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The ZW lampbrush chromosomes of birds: a unique opportunity to look at the molecular cytogenetics of sex chromosomes

S. Mizuno[a] and H. Macgregor[b]

[a] Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai (Japan), and [b] Department of Biology, University of Leicester, Leicester (England)

Dedicated to Dr. Susumu Ohno on the occasion of his 70th birthday.

This paper concerns the sex chromosomes of birds, their morphology and their molecular biology, matters that have been the principal focus of a highly successful collaborative research programme which was initiated over 10 years ago involving scientists in Japan, Russia, the USA and England.

Birds offer excellent opportunities for a study of this kind for the special reasons that they have well differentiated and strongly heteromorphic sex chromosomes and, because the female is the heterogametic sex, both these chromosomes (Z and W) can be studied at relatively high resolution in the lampbrush form that they assume during the growth of oocytes in the adult ovary. In no other group of animals is this possible.

The greater part of our research on avian chromosomes in the lampbrush form has been carried out on three species of bird: *Gallus g. domesticus* (chicken), *Coturnix c. japonicus* (Japanese quail) and *Columba livia* (pigeon), although many other species have featured from time to time in comparative aspects of our studies. *G.g. domesticus* and *C.c. japonica* belong to the same order and family (Galliformes: Phasianidae). *C. livia* belongs to the order Columbiformes.

Z and W chromosomes down the microscope

Karyotypes of most modern birds include 5 or 6 pairs of macrochromosomes and between 32 and 36 pairs of microchromosomes. An example of mitotic metaphases from male and female chickens (*Gallus g. domesticus*), with the Z and W chromosomes indicated, are shown in Fig. 1A, B.

It has been shown that vertebrate genomes are mosaics of isochores, which are compositionally homogeneous DNA segments of different GC levels and are correlated with chromosome bands (Bernardi, 1993; Saccone et al., 1997). Birds have relatively small haploid genomes, ranging from 1.2 to 2.5 pg, containing small fractions of repetitive sequences. In a finer scale than that of isochores, the interspersion period of repeated and unique DNA sequences in the avian genome is unusual. Long unique sequences alternate with long clusters of moderately repeated sequences (Epplen et al., 1978; Eden and Hendrick, 1979; Arthur and Straus, 1983). To what extent these peculiarities are reflected in the visible cytological characteristics of bird chromosomes, whether in their metaphase or lampbrush form, remains unclear.

One matter is of special importance and value, and particularly so in view of the ease and success with which it is possible to study the lampbrush chromosomes of birds: bird chromosomes, unlike those of fishes and amphibians, show G and R banding patterns that are as well defined as those of mammalian chromosomes. In almost all respects, therefore, birds present an excellent system for studies in molecular cytogenetics. They have small genomes, well characterized karyotypes, workable lampbrush chromosomes and good female heterogamety allowing both sex chromosomes to be seen in their lampbrush form. Moreover, commercially motivated molecular research on the chicken genome has generated a useful genetic map and made available a wide range of gene sequences that can be used as probes for *in situ* nucleic acid hybridization (ISH).

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Request reprints from Dr. Shigeki Mizuno, Laboratory of Molecular Biology, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981-8555 (Japan); telephone: +81 22 717 8770; fax: (+81) 22 717 8883; e-mail: smiz@biochem.tohoku.ac.jp

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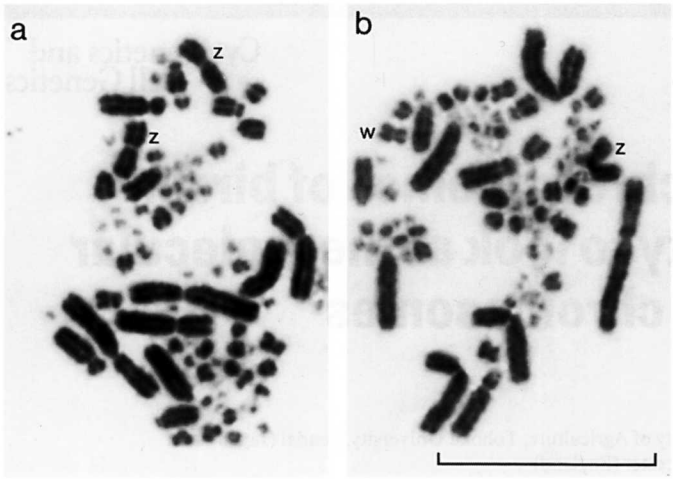
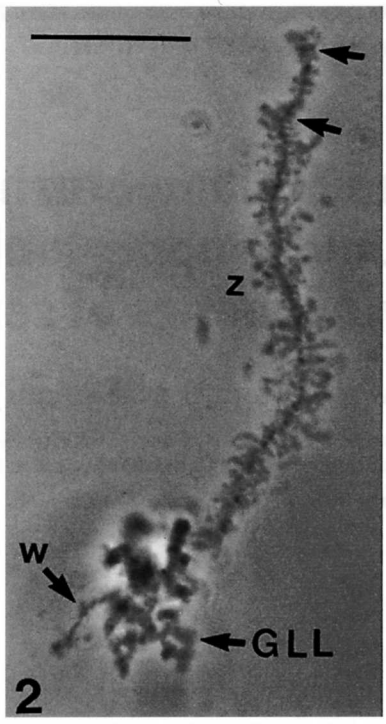


