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Compositional mapping of chicken chromosomes and identification of the gene-richest regions

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Abstract

'*Compositional chromosomal mapping*', namely the assessment of the GC level of chromosomal bands, led to the identification, in the human chromosomes, of the GC-richest $H3^+$ bands and of the GC-poorest $L1^+$ bands, which were so called on the basis of the isochore family predominantly present in the bands. The isochore organization of the avian genome is very similar to those of most mammals, the only difference being the presence of an additional, GC-richest, H4 isochore family. In contrast, the avian karyotypes are very different from those of mammals, being characterized, in most species, by few macrochromosomes and by a large number of microchromosomes. The '*compositional mapping*' of chicken mitotic and meiotic chromosomes by *in-situ* hybridization of isochore families showed that the chicken GC-richest isochores are localized not only on a large number of microchromosomes but also on almost all telomeric bands of macrochromosomes. On the other hand, the GC-poorest isochores are generally localized on the internal regions of macrochromosomes and are almost absent in microchromosomes. Thus, the distinct localization of the GC-richest and the GC-poorest bands observed on human chromosomes appears to be a general feature of chromosomes from warm-blooded vertebrates.

Introduction

Vertebrate genomes are made up of long DNA segments (several hundred kb in size), the isochores, that are compositionally fairly homogeneous, and that can be subdivided into a small number of families (for reviews see Bernardi 1995, 2000). The compositional range

covered by isochore families is different in cold- and warm-blooded vertebrates, being very narrow in the former and very broad in the latter. In fact, the genomes of cold-blooded vertebrates are mainly composed, in the vast majority of cases, by GC-poor isochores belonging to the L1 and L2 families. In contrast, the genomes of warm-blooded vertebrates show, in addition, isochores belonging to the GC-rich H1, H2, H3 and H4 families, the H4 family only being observed in the avian genomes (Cortadas *et al*. 1979, Olofsson & Bernardi 1983, Sabeur *et al*. 1993, Kadi *et al*. 1993, Cacciò *et al*. 1994). Thus, the genomes from the vast majority of warm-blooded vertebrates are characterized by the presence of about 15% of very GC-rich isochores belonging to the H2, H3 and (in the avian genomes) H4 families.

The 'single copy' DNA sequences present in the GC-rich compartments of warm-blooded vertebrates are characterized by a high level of similarity, as shown by hybridization of human H3 isochores on the GC-richest compositional DNA fractions from mammals and birds, under conditions in which the repetitive sequences were competed out (Cacciò *et al*. 1994). This suggests that single-copy sequences were strongly conserved in terms of composition during the evolution of these two classes of vertebrates. This finding is further reinforced by the high degree of similarity between orthologous coding sequences from man and chicken (Cruveiller *et al*. 2000), and from chicken and other birds (Kadi *et al*. 1993).

Avian karyotypes are generally characterized by a small number of macrochromosomes and a large number of microchromosomes. More precisely, the chicken karyotype is composed of 8 macrochromosomes, Z and W sexual chromosomes and 30 pairs of microchromosomes (Ladjali-Mohammedi *et al.* 1999). The first three chromosome pairs show a morphology and a banding pattern that are very similar among species from different orders (Takagi & Sasaki 1974, Rodionov 1997). Also ZOO-FISH analysis of chicken and emu, two species that diverged at least 80 million years ago, showed strong conservation of the karyotypes through evolution (Shetty *et al*. 1999), confirming the low rate of chromosome changes that characterize the avian genome (Burt *et al*. 1999). These conserved features are in agreement with the great uniformity of the isochore patterns from different avian genomes (Kadi *et al*. 1993, Cacciò *et al*. 1994).

In the human genome, gene concentration parallels GC levels, being low and relatively constant in GC-poor isochore families L1 and L2 and increasing over GC-rich isochore families H1, H2 and H3, the latter family being endowed with the highest gene concentration (Zoubak *et al.* 1996). 'Compositional chromosomal mapping', namely the identification of the GC levels of chromosomal bands, led to the identification, in human chromosomes, of the GC-richest $H3^+$ bands (largely coinciding with the T bands of Dutrillaux 1973) and of the GC-poorest $L1^+$ bands (Saccone *et al.* 1992, 1993, 1996, 1999, Federico *et al*. 2000), which were so called on the basis of the isochore family predominantly present in the bands. This approach is of interest because it provides information not only on the GC level of a certain chromosomal region, but also on the properties related to the GC levels. In fact, $H3^+$ bands also show the highest concentration of genes (see above), a very high level of transcriptional activity, the highest recombination frequency and an 'open' chromatin structure (for a recent review see Bernardi 2000). $L1^+$ bands are, in contrast, characterized by opposite features. Moreover, the DNA of $H3^+$ and $L1^+$ bands is replicated at the onset and at the end of the S phase of the cell cycle, respectively (Federico *et al*. 1998, 2000). In addition, the GC-richest and the GC-poorest regions do not share the same chromosomal location, the former being prevalently localized on telomeric bands (the T bands of Dutrillaux 1973) and the latter on internal bands (Federico *et al*. 2000).

