Cytogenet Cell Genet 87:278–281 (1999)

Cytogenetics and Cell Genetics

Micro- and macrochromosome paints generated by flow cytometry and microdissection: tools for mapping the chicken genome

D.K. Griffin,[a,b] F. Haberman,[b,c] J. Masabanda,[a] P. O'Brien,[b] M. Bagga,[b] A. Sazanov,[d] J. Smith,[e] D.W. Burt,[e] M. Ferguson-Smith,[b] and J. Wienberg[b,e]

[a] Department of Biological Sciences, Brunel University, Uxbridge, Middlesex (UK);
[b] Department of Pathology, University of Cambridge, Cambridge (UK);
[c] Institut für Anthropologie und Humangenetik, Ludwig-Maximilians Universität, München (Germany);
[d] The Roslin Institute, Roslin, Midlothian, Scotland (UK); and
[e] Lehrstuhl für Tierzucht, Technische Universität München, Freising-Weihenstephan (Germany)

Abstract. Despite the chicken being one of the most genetically mapped of all animals, its karyotype remains poorly defined. This is primarily due to microchromosomes that belie assignment by conventional methods. To address this problem, we have developed chromosome-specific paints using flow cytometry and microdissection. For the microchromosomes it was necessary to amplify and label DNA from single microdissected chromosomes.

Study of the domestic chicken *(Gallus domesticus)* is crucial for developmental biology and genomic investigations. The chick embryo is one of the classic models of vertebrate development because of its large size and easy accessibility, whereas the small genome size and paucity of introns and repetitive DNA in chickens make it an excellent model for the study of vertebrate genomics. For these and for commercial reasons (i.e., chickens are a significant contributor to the egg and meat industry), chickens are among the most genetically mapped of species (Burt et al., 1995). Paradoxically, the karyotype of the chicken is among the least well defined of all farm animals, primarily owing to the presence of microchromosomes. Depending on the definition given by different authors, *G. domesticus* (2n = 78) has 6–10 pairs of large macrochromosomes, including the Z and smaller W sex chromosomes in the heterogametic female (e.g., Kaelbing and Fecheimer, 1983; Auer et al., 1987; Schmid et al., 1989; Ponce de León et al., 1992). Smaller than the W are over 30 pairs of chromosomes that are very difficult to distinguish from each other. To properly assign genes and, hence, perform meaningful genetic and physical mapping, it is essential that chromosomes are adequately defined; banding studies have been limited in this regard. Despite this, Auer et al. (1987) were able to distinguish chromosomes 1–18 using counterstain-enhanced fluorescence; the smaller microchromosomes, however, remain undefined by classical means.

Recently, fluorescence in situ hybridisation (FISH) was used to define chicken chromosomes according to the gene or anonymous DNA fragment they contain (Fillon et al., 1998). Fillon and co-workers were able to distinguish 16 microchromosomes using 17 different PAC and BAC clones as molecular markers. However, since clones are merely landmarks, they will not identify rearrangements, such as translocations, between individuals, between different strains, or between different species. In our opinion, therefore, the best approach for unequivocally karyotyping chickens is to use chromosome painting, which illuminates the whole chromosome along its length.

In this paper we have generated chromosome paints for 27 chicken chromosomes. We used two approaches to accomplish this: (1) the well-established technique of flow cytometry for the larger chromosomes and (2) a novel means of generating paints from single microdissected microchromosomes for the smaller ones. In both cases the **d**egenerate **o**ligonucleotide-**p**rimed **p**olymerase **c**hain **r**eaction (DOP-PCR) was used to amplify and label the isolated chromosomes. We have demonstrated that

Received 17 May 1999; revision accepted 23 September 1999.

Request reprints from Dr. Darren K. Griffin, Department of Biological Sciences, Brunel University, Uxbridge, Middlesex UB8 3PH (UK);
telephone: 01895-274000 ext 2653; fax: 01895-274348;
e-mail: Darren.Griffin@brunel.ac.uk.

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Table 1. Details of 11-colour FISH experiments

Chromosome	1	2	3	4	5	6	7	8	9	?[a]	Z
Label	Cy3	Cy5	Bio	Dig	Cy5 Bio	Cy3 Cy5	Cy3 Bio Dig	Cy3 Cy5 Bio	Cy5 Dig	Bio Dig	Cy3 Dig
Volume placed in pool (µl)	6	6	4	4	4	4	2	2	2	2	4

[a] Unidentified microchromosome.

these chromosome paints are essential tools for mapping clones throughout the avian genome in multicolour labeling strategies. Adaptations of such strategies will eventually define the avian karyotype in full.