Fig. 1. Giemsa-stained metaphases of (**a**) male, and (**b**) female chickens, showing ZZ- and ZW-sex chromosomes. The bar represents 10 μm. Micrographs kindly provided by Indrajit Nanda and Michael Schmid.



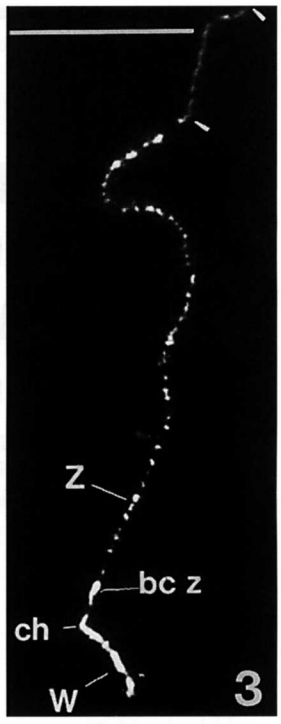


Fig. 2. Phase contrast micrograph of a lampbrush sex bivalent from *Gallus domesticus.* Z and W, axes of the Z and W lampbrush chromosomes. In chicken, giant lumpy loops (GLL) arise from a position near the chiasma. Arrows show the relatively condensed region with short loops towards the end of the free arm of the Z chromosome. Scale bar = 20 μm.

Fig. 3. Sex bivalent from chicken after staining with DAPI. Z and W, axes of the Z and W chromosomes. ch, estimated position of the chiasma. bc Z, the large bright chromomere on Z. Arrows indicate limits of the region of short loops near the end of the free arm of the Z chromosome. Scale bar = 20 μm.

Lampbrush chromosomes (LBCs) are greatly elongated diplotene bivalents found in the growing oocytes of all animals except mammals, some insects and some reptiles (Callan, 1986). The large size of these chromosomes and the intense RNA transcription that is taking place simultaneously on hundreds of lampbrush loops makes them particularly useful for locating gene sequences by ISH. This is specially true in relation to animals with small genomes, where many of the mitotic chromosomes are simply too small to work with. In the lampbrush form, even the smallest of chromosomes is easily visible and shows useful linear differentiation. In mitotic metaphases from birds, the largest macrochromosomes are about 10 μm in length and the smallest microchromosomes about 1 μm. In the lampbrush form, the lengths of these chromosomes are 20–30 fold greater.

The sizes of oocytes used for lampbrush studies differ from species to species: 1–2.5 mm diameter has been found to be best for chicken, 0.5–0.75 mm diameter for Japanese quail, 0.5–1.5 mm diameter for pigeon. LBCs are isolated manually employing the standard techniques initially developed for LBCs of amphibians (see Macgregor and Varley 1988), suitably modified for oocytes of birds (see Solovei et al., 1993). Preparations of LBCs can be examined directly and unfixed with a phase contrast microscope or fixed in formaldehyde and 70% ethanol prior to fluorescent staining with DAPI (4′,6-diamidino-2-phenylindole) or processing for fluorescence *in situ* hybridization (FISH).

The ZW lampbrush bivalent, when seen by phase contrast microscopy in a freshly made and unfixed LBC preparation, looks like a univalent (Solovei et al., 1993). Most of it has a typical lampbrush organization but the terminal one fifth consists of a relatively thick condensed axis carrying only a few very small lateral loops and, often, no discernible loops at all (Fig. 2). The region with normal lampbrush appearance is known to be the Z chromosome. The short thick loopless region is the W chromosome. The point at which these two chromosomes are joined by a single chiasma is usually marked by two adjacent pairs of large conspicuous loops, one belonging to the end of the W and the other marking the end of the Z. The limits of Z and W chromosomes and the gross differences in the axial organizations of these two chromosomes are best seen in DAPI stained preparations (Figs. 3, 4).