In the present work, we studied the chicken genome by *in-situ* hybridization of fractionated DNA on mitotic and meiotic chromosomes, in order to obtain a 'chromosomal compositional map' of chicken chromosomes.

Materials and methods

DNA fractionation

Compositional fractions of chicken genomic DNA were obtained by preparative ultra-

centrifugation in Cs_2SO_4 density gradients in the presence of the AT-specific DNA ligand BAMD (3,6-bis (acetato-mercurimethyl) dioxane; (Thiery *et al.* 1976, Kadi *et al.* 1993). Briefly, this was done at 20°C and 40 000 rpm for 65 h, using a BAMD/nucleotide molar ratio, rf = 0.14. The relative amount of DNA in each fraction and its modal buoyant density are shown in Table 1.

Chromosome preparations

Mitotic chromosomes. Primary fibroblast cell lines were isolated from leg muscle of young domestic chicken (*Gallus gallus domesticus*). The tissue was minced and trypsinized and the cells, collected after centrifugation, were cultured in RPMI-1640 medium supplemented with 10% foetal calf serum, 2 mmol/L L-glutamine, 100 IU penicillin, 100 μg/ml streptomycin, and then incubated at 39°C in 5% CO_2. Monolayer cultures were treated with colcemid (final concentration 0.05 μg/ml) for 30 min, detached with trypsin for 10 min at 37°C, and then resuspended in hypotonic solution (75 mmol/L KCl) for 20 min at 37°C. Conventional methanol/acetic acid fixation and slide preparation were then carried out.

Meiotic lampbrush chromosomes. The meiotic lampbrush chromosomes were isolated manually from the previtellogenic/early vitellogenic oocytes of laying chickens as described by Solovei *et al.* 1993. Briefly, the nuclei of 1.2–2.5-mm oocytes were removed in '5:1+phosphate' Gall's solution, a medium containing 83 mmol/L KCl, 17 mmol/L NaCl, 6.5 mmol/L Na_2HPO_4, 3.5 mmol/L KH_2PO_4, pH 7.0 (Gall *et al.* 1981), by needle dissection, the nuclear envelope being removed manually with tungsten needles in 1/4 strength. After the nuclear content dispersion, the operation chamber was centrifuged at 2000–2500*g* for 15 min to stick the lampbrush chromosomes firmly to the slide. Lampbrush chromosomes were then fixed in 2% formaldehyde and 70% ethanol. Identification of lampbrush macrobivalents was carried out by using the cytological map of the chicken lampbrush chromosomes (Chelysheva *et al.* 1990).

In-situ *hybridization and detection*

The *in-situ* hybridization protocol used was that previously described (Saccone *et al.* 1992, 1993) with some modifications. Briefly, hybridizations were performed with 150 ng of each biotin-labelled fraction. In the case of simultaneous hybridization of the L1 and H4 isochores, 150 ng each of digoxigenin- and biotin-labelled DNA fraction P (pellet) and 7, respectively, were used. In all cases, preannealing (60 min at 37°C) was performed with 100-fold excess of chicken sonicated genomic DNA, in order to avoid hybridization due to the repetitive sequences present in the probe. Hybridization conditions and detection of the biotin-labelled probes were according to Saccone *et al.* (1992). Detection of the simultaneously hybridized biotin- and digoxigenin-labelled probes was obtained by avidin–TRITC and antidigoxigenin–FITC antibody, respectively. After hybridization, chromosomes were stained with propidium iodide (single biotin-labelled probe) or DAPI (simultaneous hybridization of biotin- and digoxigenin-labelled probes). At least ten metaphases for each hybridized fraction were analysed, and the signals were correlated with the chromosomal bands by measuring the relative distance from the telomeres. Subsequent G banding with Wright's stain method (Saccone *et al.* 1992) was also performed after hybridizations to exactly establish and confirm the hybridized chromosomal bands.

Results

Chromosomal localization of the chicken H4 and L1 isochores

An overview of the chromosomal distribution of all the chicken isochore families is presented in Table 1.

Fraction 7 was used to localize the H4 isochore family, since it represented about 50% of this family. This fraction (like fractions 3–6; see

Table 1. Compositional features and chromosomal distribution of DNA fractions.

