC can be displayed in a histogram where the x-axis represents the average SC length divided in 0.5-μm intervals and the height of each bar shows the number of MLH1 foci observed per interval (Fig. 2). There are regions with focus frequency considerably lower and higher than the average (the horizontal line in the graph), particularly there are scarce foci around the centromere. The paucity of foci in this region is shown by the fact that only 13 foci out of 552 were found within 1.6 μm around the kinetochore. The histogram is represented in comparison to the standard RGB idiogram of the chicken (Ladjali-Mohammedi et al., 1999) to show the correspondence of the centromere position on SC 1 and on its mitotic counterpart.

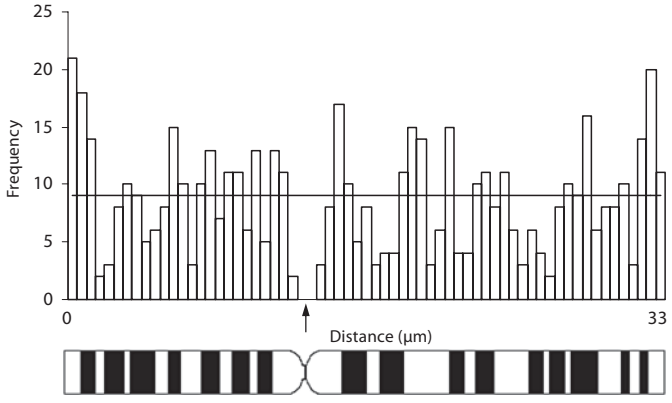

Fig. 2. MLH1 foci along SC 1. The histogram represents the frequency distribution of MLH1 foci scored in 62 oocytes. The x-axis represents the distance on the SC from the kinetochore (arrow) to each telomere, positioned at 0 and 33 μm. Each interval represents a 0.5-μm segment of the SC and the y-axis indicates the frequency of MLH1 foci on each 0.5-μm interval. No foci were observed in the two intervals close to the centromere. The horizontal line indicates the average number of foci. Notice the correspondence between the mitotic chromosome and the pachytene bivalent regarding the centromere position. Additional information about the construction of this frequency histogram can be found in Materials and methods.

Localization of single-copy sequences on SC spreads

After the FISH procedure, a signal was observed almost invariably associated with the longest SC in pachytene oocytes (Fig. 3), as well as double signals in somatic nuclei present in the spreads. Both target sequences localize to the short arm of SC 1: N20 is closer to the telomeric end and B20 is around the middle of the arm (Fig. 3A, B). Two threadlike signals extend from the SC marking the location of the sequence on the pachytene bivalent. In most nuclei (76%), the