The fully extended Z-LBC in chicken is 70–100 μm long in fixed and stained preparations. There are three distinct regions along the length of the chromosome (Fig. 5A). Region I occupies about 17% of the chromosome and lies at the free end. It is characterized by closely packed chromomeres bearing uniformly short loops that extend only 2–3 μm from the chromosome axis. Region II occupies the central part of the chromosome and constitutes about 60% of it. It is characterized by large chromomeres that are more widely spaced along the axis and loops that are at least twice as long as those in region I. In region III, which is about 25% of the chromosome, the axis consists of very small chromomeres bearing loops of variable length. Var-

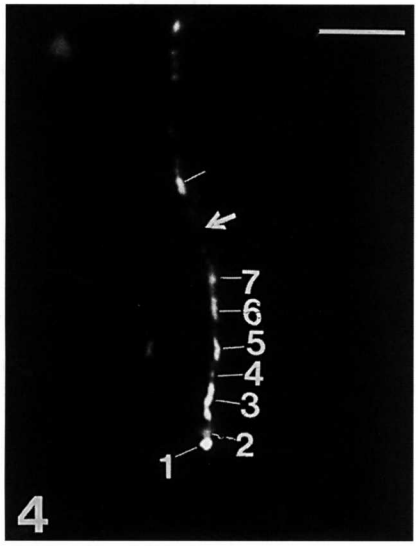


Fig. 4. Chicken W-LBC after staining with DAPI. 1–7, numbers assigned to chromomeres. Arrow shows the position of the chiasma. Immediately above the arrow is the first bright chromomere of the Z chromosome. Scale bar = 5 μm.

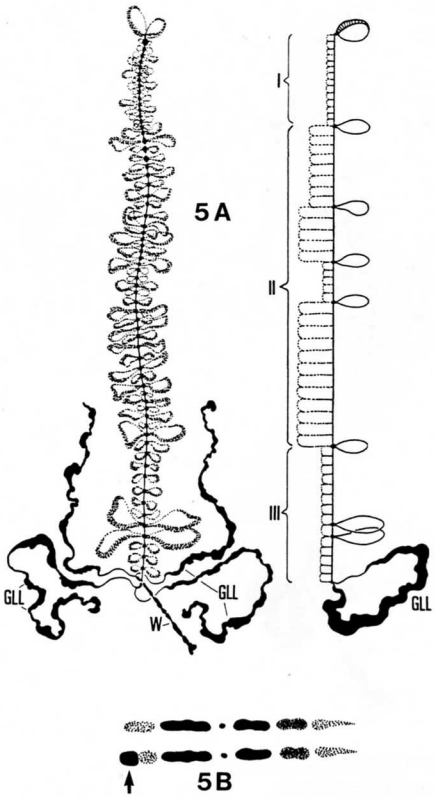


Fig. 5. (**A**) Representative scale drawing of the chicken ZW bivalent, left, together with an LBC map showing the locations of the main marker loops and regions. GLL, giant lumpy loops located at the chiasmate ends of the Z and W chromosomes; the bases of these loops mark the approximate position of the chiasma. The three regions of the Z chromosome are labeled I, II and III. (**B**) The arrangement and shapes of the chromomeres of the chicken W-LBC from a DAPI stained preparation. The lower diagram shows all 7 chromomeres present; the upper diagram shows the arrangement when the bright chromomere 1 (arrow) is absent.

ious loops of distinctive appearance are distributed along the length of the Z chromosome and serve as a basis for the lampbrush map of this chromosome (Solovei et al., 1993). The centromere on the chicken Z-LBC lies within a small region that is characterized by loops which are relatively small and compact in appearance (Solovei et al., 1993). A cytological map of the chicken Z chromosome is shown in Fig. 5A.

The W-LBC of chicken is 9–14 μm long. It is formed by a dense chromomeric axis that appears to carry no lateral loops or has minute loops that are hard to resolve with the light microscope. The axis of W-LBC consists of several densely packed chromomeres that are larger and brighter, after staining with DAPI, than any of the chromomeres in Z-LBC (Fig. 4). At the stage of fully developed LBCs, from the oocytes 0.5–1.5 mm diameter, the arrangement of chromomeres in the W-LBC is absolutely consistent from one preparation to another and each chromomere is individually recognizable. In quail and turkey, the W is of medium size in relation to the rest of the karyotype and in its lampbrush form it is strongly condensed and totally lacking in discernible chromomeric organization.

There are two kinds of W-LBC in chicken: with 6 or 7 visible chromomeres (Figs. 4, 5B). The difference between them resides in the presence or absence of a very bright chromomere at the extreme end of the free W arm. The other six chromomeres have the same appearance and arrangement in both kinds of W-LBCs.