Fr.[1]	DNA (%)	ρ^0 (g/cm^3)[2]	Isochore family (%)						Localization			Figure
			L1	L2	H1	H2	H3	H4	Macrochromosomes		Microchromosomes	
									Telomeric bands	Internal bands		
P	31.9	1.6978	31.9						No signals	Many G bands	No signals	1
1	16.2	1.6998	10.0	6.2					No signals	All – diffuse	No signals	5A
2	14.5	1.7023		14.5					No signals	All – diffuse	Almost all	5B
3	11.7	1.7041[3]		4.4	7.3				All	Many G and R bands	Almost all	5C
4	9.8	1.7066[3]			6.7	3.1			All	Many R bands	Almost all	5D
5	7.1	1.7098[3]				6.9	0.2		All	Some R bands	Almost all	5E
6	7.4	1.7135[3]					5.8	1.6	All	Some R bands	Almost all	5F
7	1.4	1.7190[3]						1.4	Almost all	Few and weak	Almost all	1

[1]Fraction number; P = pellet. [2]Modal buoyant density. [3]Satellite DNAs are also present.

Table 1) also contained a satellite DNA that did not interfere with the hybridization results since it was largely located on the heterochromatic region of the Z chromosomes. Pellet DNA and fraction 1 were used to localize the L1 isochore family.

Hybridizations with fraction 7 (H4 isochore family) on mitotic chromosomes can be summarized as follows (see also Table 1): (1) a large majority of microchromosomes were completely covered by the hybridization signals (Figures 1 & 2A); (2) a number of microchromosomes showed signals only on about half of their length (Figures 1 & 2B); (3) a few microchromosomes showed very weak or no hybridization signals (Figures 1 & 2C); (4) macrochromosomes showed intense hybridization signals on almost all telomeric bands, the signals being very low or absent only in some telomeres (Figures 1 & 3); macrochromosomes showed, in addition, hybridization signals on some internal R bands (Figures 1 & 3); (5) the telomeric region of the q arm of the Z chromosome, known to be heterocromatic and GC-rich (Mizuno and Macgregor 1998), showed intense hybridization with the H4 isochore family DNA, indicating that the macrosatellite pFN-1 DNA sequences (Hori *et al*. 1996) present in fraction 7 (see Table 1, Figures 1 & 3) were not completely competed out. Thus, our competition procedure was unable to prevent the hybridization of the repetitive sequences present in the GC-rich probes and the Z-heterochromatin DNA, as it was also observed when using chicken painting probes (Guillier-Gencik *et al*. 1999). It should be pointed out that hybridization with H4 isochores on telomeric bands of macrochromosomes are not due to the macrosatellite pFN-1 (Hori *et al*. 1996),