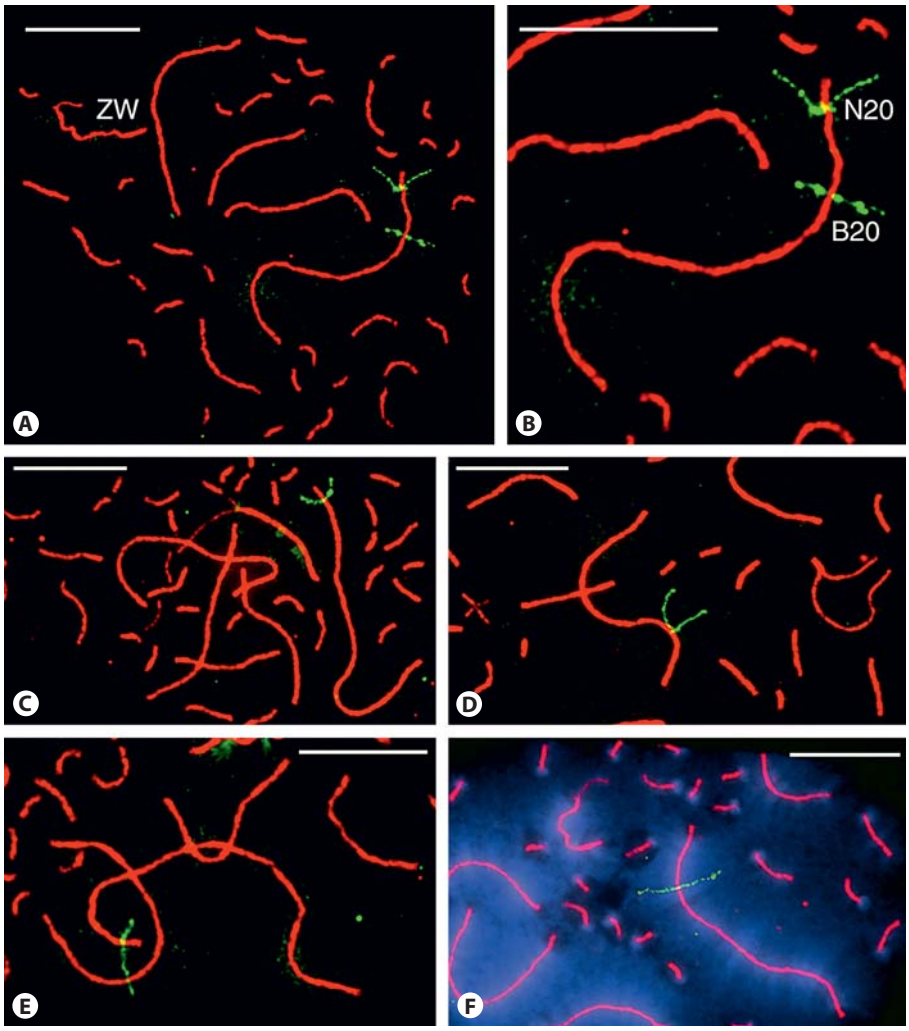

Fig. 3. FISH and immunostaining on SC spreads. (**A**) Complete SC set showing the signals of two BACs hybridizing to SC 1. (**B**) Enlargement of SC 1 shown in **A**. The signals extend on both sides of the SC crossing it at a single point that corresponds to the base of the chromatin loops. The signal closest to the telomere corresponds to BAC 135N20 and the other corresponds to BAC 174B20. (**C**–**E**) The varying arrangement of the homologous chromatin loops after the spreading is shown by the presence of symmetric signals on both sides of the SC, on one side of the SC or by asymmetric signals. (**F**) In overlapped images of FISH, immunostaining and DAPI, the chromatin (blue) extends beyond the signal of the target sequence (B20). The size of the insert of this BAC is about 178 kb.

Table 1. Position of the BACs on the meiotic bivalent 1 and their predicted map assignments on the basis of MLH1 focus distribution

BAC	n[a]	Distance to pter[b]	MLH1 foci[c]	Linkage map position (cM from pter)	
				Cytogenetic map	Genetic map
N20	69	5.5 ± 0.48	57	46	N/A[d]
B20	70	21.1 ± 0.96	134	108	118

[a] No. of nuclei with FISH signals.
[b] Percent length of the SC 1 from the telomere of the short arm.
[c] No. of MLH1 foci in 62 nuclei from pter to the average positions of the BACs.
[d] N/A: not available.

'threads' extended on both sides of the SC (Fig. 3A–C). In the rest of the nuclei the signals were on one side of the SC probably due to random positioning of the chromatin loops after the drying-down procedure (Fig. 3D). In most cases both signals had similar lengths but they also could be asymmetric, as shown in Fig. 3E. This appearance might be due to fold backs of one loop pair. Another reason for the presence of asymmetric signals could be incomplete hybridization at the distal end of the loops, although this seems unlikely because no signals were observed lacking a point of contact with the SC, as expected if a similar hybridization failure affected the proximal segment of the loops. Because of the mentioned signal asymmetry and also because the resolution of the method prevents the distinction of single