The position of the chiasma is marked by two pairs of "giant lumpy loops" (GLL) stretching out from the telomeres of the Z and W chromosomes (Fig. 5A). The ends of the GLLs very often hang free, rather than being tacked back onto the chromosome to form typical closed loops (Fig. 6A). In such cases, the true telomeres of each of the chromatids that form the LBC must lie at the extreme tips of the free-hanging loops (Fig. 6B).

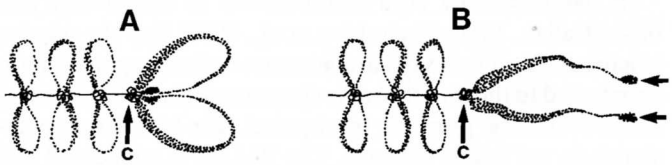


Fig. 6. Arrangement of open ended telomeric loops on lampbrush chromosomes. In A, the loops are folded back onto the terminal chromomere (c) in the manner that is normal for most LBCs. In B the telomeric loops hang free from the terminal chromomere. Arrows indicate ends of the meiotic chromatids.

Such "open-ended" telomere loops are a peculiar and extremely interesting feature of bird LBCs and a more detailed discussion of their significance can be found in Solovei et al. (1994, 1995) and Macgregor et al. (1996). As a rule, the Z/W chiasma involves the axes of the chromosomes, very close to the telo-

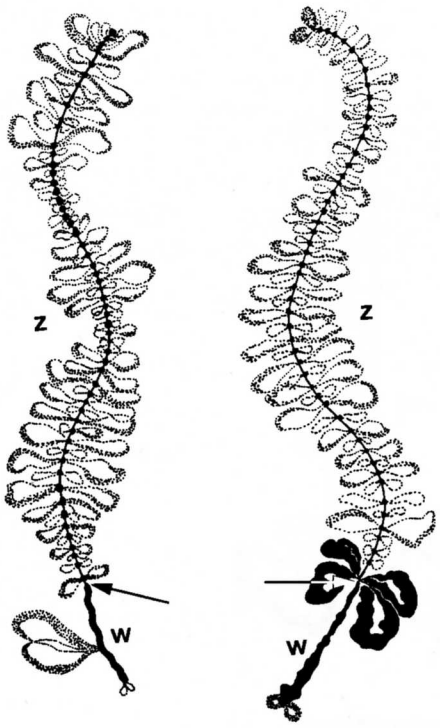


Fig. 7. ZW bivalents of quail (left) and turkey (right). Arrows indicate the positions of the chiasma.

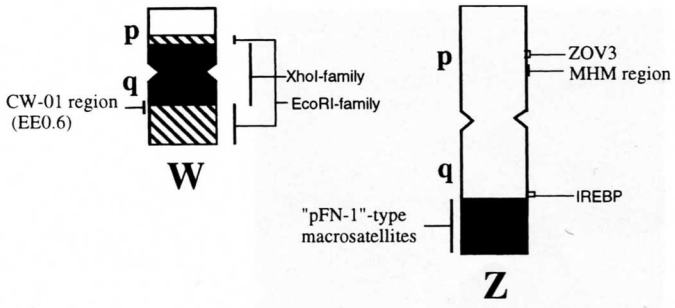


Fig. 8. Mitotic W and Z chromosomes of chickens showing locations of genes and sequence regions which are mentioned in the text. *IREBP* was formerly known as *ACO1*. Exact locations of *VLDLR* on the Z chromosome and of *CHD1* and *ASW* on the W chromosome have not been reported.

meres and near to the chromomere from which the GLLs originate.

Sex lampbrush bivalents of quail and turkey are shown in Fig. 7. The most noticeable differences between them relate to the nature of the telomere loops, the presence or absence of distinctive loops along the length of the W chromosome and the general compactness of the W chromosome axis. Full descriptions of these can be found in Solovei et al. (1993).

It has repeatedly been demonstrated in amphibians and birds (Callan, 1986; Chelysheva et al., 1990) that the relative lengths and arm ratios of chromosomes in the lampbrush form correspond to those of the same chromosomes at mitotic metaphase or meiotic prophase (pachytene). The bird sex chromosomes do not follow this rule. The W-LBC is condensed and shortened in comparison with the Z, which is typically chromomeric and lampbrushy. In chaffinch, for example, the W is less than a quarter the length of the Z, although at mitotic metaphase the relative lengths of these two chromosomes are 8.9 and 11.1 respectively (Ray-Chaudhuri, 1973). The estimated position of the centromere in the W lampbrush chromosome agrees well with the known position for the corresponding chromosome in metaphase (Solovei et al., 1993).