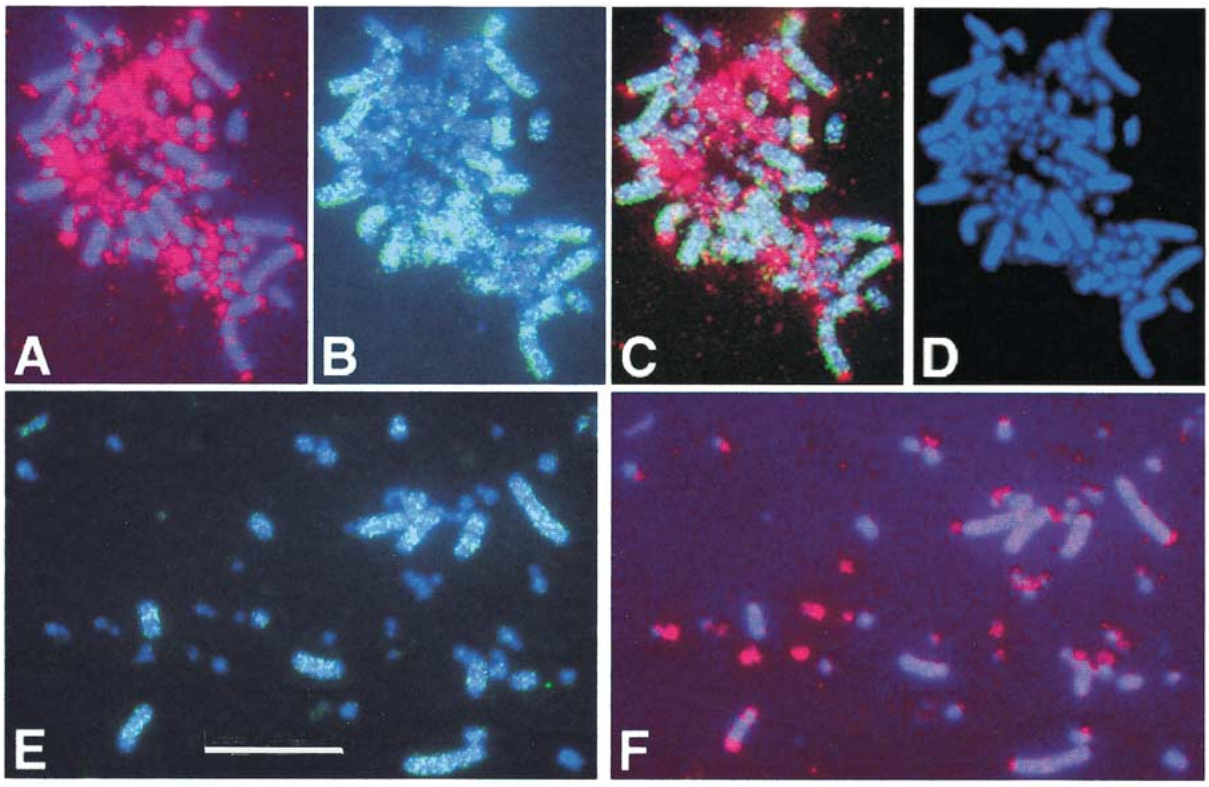


Figure 1. Localization of L1 and H4 isochores. *In-situ* hybridization with pellet and fraction 7 DNA labelled with digoxigenin (green signals are due to fluorescein) and biotin (red signals are due to rhodamine), respectively. Chromosomes were stained with DAPI. **A**, **B**, **C** and **D**: the same metaphase showing hybridization of H4, L1, H4+L1 isochores and DAPI staining, respectively. **E** and **F**: the same (partial) metaphase showing hybridization of H4 and L1 isochores, respectively. Scale bar = 10 μm.

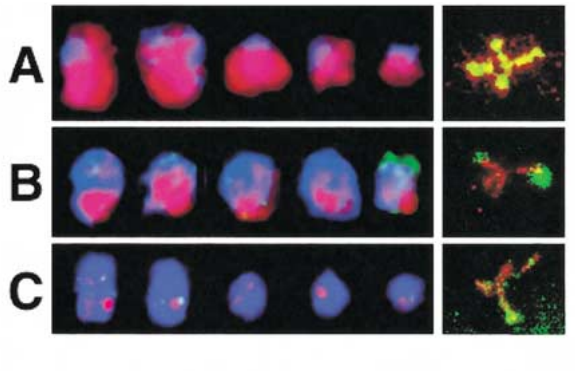


Figure 2. Hybridization of L1 and H4 isochores on microchromosomes. Representative chicken microchromosomes showing the different hybridization types observed with the same probes used in Figure 1. In the case of mitotic (left) and meiotic (right) chromosomes the H4 signals are red and yellow/green, respectively. (**A**) H4 signals covering most of the microchromosomes. (**B**) H4 signals covering half or less of the microchromosomes. (**C**) H4 signals almost absent from the microchromosomes. In the mitotic chromosomes, L1 signals (green) are almost absent. Lampbrush chromosomes were only hybridized with the H4 isochores and stained with propidium iodide (red).