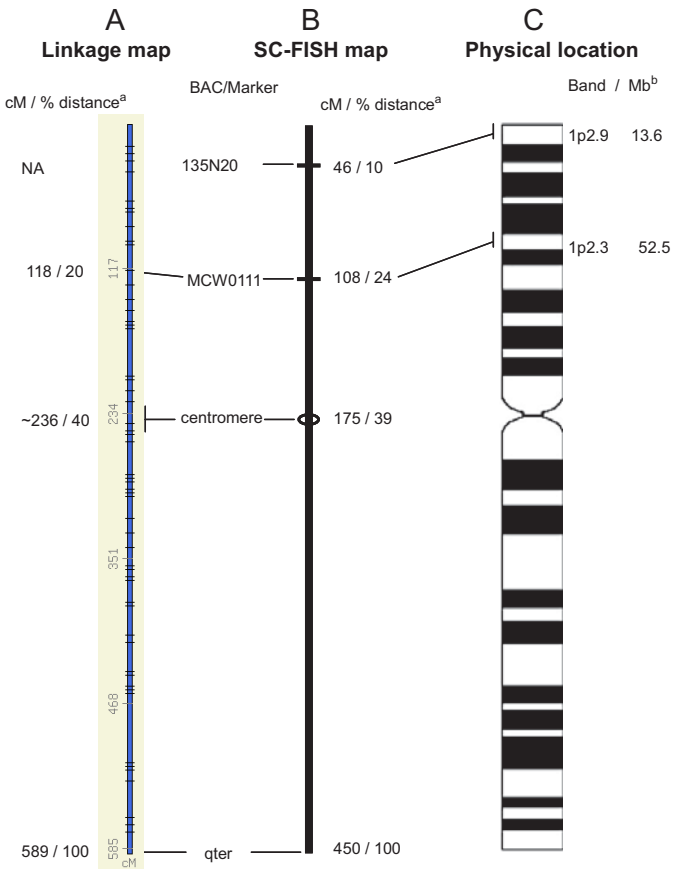

Fig. 4. Comparison of the SC-FISH map of GGA1 and the corresponding linkage map. (**A**) Linkage map of chromosome 1 showing the position of some microsatellite markers (thin lines), including marker MCW0111 that is part of the insert of the BAC 174B20. The second BAC, 135N20, does not contain any genetically mapped marker; therefore its position is not available in cM (NA). The distance in cM from pter to the centromere was calculated from the plots of genetic vs. physical distances in Schmid et al. (2005). The genetic linkage map is available from the ChickMap database at the Waageningen University site (http://lx1b.zod.wau.nl/cmap/). (**B**) Summary of the SC-FISH map showing the genetic and relative distances from pter to each BAC and to the centromere. The cM values derivate from the MLH1 focus map (see Results). The percent distances of the BACs differ slightly from those calculated on the SC (Table 1) because in this map they are relative distances of the map length in cM. There is a good correspondence of the relative distances from pter to the microsatellite and the centromere in both maps. (**C**) Standard idiogram of GGA1 showing the physical location of the target sequences on the mitotic chromosome and the distances in Mb estimated from their locations on the SC. [a] Distances calculated as percent of the total map length in cM. [b] Distance in Mb from pter.

loop pairs it is considered that the length of the BAC inserts in Mb cannot be used to estimate the DNA content in a single loop with certainty. When the DAPI image is overlaid to the FISH and immunostaining images, the counterstained DNA often extends beyond the signal termini (Fig. 3F). It is interesting to note that in most nuclei there were two signals associated with the SC instead of the four expected indicating that sister loops are held together during pachytene.

Positions of the target sequences on the cytogenetic SC map

The target sequences can be mapped very accurately on SC spreads because the bases of the chromatin loops are attached to the lateral elements of the synaptonemal complex and the FISH signals cross the SC at a defined point. The linearity of the SCs represents an advantage to establish the physical position of the sequences on the chromosome relative to the centromere or the telomeres. Because little variation was found in the centromeric index of SC 1, the relative position of each probe was determined as a percentage of the total SC length. This also allows a direct comparison with cumulative genetic distances that are represented following one direction from the most distal marker on the short arm in linkage maps. The location of each signal in respect to the telomere of the short arm varied within a small range, with most values grouped tightly around the mean (Table 1). To obtain the map length in cM from pter to each target sequence, the number of MLH1 foci scored in the interval from the telomere to the average position of the signals was divided by the total number of SCs analyzed for focus distribution, then multiplied by 50 map units per MLH1 focus. The values obtained from these measurements are summarized in Table 1. Figure 4 compares the consensus linkage map (http://lx1b.zod.wau.nl/cmap/) and the cytogenetic SC map of GGA1 with the relative distances (in percent) and the absolute distances (cM) from pter to each molecular marker and to the centromere.