The relative lengths of the Z-LBC and the Z-mitotic chromosome are the same, but their arm ratios differ. It is impossible to measure arm ratios of Z-LBC in turkey and quail, but, if the position of the centromere has been correctly estimated, then in pigeon, sparrow and chaffinch the two arms of the Z-LBCs are obviously of different lengths, whereas mitotic Z chromosomes from these species are decidedly metacentric. In chicken, the mitotic Z chromosome is very slightly sub-metacentric (non-heterochromatic arm/heterochromatic arm is 0.893 to 0.970 depending upon stages [Ponce de Leon et al., 1992] or the centromere index [CI] is 0.464 [Saitoh et al., 1993]) and it has been shown that the single recombination nodule is situated on its shorter arm (Solari et al., 1988; Solari, 1992). The Z-LBC appears more decidedly sub-metacentric (CI 0.45). We consider that such differences as exist in arm ratios may result from a non-uniform pattern of condensation along the axis of the Z-LBC. The sub-telomeric portion of the free arm of the Z-LBC in chicken (region I, Fig. 5A), pigeon, sparrow and chaffinch is always occupied by large, brightly DAPI-positive and closely packed chromomeres with short loops, and this may signify a greater degree of general condensation of chromatin than in other parts of the LBC.

In general, there is a good correspondence in birds between blocks of C-band heterochromatin in mitotic metaphase chromosomes and relatively condensed regions with short loops or no loops in LBCs (Rodionov et al., 1989). It is known that the W chromosome in chicken consists almost entirely of Giemsa C+ material (Pollock and Fechheimer, 1981). The same applies to several other species of birds (see Solovei et al. [1993] for all references on this topic). In the chicken, a large heterochromatic block occupies a terminal portion of the long arm of the Z (Pollock and Fechheimer, 1981) and this would seem to correspond to region I of the Z-LBC (Fig. 5A). Quail has a smaller interstitial C-band (de la Sena et al., 1991) which may correspond to region II of its Z-LBC (Solovei et al., 1993).

Two morphological aspects of the W chromosome of chicken need special consideration. The first is its size. The W chromosome at mitotic metaphase measures about 1.5 × 0.5 μm, estimated from the authors' photomicrographs and from scaled photographs of Giemsa-stained metaphase chromosomes published by Ohno (1967). In its lampbrush form, when all its chromomeres are distinguishable but lie close to one another, its DAPI-stained image measures between 8 and 12 μm in length and each chromomere is about 0.5 μm wide. Lampbrush chromomere 3, the largest, varies in length from 2–3.5 μm in DAPI preparations. We do not know why the W

Table 1. Chromosome-specific repetitive DNA families made up of tandem repeats of 21-bp basic repeating units in the order Galliformes

Species	Repetitive family	Consensus sequence[a]	Identity[b] (%)		Localization (chromosome)	Major restriction fragments (kb)
			CBU	MRF		
Gallus domesticus (chicken)	*Xho*I	GAAAATA(C/A)CACNTTTTCTCCC	100	100	W	*Xho*I 0.7, 1.1
	*Eco*RI	GAAAAT(A/C)(C/A)CNCNTTT(C/T)NCCCC	81	68	W	*Eco*RI 1.2
	CNM[c]	(1)GCGTTTTCTCTT CGCAAATCC(21) (22)CCCATTTAAGC(C/A) GAAAATCAC(42)	57 67		micro(s)	*Eco*RI 1.2[d] *Bst*UI[e]
Meleagris gallopavo (turkey)	*Pst*I	AGAAATA(T/C)(G/C)NCATTTTCTCCC	81	63	W	*Pst*I 0.4
Phasianus versicolor (pheasant)	*Taq*I	AGAAATANNNN(A/C)TTTTCTCCC	67	57	W[f]	*Taq*I 0.5

[a] Consensus sequence of the basic 21-bp unit. N; any base. An alternative base is shown in parentheses.
[b] CBU; identity of consensus basic units regarding N as a mismatched base. MRF; overall identity of major restriction fragments determined by nucleotide sequencing.
[c] CNM; isolated as chicken nuclear membrane-associated DNA. Basic unit is an imperfect dimer of 21-bp sequences.
[d] A part of CNM repeats is digested into 1.2-kb fragments with *Eco*RI.
[e] Although the *Bst*UI site (CGCG) is present in the tandemly repeated sequence, a large fraction of the genomic DNA is resistant to the digestion, which is most likely due to methylation.
[f] Related sequences are present elsewhere in the genome, although they are not digested into 0.5-kb fragments with *Taq*I.

is so much bulkier in its lampbrush form than it is at metaphase but it is certainly a real phenomenon that merits further careful investigation.