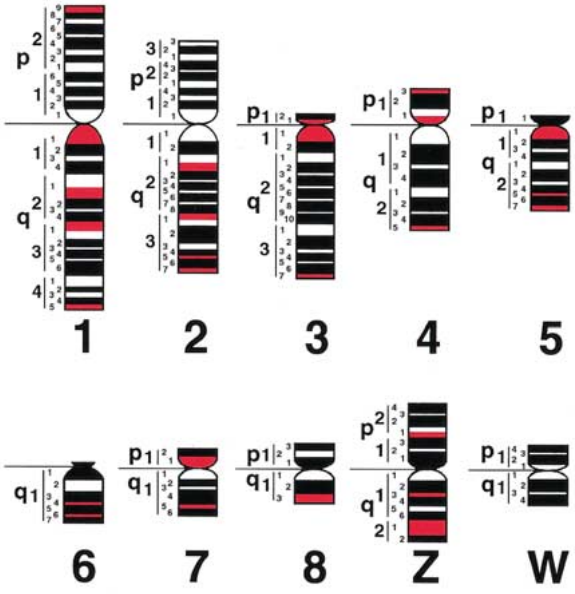


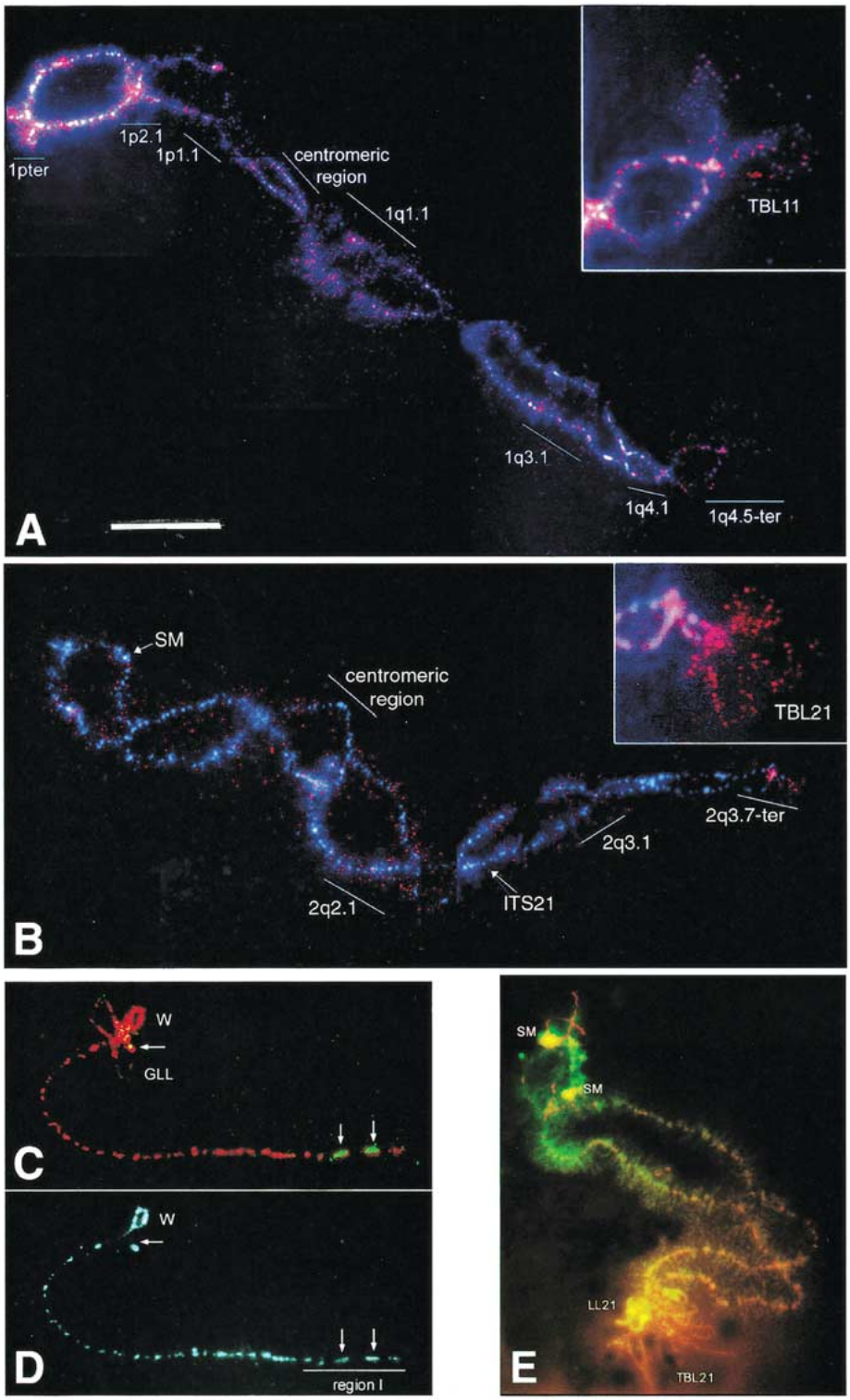
Figure 3. H4 isochore family distribution on the largest macrochromosomes. G-banded ideograms of the largest chicken chromosomes (from: Ladjali-Mohammedi *et al.* 1999) showing the distribution of the H4 isochores (red bands) as assessed by *in-situ* hybridization of fraction 7 on mitotic chromosomes. Some H4-containing bands are incompletely coloured in red, indicating that hybridization signals were not as wide as the corresponding R band.

no correlation being observed between the type and the number of hybridization signals obtained with the isochore DNA and the macrosatellite DNA.

Hybridizations with the pellet DNA (L1 isochore family) on mitotic chromosomes showed the following (see also Table 1): (1) a large number of hybridization signals were observed on macrochromosomes and more precisely on internal bands, generally G-bands, that did not coincide with those hybridized with the H4 isochores (Figure 1; see also Figure 5A for hybridization with the fraction 1 largely containing L1 isochores); (2) the telomeric regions of the macrochromosomes and almost all the microchromosomes showed no signals (Figure 1); (3) only a few microchromosomes presented weak hybridization signals (Figures 1 & 2).

Thus, hybridization patterns of the H4 and L1 isochores did not share the same localization on mitotic chromosomes, the former being prevalently located on microchomosomes and

Figure 4. Distribution of the H4 and L1 isochores on lampbrush chromosomes. (**A**) DAPI-stained lampbrush chromosome 1 hybridized with the digoxigenin-labelled H4 isochores (fraction 7) detected with Cy3-labelled antibody (red signals). H4 signals are observed in the lateral loops of both internal R bands and telomeric regions. Scale bar = 20 μm. The subpanel shows 1q4-qter region carrying H4-positive telomeric bow-like loops (TBLs). Landmark regions are indicated. (**B**) DAPI-stained lampbrush chromosome 2 hybridized as in **A**. SM = spaghetti marker; ITS21 = internal TTAGGG-positive site located in the middle of 2q2 region. The subpanel shows 2qter region carrying H4-positive telomeric bow-like loop (TBL21). Note there are no H4-positive signals on the 2p-telomere (see Figure 3 for comparison with the mitotic chromosome H4-positive banding pattern). (**C**) Propidium iodide-stained lampbrush sex bivalent ZW hybridized with the biotin-labelled H4 isochores (fraction 7) (green signals). H4-positive signals are located in the heterochromatic region 1 of the chromosome Z (see Solovei *et al.* 1993, Mizuno & Macgregor 1998). W = sex chromosome W; GLL = giant lumpy loops. (**D**) The same chromosomes shown in C stained with DAPI. Arrows point to the H4-positive chromomeres of region 1. (**E**) Propidium iodide-stained lampbrush bivalent 2 hybridized with the biotin-labelled fraction 1 (L1 isochore) (yellow/green signals). Note the very intense hybridization signals on subtelomeric loops LL21 and the absence of hybridization on telomeric loops TBL21.