Localization of the target sequences on mitotic chromosome 1

FISH on mitotic chromosomes was done to compare the resolution with the pachytene bivalents and to confirm the position of the target sequences on the short arm of chromosome 1. The main observation concerning this procedure is that the signal corresponding to the most distal sequence (N20) locates practically at the end of the chromosome (Fig. 5A). By comparison, in SC spreads a considerable distance is observed between the signal and the end of the synaptonemal complex of bivalent 1 (Fig. 3). This result depicts the higher resolution of the pachytene bivalents compared to mitotic chromosomes, even if well elongated metaphases are used. As expected, the BAC B20 maps around the middle of the short arm and its relative position is 0.2 with respect to the total chromosome length (Fig. 5B).

Discussion

The cytogenetic SC map compared to the genetic linkage map

According to the last report on chicken genes and chromosomes 193 loci have been mapped both physically and genetically in the chicken genome, with 19 of these loci belonging to chromosome 1 (Schmid et al., 2005). Most of the physical localization of the markers was done using FISH on mitotic chromosomes (reviewed in Schmid et al., 2000). In the present work, FISH in combination with immuno-

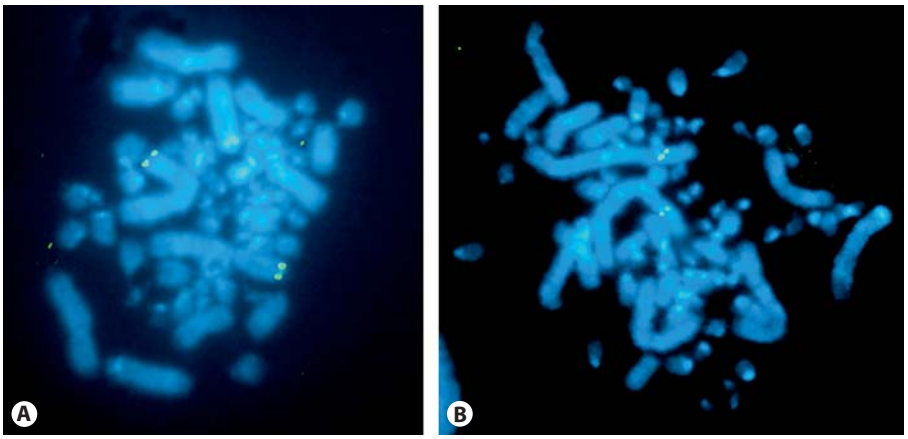

Fig. 5. Localization of the target sequences on mitotic chromosomes. (**A**) The BAC N20 is located at the end of the short arm of chromosome 1 and no distal segment is visible with DAPI staining. (**B**) The signal corresponding to BAC B20 is located around the mid region of the same arm.

staining is used for the first time to locate non-repetitive sequences on avian SC spreads. The relative distances from pter to the microsatellite MCW0111 and to the centromere calculated from FISH data and the MLH1 crossover map correspond well with the relative positions in the consensus linkage map (Fig. 4). The difference in cM between the SC-FISH map and the linkage map is only 10 cM up to the position of the microsatellite marker. However, the absolute values in cM for the short arm and the total length of bivalent 1 based on MLH1 data are about 1.3 times shorter than those in the consensus linkage map, with the consequence that the cM distances in both maps will differ considerably, especially for the long arm. In spite of the difference in these absolute values, the possibility to overlap directly the genetic distances and the physical location of markers make SC-FISH a valuable tool to predict the position of markers that have not been genetically mapped yet or to solve contradictory data about the order of molecular linkage markers. The difference of total map lengths between the SC map and the linkage map cannot be ascribed to the transient nature of the MLH1 foci because in chicken oocytes foci do not vary significantly throughout pachytene as shown by their stable number on SCs from early to late substages (Pigozzi, 2001). In addition, chiasmata on lampbrush bivalents represent undoubtedly stable markers of crossing over and their numbers, known for the largest bivalents, are close to MLH1 foci (Rodionov et al., 1992; Pigozzi, 2001). These results strongly suggest that some factors are acting to cause overestimation of linkage map values, a common observation in many organisms when recombination frequencies are compared using cytological data vs. linkage data from molecular markers (Lincoln and Lander, 1992; Sybenga, 1996).