The second W chromosome problem is the matter of lampbrush loops and transcription of W chromosome DNA. No lampbrush loops can be seen on the chicken W chromosome with a light microscope, except for the giant loops at its chiasmate end. The chromosome appears virtually naked as compared with other LBCs in the set. Sometimes the W chromomeres seem to be lightly clothed in a fine "fuzz" such as might represent a number of exceedingly short loops, but this is not the usual situation. Resolution of this matter is of some importance with regard to views concerning the possible transcription of W-DNA sequences during the lampbrush phase. In other species of bird there definitely are loops on the W-LBC. There are conspicuous marker loops in the middle of the W chromosome in quail and lumpy loops at its free end in turkey. There are many small loops along the W-LBCs of sparrow, chaffinch and pigeon (Solovei et al., 1993). The reality and significance of this kind of interspecific variation should be examined with a combination of light and electron microscopy and DNA/RNA-transcript ISH.

Z and W chromosomes at the molecular level

Genus-specific repetitive sequences: their relation to heterochromatin and chromomeric organization

Two major blocks of Giemsa C-staining heterochromatin are present on the chicken ZW pair: a large part of the W chromosome and one end of the Z chromosome, suggesting that these two regions consist of repetitive DNA sequences. The *Xho*I and *Eco*RI families of repetitive sequences are the two major families that are represented in the W heterochromatin of chicken (Suka et al., 1993). The principal repeating units of the *Xho*I family are 0.7 and 1.1-kb *Xho*I fragments which together account for about 2.1×10^4 kb (Tone et al., 1984) in the White Leghorn chicken. Similarly, an *Eco*RI 1.2-kb fragment is the major repeating unit of the *Eco*RI family and its repeat accounts for a maximum of about 1.1×10^4 kb (Saitoh et al., 1991). These two sequence families, together account for about 32 Mb, or roughly 65% of the DNA in the W chromosome of the chicken (Saitoh and Mizuno, 1992). To judge from the results of FISH experiments on the mitotic W chromosome, it seemed that the major fraction of the *Eco*RI family occupied one entire arm and a minor fraction was located at the middle of the other arm, and that the *Xho*I family was present in a pericentric region (Saitoh and Mizuno, 1992) (Fig. 8). FISH experiments with Z-, W-LBCs showed that the two sequence families occupy different chromomeres. The *Xho*I family is in chromomere number 3 (refer to Figs. 4 and 5B). Most of the *Eco*RI family is in chromomere number 1 and a minor fraction of it is in the terminal region of chromomere number 5 (Solovei et al., 1993; 1998; Ogawa et al., 1997).

Towards one end of the arm of the W chromosome that contains the minor fraction of the *Eco*RI family, there is a relatively large area that is lacking in both these repetitive families (Saitoh and Mizuno, 1992) (Fig. 8) and does not form heterochromatin (Suka et al., 1993). It is this end of the W chromosome that pairs with the non-heterochromatic end of the Z chromosome in the prophase of female meiosis (Solovei et al., 1993; Hori et al., 1996).

The sequence similarity of *Xho*I and *Eco*RI family sequences is about 68% (Table 1). Therefore, they do not cross-hybridize under conditions of hybridization that allow about 10% base-pair mismatch (Saitoh et al., 1991). Under such

hybridization conditions they are detectable only in the genus *Gallus* (domestic and jungle fowls). However, by applying conditions of extremely low stringency, female-specific and *Xho*I-family-related repetitive families can be detected in two other species in the order Galliformes: the *Pst*I family of turkey (*Meleagris gallopavo*) and the *Taq*I family of pheasant (*Phasianus versicolor*) (Saitoh et al., 1989). These sequence families consist of tandem repeats of an average 21-bp basic unit, equivalent to two helical turns of a B-form DNA. In all of these repetitive sequences, there are A and T stretches separated from one another by about 10 bp in almost every 21-bp unit (Table 1). This arrangement probably underlies the tendency for these repetitive sequences to behave as strongly curved molecules.