1pter
1p2.1
1p1.1
centromeric region
1q1.1
TBL11
1q3.1
1q4.1
1q4.5-ter
A
SM
centromeric region
TBL21
2q3.7-ter
2q3.1
ITS21
2q2.1
B
W
GLL
C
W
D
region I
SM
SM
LL21
TBL21
E

telomeric bands of the macrochromosomes, and the latter, conversely, being located on the internal regions of macrochromosomes (Figure 1). Generally, the microchromosomes lacking H4 isochores were 3–5 pairs per metaphases.

The use of meiotic lampbrush chromosomes confirmed, also at this higher resolution, the distribution of the H4 isochores in the telomeric and some internal regions of the chicken macrochromosomes (Figure 4A, B). In fact, H4 isochores were prevalently found, in the lampbrush autosomes, in both telomeric bow-like loops (TBLs), and in the lateral loops corresponding to the internal R-bands identified in the mitotic chromosomes (see Figures 3 & 4A, B). In addition, the heterochromatic region 1 of the sex bivalent ZW showed hybridization signals (Figure 4C). In the case of microbivalents, hybridization with fraction 7 was in agreement with the hybridizations on mitotic chromosomes. In fact, we observed microbivalent lateral loops completely covered by the signals, along with microbivalents with signals covering about half the microchromosomes and others with only a few labelled loops. The distribution of L1 isochores on lampbrush chromosomes indicated: (1) the absence of signals on microchromosomes (data not shown) and on telomeric TBL loops of the macrobivalents (Figure 4E); and (2) hybridization signals on the internal regions and some marker loops of the macrobivalents, such as LL21 (Figure 4E).

Chromosomal localization of the L2, H1, H2 and H3 isochores

L2 isochores were present in fraction 1 (together with L1 isochores), 2 and 3 (together with H1 isochores). In other words, fraction 2 hybridization (Figures 5B & 6) indicates the distribution of the L2 isochore family on the chromosomes, fractions 1 and 3 being largely composed of L1 and H1 isochores, respectively. Fraction 2 hybridization signals cover almost all the macrochromosome arms except for many telomeric regions and some internal bands that coincided with the bands containing the H4 isochores (see above). Moreover, almost all the microchromosomes showed hybridization signals, a small number of them being depleted of signals (Figure 5B**)**

H1 isochores were present in fractions 3 (together with L2 isochores) and 4 (together with H2 isochores). In both fractions, the majority of DNA belongs to the H1 isochores. Fraction 3 showed hybridization signals (Figure 5C) on almost all the chromosomal regions both on micro- and macrochromosomes, indicating the wide distribution of this DNA fraction, which contains L2 and H1 isochores. In the case of fraction 4, the hybridization signals became more evident in all the telomeric regions of the macrochromosomes and on almost all the microchromosomes (Figure 5D). The internal regions of the macrochromosomes also showed weak signals along their arms but some regions were endowed with high intensity of signals, all coincident with the regions hybridized with the GC-richer isochore families (Figure 5D & 6).

H2 isochores were present in fraction 4 (along with H1 isochores, see above) and in fraction 5. In the latter fraction the amount of adjacent H3 isochore family is negligible. We can, therefore, assume that fraction 5 is only composed of H2 isochores. In the latter case, the chromosomal distribution of the hybridization signals (Figure 5E) was very evident in the telomeric regions of the macrochromosomes, as well as in some of their internal bands. Moreover, almost all microchromosomes showed intense hybridization signals.

H3 isochores were almost completely present in fraction 6 together with H4 isochores, but in a larger amount compared with the latter (see Table 1). The hybridization pattern observed with fraction 6 (Figure 5F) was very similar to that observed with fraction 5, all the telomeric regions and almost all the microchromosomes being hybridized. In this case, the hybridized internal R bands of the macrochromosomes were very evident. The pattern of hybridization shown by H3 isochores is also very similar to that of H4 isochores, only some bands from macrochromosomes not hybridizing H4 isochores (see Figure 6).