Materials and methods

Cell culture and preparation

Metaphase chromosome preparations were generated by standard protocols using chicken or goose fibroblast cell cultures established from 5- to 7-day-old embryos.

Fluorescence-activated chromosome sorting (FACS)

Chromosomes were prepared for flow sorting as described previously (Carter et al., 1992). The preparations were spun briefly (100 *g* for 1 min) to remove any debris, and then the supernatant was stained with 2 µg/ml Hoechst 33258 (Sigma) and 40 µg Chromomycin A_3 (Sigma). Bivariate flow karyotypes were generated on a FACStar Plus dual-laser flow cytometer (Beckton Dickinson) equipped with two 5-W argon ion lasers. Figure 1 shows the bivariate flow karyotypes for the chicken. Approximately 400 chromosomes from the relevant peak were sorted into a 0.5-ml Eppendorf tube containing water.

Microdissection of microchromosomes

Chromosome preparations on cover slips were stained with 10 % Giemsa, and individual chromosomes were lifted from the glass using a glass needle attached to a micromanipulator and an inverted microscope (at 1,000× magnification). The needle was then broken in a tube containing 10 µl of sterile distilled water prior to PCR amplification (e.g., Guan et al., 1994).

DOP-PCR

A primary round of DOP-PCR amplification was performed on these chromosomes (Carter et al., 1992). For microdissection experiments, thin-walled tubes were used, and a "hot-start" strategy was employed, i.e., the enzyme was added after the initial denaturation step. From each of these primary DOP-PCR reactions, 1–2 µl was used as a template for a secondary DOP-PCR amplification incorporating labeled dUTP. This facilitated amplification and labeling of the relevant chromosome, thereby creating a chromosome paint (Carter et al., 1992).

Labeling of cosmids

For cosmid mapping experiments, clones isolated by screening cosmid libraries with cDNAs from the genes CCNC (cyclin C), INS (insulin), IGF1 (insulin growth factor 1), CCNE (cyclin E), and ETS1 (avian erythroblastosis virus e26 oncogene 1) (further details are available at www.ri.bbsrc.ac.uk/chickmap/) plus 25 anonymous cosmids were labeled with digoxigenin-dUTP, biotin-dUTP, and/or fluorescein-dUTP by nick translation (Boehringer Mannheim).

FISH

Metaphase chromosome preparations were dehydrated and then aged overnight at 37 °C. Standard protocols were employed. Chromosomal DNA was denatured for 1 min in 70 % formamide, 2 × SSC (65 °C); and the probe was denatured at 65 °C for 10 min in standard hybridisation buffer. After 1–3 d hybridisation, the preparations were washed twice (5 min each) in 50 % formamide, 2 × SSC, then twice (5 min each) in 0.1 × SSC at 42 °C. Detection of labeled probe was made possible with Cy3-conjugated avidin (diluted 1:500 in 4 × SSC, 0.1 % Tween 20, and 1 % BSA) for biotinylated probes, or with FITC-conjugated anti-digoxigenin antibodies (1:200 dilution) for digoxigenin-labeled probes. Finally, the slides were counterstained and mounted using DAPI/Vectashield anti-fade medium (Vector Laboratories).

Table 2. Composition of the pools

Pool	Chromosomes in pool	Label	Volume in final precipitate (µl)[a]
1	1, 6, 7, 8, Z	Cy3	10
2	2, 5, 6, 8, 9	Cy5	10
3	3, 5, 7, 8, ?[b]	Bio	6
4	4, 7, 9, ?[b], Z	Dig	5

[a] 10 µl of secondary product ≈ 1 µg DNA.
[b] Unidentified microchromosome.

Eleven-colour FISH

Table 1 shows the labeling strategy for each chromosome. In each case a pool of primary DOP-PCR products from selected chromosomes were precipitated with ethanol and resuspended in 10 µl of water. Table 2 shows the composition of the pools. This provided a template for a secondary DOP-PCR labeling with digoxigenin, Cy3, biotin, or Cy5-dUTP (Amersham, Boehringer Mannheim). The products were then pooled and ethanol precipitated with an excess of unlabeled chicken DNA prior to FISH. FISH proceeded as above, except that Cy3.5-conjugated avidin was used to detect biotinylated probes. All images were captured by a cooled black-and-white CCD camera (Sensys KAF 1400, Photometrics) driven by Vysis/Digital Scientific SmartCapture software or QFISH software (Leica).

Results

FACS experiments

Our flow-sorting-based experiments were successful in generating chicken chromosome paints from autosomes 1–9, the Z chromosome, and a smaller microchromosome. We also generated three paints that recognise two pairs of different microchromosomes, two paints that recognise three pairs of microchromosomes, and one that recognises approximately 10 pairs. No further microchromosomes were resolved in the flow karyotype (Fig. 1).