Interestingly, the CNM (chicken nuclear membrane associated) repeat family, which accounts for about 10% of the genomic DNA of the chicken and is distributed on many microchromosomes (Matzke et al., 1990) consists of 41–42 bp repeating units, whose sequences are imperfect dimers of the 21-bp basic unit that we refer to in this paper (Table 1). The CNM repeat family is also found only in the genus *Gallus*, under standard conditions of nucleic acid hybridization. Recent studies with FISH indicate that the CNM repeats are present on as many as 20 different microchromosomes of chicken (E. R. Gaginskaya, personal communication). These observations suggest that an original pool of 21-bp sequences, perhaps present in an ancestral species of the order Galliformes, was diversified into related sequences or imperfect dimers and some of these members were further amplified on different chromosomes during the divergence of genera and species.

Why should the 21-bp unit have been utilized so successfully to form chromosome-specific repetitive families that are the basis of heterochromatin? We suggest that tandem repeats of this two-helix-turn unit result in regular appearances of A and T clusters, strong DNA curvature, and frequent exposure of CpG dinucleotides. The latter are present between the A and T clusters and a target for cytosine methylation. The first two factors seem to facilitate the formation of a regular array of nucleosomes. This, in turn, leads to the formation of heterochromatin through regularly repeated accumulation of non-histone chromatin proteins that specifically recognise the properties of the 21-bp unit.

In contrast to W heterochromatin, the terminal heterochromatin of the chicken Z chromosome contains pFN1 (fibroblast-derived *Nhe*I fragment)-type macrosatellite DNA repeats, in which a 24-kb *Nhe*I unit is repeated about 830 times (Hori et al., 1996). This 24-kb unit is a non-curved molecule and has no internal repeat structure. It consists of a mosaic of different segments, whose order and interspersion with other sequences may be different from one repeat to another. The only common feature between those sequences constituting the W heterochromatin and the Z-terminal heterochromatin is rather extensive cytosine methylation of CpG dinucleotides. Sequences of most of the segments constituting the 24-kb unit are found only in the genus *Gallus*, but one segment consists of a sequence common to several species in the order Galliformes.

In the present-day chicken population, two aspects of variation in W heterochromatin have been observed. First, the genomic content of *Xho*I family sequences varies among different breeds. According to our preliminary survey, the highest was about 4×10^4 copies of the 0.7-kb units per genome of the Taiwan native fowl and Brown Leghorn (for this calculation all the hybridizable sequences with the *Xho*I 0.7-kb probe were assumed to consist of 0.7-kb repeats). White Leghorn has about 3.0×10^4 copies. Black Minorca and Red jungle fowl have about 2.5×10^4 copies (H. Saitoh and S. Mizuno, unpublished). Fayoumi chicken has the lowest with about 0.5×10^4 copies (Tone et al., 1984). The low content of the *Xho*I family sequences in Fayoumi correlates with a much smaller W-LBC chromomere number 3 (Solovei et al., 1998).

The second feature of W heterochromatin variation is that there seem to be two different sizes of the *Eco*RI-repeat family among individuals of the present-day female chicken population. One has about 4,000 copies of the 1.2-kb unit per genome. The other has about 700 copies per genome (Saitoh et al., 1991). These two types were found in populations of White Leghorn (Saitoh et al., 1991) and English Rhode Island Red (Solovei et al., 1998). In chickens with the small *Eco*RI-repeat family, W-LBC chromomere number 1 is so small as to be almost undetectable (Solovei et al., 1998).

We are disposed to attach special significance to the fact that a 32% difference in the overall nucleotide sequences between the *Xho*I and *Eco*RI family sequences is enough to cause structurally independent behavior of these two repetitive families the consequences of which can actually be seen at the level of the chromosome. The two families do not coexist over a distance of at least 1–2 Mb (Saitoh et al., 1991). Moreover, our recent findings show that each family forms a distinctly different lampbrush chromomere and they suggest that each family exists on its own, in its own territory, on a large molecular scale. Hence the existence of W and Z chromosome-specific repetitive families or chromomeres may be important factors in promoting independence of sex chromosome arms and conserving genes and unique sequences by preventing recombination between W and Z chromosome arms and discouraging intrachromosomal exchange between major chromosome segments.

The evolutionary conservation of genes and unique sequence regions

The W chromosome

A functional gene on the avian W chromosome that controls female sex determination or differentiation has not been identified. A search for chicken gene homologues of human SRY or mouse *Sry* (Sinclair et al., 1990; Koopman et al., 1991) by Southern blot hybridization with the mouse *Sry* probes showed that these probes hybridized to many restriction fragments but none was specific to either male or female sex (R. Kunita and S. Mizuno, unpublished). In triploid chickens having 3A + ZZW chromosome constitution, the left gonad develops into an ovotestis and the right gonad into a testis at hatching (Ohno et al., 1963; Lin et al., 1995). In these chickens, however, the left gonad follows a nearly normal female pattern of development in