The above results could be summarized (see Figures 5 & 6) as follows: (1) L2 and H1 isochores are distributed on all the micro- and macrochromosomes with a larger amount of the L2

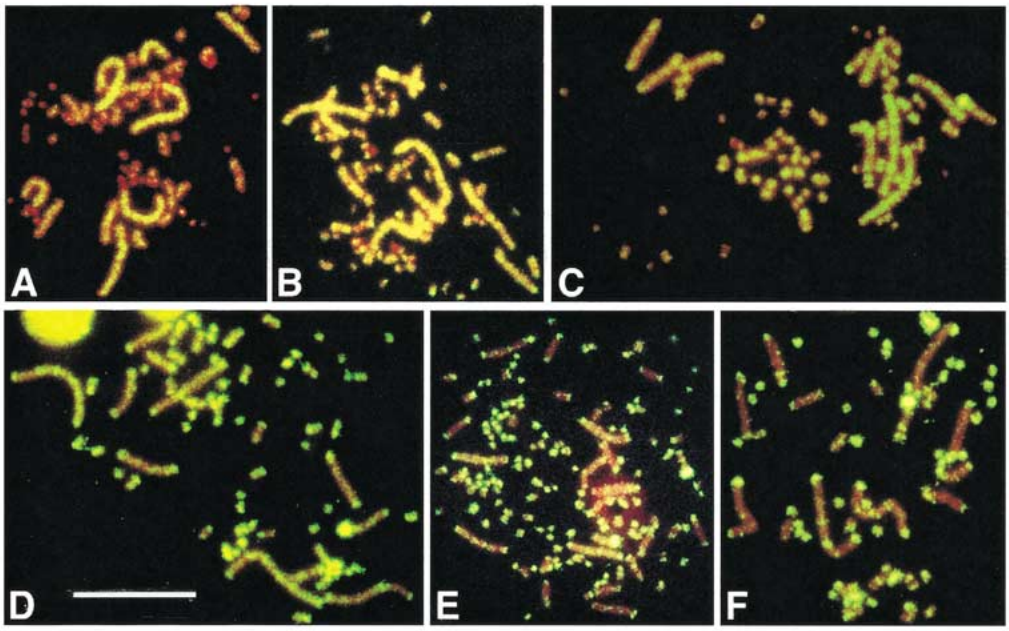


Figure 5. Hybridizations of L2, H1, H2 and H3 isochores on mitotic chromosomes. **A–F**: *In-situ* hybridizations of fractions 1–6, respectively. Each fraction was biotin-labelled and detected with avidin–FITC (yellow–green). Chromosomes were stained with propidium iodide (red). Scale bar = 10 μm.

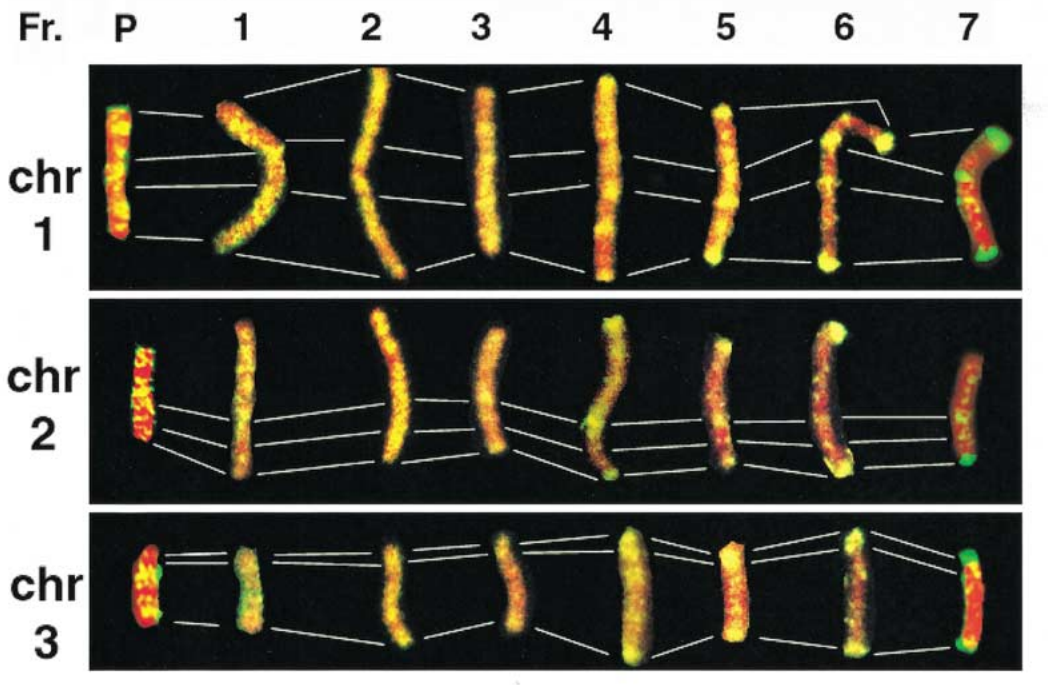


Figure 6. Hybridizations on macrochromosomes. Representative chicken macrochromosomes showing the distribution of the hybridization signals observed with the compositional DNA fractions indicated above each chromosome. The white lines indicate the GC richest chromosomal regions, namely the bands hybridized with the H4 isochores..