Eleven-colour FISH

Figure 2 shows a multicolour image in which all chromosome paints generated by flow sorting were detected in a single experiment.

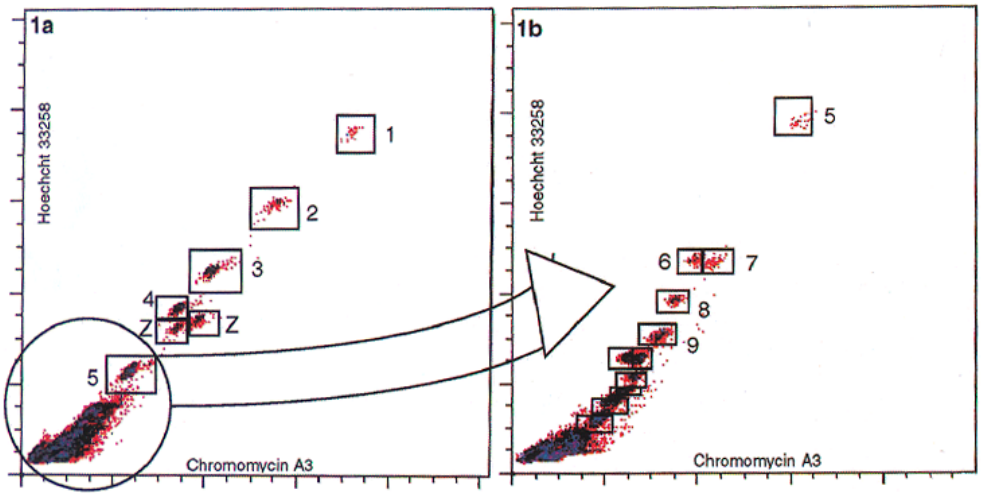

Fig. 1. Flow karyotype of the chicken. (**a**) Larger microchromosomes. (**b**) Higher resolution, showing detection of smaller chromosomes.

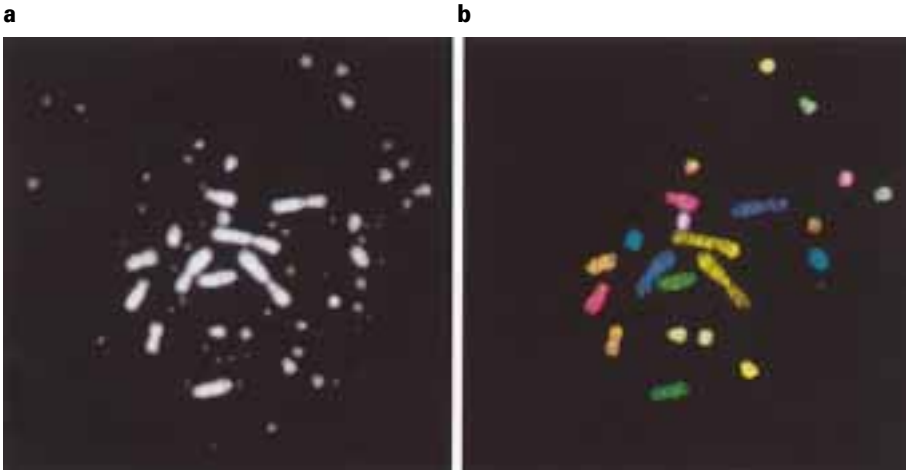

Fig. 2. Eleven-colour chromosome painting of chicken metaphase spread (**b**). DAPI-counterstained chromosomes are shown in (**a**).

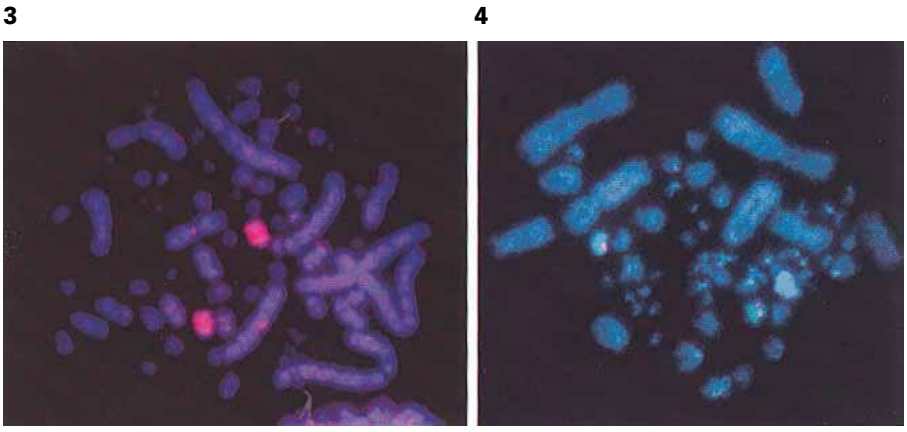

Fig. 3. Chromosome paint made from a single microdissected microchromosome, showing painting of a medium to large microchromosome (consistent with the size of chromosomes 10–15).