A comparison of physical vs. recombination data generated by this SC-FISH procedure shows that the relative position of the sequence closer to the centromere (N20) represents 5.5% of the SC length but it is 10% of the MLH1 crossover map. On the other hand, more linear correlations are observed for the physical and cM distances of B20 and the centromere (Table 1; Fig. 4B). Lack of linearity between physical and genetic positions can be expected for those markers closer to the telomere because the recombination frequency is above the average towards the end of the bivalent, and therefore markers will tend to appear more spread in the genetic map even if they are not very distant on the chromosome. The plot of genetic vs. physical distances for GGA1 shows a linear relationship all along the bivalent, but only two markers are included within the first 80 cM from pter (Fig. 4 in Schmid et al., 2005). More detailed conclusions about the relationship between physical and genetic distances could be drawn once more markers become both physically and genetically mapped.

Resolution of the method and applications of the cytogenetic SC map

The resolution of FISH on pachytene chromosomes from plants such as rice and maize has been estimated to range from 1.2 Mb to 100 kb; that is much better than the resolution achieved on mitotic chromosomes in these species (Wang et al., 2006; reviewed in Jiang and Gill, 2006). The present results show that FISH on chicken SCs also offers an excellent resolution compared to mitotic chromosomes. A stretch of 1.8 μm can be measured between the signal closer to the telomere (N20) and the end of the synaptonemal complex while in mitotic chromosomes the same signal is nearly telomeric (compare Figs. 3B and 5). In the latest available assembly (May 2006) the BAC 135N20 is located at 3.3 Mb from the end of chromosome 1. However, the assembly at this chromosome region is still under construction and extending towards the telomere since this same marker was at only 0.3 Mb from the chromosome end in the previous assembly of the chicken genome (February 2006). Given the size of 250 Mb estimated for GGA1 (Smith and Burt, 1998), the SC segment from the telomere to N20 probably comprises ~13 Mb (5.5% of 250 Mb), assuming that the size of the DNA loops attached along the SC is relatively constant in a given species (Kleckner, 2006). The limit of resolution of light microscopy is ~0.25 μm, so potentially sequences located 7 times closer (1.8 μm divided by 0.25 μm) could be resolved. The equivalent in Mb based on the 13 Mb estimated here for the distance from N20 to the telomere would be then a resolution of 1.8

Mb using the large BAC inserts. This higher resolution provided by SC-FISH can be especially useful to map simultaneously several markers on the shortest microbivalents that are on average 1.5 μm long in this species. Furthermore, chicken microbivalents are acrocentric and the centromeric end can be identified as the brighter end with DAPI staining in SC spreads (see Fig. 3F), providing an additional landmark for FISH and immunostaining procedures.

The present results show that FISH on chicken SC spreads in combination with immunostaining is a reliable method to map single-copy sequences on meiotic chromosomes, and this procedure might be equally suitable in other birds as well. This method can help to map single-copy sequences directly on chromosomes and also to order BAC contigs solving problems associated with physical mapping from genome projects. Since MLH1 foci maps are a representation of the distribution of crossing over along the pachytene bivalents, they can be combined with FISH as a source of recombination data for each linkage group to compare with classic or molecular linkage maps. The largest SCs (1 to 8) in the chicken can be reliably identified by length and kinetochore position and their recombination maps have been obtained scoring recombination nodules (Rahn and Solari, 1986) or MLH1 foci in oocytes (Pigozzi, unpublished). Bivalents 9–38 instead cannot be identified solely by their lengths and kinetochore position but they are invariably present in SC spreads from oocytes or spermatocytes. In the chicken, it should be possible to obtain specific recombination maps for medium- and small-sized bivalents using chromosome-specific probes simultaneously or in succession with MLH1 and SC immunostaining. Specific probes are available to identify each chromosome pair in the groups B and C of the chicken karyotype, which include the smaller macrobivalents and all microchromosomes (Fillon et al., 2003; Masabanda et al., 2004). A combination of multiple color FISH and immunostaining has been used to obtain the MLH1-focus maps for each bivalent in mouse and human spermatocytes (Froenicke et al., 2002; Sun et al., 2004; Codina-Pascual et al., 2006).

In addition to serve as a means to anchor the genetic and cytological positions of genes and molecular markers, the combination of MLH1 focus counts and FISH on SCs can also give a better understanding about the relationships between genome structure, gene density and the distribution of crossing over among Galliformes, an avian group where several species have ongoing genome-mapping projects.

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