and H1 in the macro- and microchromosomes, respectively; (2) H2 isochores are largely present on telomeric bands of macrochromosomes, as well as on some intercalary bands largely coincident with those containing H4 isochores; moreover, the microchromosomes are largely composed of this isochore family; (3) the distribution of H3 and H2 isochores is largely coincident, the only difference being a higher spread of signals on the internal bands of the macrochromosomes observed with the H2 isochores (compare Figure 5E, F and the hybridization on the chromosomes 1, 2, and 3 shown in Figure 6).

The L2 and H1 isochore families were, therefore, localized on all chromosomal bands, on both G and R bands, whereas the H2 and H3 isochores hybridized exclusively on a number of R-bands, the large majority being telomeric. Concerning microchromosomes, they were dominantly composed of isochores belonging to the H4, H3 and H2 families and to a lesser extent to the H1 and L2 families, indicating the high GC level of these chromosomes.

Discussion

In the present work, we localized all the chicken isochore families on metaphase chromosomes and defined the 'compositional mapping' of chicken chromosomes, an approach allowing the study of chromosomal features correlated with the DNA composition, namely the gene concentration, the recombination level, the replication timing, the chromosomal band type, etc. An important finding concerns the completely different chromosomal distribution of the GC-richest and the GC-poorest isochores, namely of the isochore families endowed with the most contrasting compositional features. To refine this latter result, oocyte-derived lampbrush chromosomes were also hybridized with the L1 and H4 isochore DNAs. This type of chromosome was described in avian species, such as chicken, pigeon and turkey, and was used to obtain higher resolutions than that obtainable with mitotic chromosomes. Indeed, the mean length of chicken chromosome 1 at the lampbrush stage is 33 times longer than the same chromosome in metaphase of meiosis and 15 times higher than diakinesis/metaphase I in the spermatocytes (Chelysheva *et al.* 1990).

The DNA content of eight macrochromosomes and sex chromosomes is 77–82% of the chicken genome, microchromosomes only comprising 18–23% of genomic DNA (Smith & Burt, 1998). Macrochromosomes exhibit opposite cytochemical and molecular features compared to microchromosomes. After GC-specific chromomycin A_3 staining, both macro- and microchromosomes fluoresce brightly. On the other hand, AT-specific staining with DAPI/AMD or Hoechst 33258/AMD is followed by bright fluorescence of macrochromosomes, whereas the majority of microchromosomes are dull (Auer *et al.* 1987, Schmid & Guttenbach 1988, Rodionov *et al.* 1989). All chicken microchromosomes, or at least a large number of them, replicate during the first half of S phase whereas there are both early and late replicating regions in all chicken macrochromosomes (Carlenius *et al.* 1981, McQueen *et al.* 1998, Schmid *et al.* 1989, I. Solovei, personal communication). Chicken microchromosomes comprise histones that are much more strongly acetylated than those from macrochromosomes. Moreover, microchromosomes contain larger amounts of CpG islands (McQueen *et al.* 1996, 1998). Chicken microchromosomes show a 2–4-fold higher recombination rate when compared with the macrochromosomes (Rodionov *et al.* 1992, Schmid *et al.* 2000).

As expected, the different properties of macro- and microchromosomes were also evident at the compositional level. In fact, H4 isochores were predominantly located in microchromosomes as well as in a number of chromosomal bands of macrochromosomes located on the telomeric regions. It should be noted that the location of the GC-rich isochores in the telomeric bands (more precisely in the T-bands) was previously demonstrated by *in-situ* hybridization of isochores in mammals (Saccone *et al.* 1992, 1997, 1999) and by hybridization of the telomeric repeat (TTAGGG)n in fractionated mammalian and avian genomes (unpublished results). In the present case, also the H4-enriched bands of the microchromosomes could be considered as T-bands (Dutrillaux 1973), being located near the telomeres. Moreover, the lowest GC level,

coinciding with the L1 isochore distribution, was observed on some internal G-bands of macrochromosomes that generally did not coincide with the telomeric GC-richest bands.

Our data refine previous results obtained on the basis of hybridization of CpG islands that indicated the microchromosomes as the gene-richest chromosomes (McQueen *et al*. 1998, Clark *et al*. 1999, Smith *et al*. 2000). In addition, they show that the telomeric regions of macrochromosomes are endowed with the same gene density observed in microchromosomes. Moreover, they provide the localization of the gene-poorest regions on chromosomes. Needless to say, the chromosomal identification of the GC-richest/gene-richest regions indicates the chicken chromosomal regions that could be considered priority targets in sequencing.

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