Fig. 4. Co-hybridisation of an anonymous cosmid and chromosome paint (chromosome 9).

Mapping of cosmid clones

A systematic mapping study of 29 cosmid clones in conjunction with dual and multicolour chromosome painting was successful in assigning 19 clones to specific chromosomes. Although chromosome assignments could be easily made for chromosomes 1–5 on the basis of size and centromere position, multicolour hybridisation with the chromosome paints was particularly useful in assigning clones to the others. In total, one clone mapped to chromosome 1, three to chromosome 2, three to chromosome 3, two to chromosome 4, two to chromosome 5, one to chromosome 6, three to chromosome 7, one to chromosome 8, two to chromosome 9, and one to the Z chromosome. Ten clones remained unidentified on microchromosomes from which we do not yet have paints.

Microdissection experiments

We generated chromosome paints from 16 different individual chicken microchromosomes. We also performed similar experiments on goose metaphase spreads for which we made microdissected paints for the W chromosome and three microchro-

mosomes. Cross-species painting revealed that these paints (except for the W chromosome paint) hybridise equally well to both goose and chicken metaphase chromosomes. Figure 3 shows one of these paints on a medium to large microchromosome.

Discussion

The aim of the present study was to develop strategies that will, in time, produce probes for all chicken chromosomes, as well as subregional paints. Genetic and physical mapping for all species requires the unequivocal identification of chromosomes; however, this has been a barrier to the progression of mapping of the avian genome due to the presence of microchromosomes. Dual and multicolour experiments (e.g., Fig. 4) incorporating both isolated clones and chromosome paints will eventually lead to the isolation of any clone, and co-localisation of two or more clones will indicate genetic linkage.

Flow cytometry, while successful in isolating some of the larger microchromosomes, was clearly limited in its sensitivity for isolating the smaller ones. Nevertheless, we were able to demonstrate, using these paints, how multicolour strategies will eventually be used to define all chicken chromosomes. A systematic study of 29 cosmid clones in conjunction with dual and multicolour chromosome painting strategies led to the assignment of 19 of the 29 clones. Some, however, remain unassigned. To circumvent this problem, we developed a means of generating chromosome paints by amplification of a single microdissected chromosome. It is essential that PCR techniques are sensitive enough to amplify only a single chromosome, as most are indistinguishable under the microscope, and, hence, it is not practicable to locate the same chromosome on a different metaphase. This represents a significant advance in the field of chicken gene mapping, as, in previous studies, microdissected paints were made from a template of several isolated chromosomes (e.g., Ponce de León, 1996; Zimmer et al., 1997). In other words, although chromosome paints generated by microdissection of larger chicken chromosomes are reported in the literature, this study is the first, to our knowledge, to report the generation of chromosome paints from single microdissected microchromosomes. Furthermore, the combination of these two approaches has, we believe, produced the most comprehensive definition of the avian karyotype by chromosome painting to date.

In general, paints made from flow-sorted preparations were brighter and more specific than those made from microdissected chromosomes. This is almost certainly because a template of 400 chromosomes was used in the former case, whereas one chromosome was used for the latter. However, our experiments indicate that the microdissection approach is not limited by size, as we succeeded in making a chromosome paint from one of the smaller microchromosomes.

Chromosome paints have been used extensively in the study of comparative gene mapping and mammalian genome evolution. Similar work in birds will form the basis of our further studies. Indeed, these paints are currently being used for such investigations in related avian species (Wienberg, Griffin, et al., manuscript in preparation) and in distantly related birds, such as the ostrich and emu (Shetty et al., 1999). Chicken/human cross-species chromosome painting to establish interspecific synteny between these two extensively mapped vertebrates may ultimately become a reality. Furthermore, unlabeled paints represent libraries from which chromosome-specific sequences can be isolated (e.g., Ponce de León, 1996; Zimmer et al., 1997).

The strategies developed here will be extended to include more, and smaller, chromosomes and will ultimately allow us to define all avian chromosomes on the basis of molecular cytogenetics. There are a number of multicolour labeling strategies in the literature for the identification of human chromosomes (e.g., Schrock et al., 1996). Utilising this novel technology will eventually lead to a fully analysable chicken karyotype in a single experiment.

Acknowledgements

The authors are grateful to I. Solovei and R. Fries for preparing and sending the chicken and goose cell lines.

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