the early embryonic stages (Sheldon and Thorne, 1995). These results suggest that a gene inducing the early development of the female left gonad is present on the W chromosome, although its determinant role is not as strong as in male determination by SRY/*Sry* in mammals. So far, two genes have been noted to be linked to the avian W chromosome: a homologue of mouse chromo-helicase-DNA binding (*CHD1*) gene (Ellegren, 1996) and an *ASW* gene (presumed avian sex-determining on W; A.H. Sinclair: presented at the Australian Poultry Science Symposium, February, 1997, Sydney). *CHD1* has a counterpart gene with a very similar sequence elsewhere in the genome. Several restriction fragments from the W-linked gene, *CHD1-W*, are identified among the *Taq*I-digested genomic DNA fragments in several carinate birds but not in ostrich, a ratite bird (Ellegren, 1996). The *ASW* gene is expressed in various female embryonic tissues, including the genital ridge (Sinclair, as above).

Genomic libraries from a single chicken W chromosome have been constructed by applying laser microbeam irradiation to destroy all other chromosomes, amplifying DNA in the remaining W chromosome by the single unique primer PCR (Hadano et al., 1991; Saitoh and Ikeda, 1997) and selecting clones which were not reactive to *Xho*I and *Eco*RI family sequences. By using one of these clones as a probe, genomic clones covering a non-repetitive region of about 25 kb (the CW01 region of the W chromosome) were isolated from a genomic library of the female chicken. The CW01 region was located by FISH to the region between the *Xho*I and *Eco*RI family domains on the mitotic W chromosome (Fig. 8), or between the chromomere 1 and 3 on the W-LBC (Ogawa et al., 1997). A subregion of about 3.8 kb in the CW01 region was found to be conserved and W-linked across orders of carinate birds. The EE0.6 sequence (*Eco*RI 0.6-kb fragment) within this subregion of chicken and the homologous sequences in duck (*Anas plathynchos domestica*), rock dove (*Columba livia*) and Oriental white stork (*Ciconia boyciana*) show 82, 78, and 81% identities, respectively (Ogawa et al., 1997; Itoh et al., 1997).

We consider that the chicken EE0.6 may be useful as a universal probe for sexing carinate birds by Southern blotting (Ogawa et al., 1997). An EE0.6-related sequence is also present on the Z chromosome and sequences of EE0.6-W and EE0.6-Z are highly similar in certain species; e.g. 92% identity in Oriental white stork (Itoh et al., 1997). In the latter case, a suitable set of primer sequences selected from those W- and Z-derived sequences could produce a PCR product only from the genomic DNA of the female (Itoh et al., 1997). Although the chicken EE0.6 contains a putative exon, ET15, flanked with functional splice acceptor and donor signals, this sequence is likely a part of a pseudogene in present-day birds because of the facts that every reading frame in the chicken ET15 contains a stop codon and that the AG in the splice acceptor signal in the Oriental white stork EE0.6 is changed to GG (Itoh et al., 1997).

The Z chromosome

Although the Z chromosome is the fourth to fifth largest chromosome in bird karyotypes and about twenty Z-linked phenotypic traits have been mapped (Bitgood, 1993), the number of genes that have been physically mapped on this chromosome remains small (CHICKGBASE http://www.ri.bbsrc.ac.uk/chickmap/chickgbase/chickgbase.html). The Z-linkage of cytosolic aconitase gene (*ACO1*) was demonstrated by sex-linked electrophoretic patterns of its isozymes in guinea fowl (*Numida meleagris*), chicken, house sparrow (*Passer domesticus*), cockatoo (*Cacatua sanguinea, Calyptorhynchus funereus*) (Baverstock et al., 1982) and zebra finch (*Poeohila guttata*) (Lacson and Morizot, 1988). It has since been shown that a single gene encodes a protein of dual functions; cytosolic aconitase and iron-responsive element-binding protein (IREBP) (Klausner and Rouault, 1993). The latter function is to inhibit translation of ferritin mRNA and to stabilize transferrin receptor mRNA by binding to the iron-responsive element in the untranslated region of each mRNA when the cell is depleted of iron (Klausner and Harford, 1989). cDNA and partial genomic clones for chicken *IREBP* were obtained by using a partial cDNA fragment of human IREBP as a probe and the gene was located by FISH very close to the terminal heterochromatin on the chicken Z chromosome (Saitoh et al., 1993) (Fig. 8). The deduced amino acid sequence of chicken *IREBP* shows 88% identity to the mouse *Irebp* and includes 17 of the 20 active site residues present in the pig heart mitochondrial aconitase (Saitoh et al., 1993).

The *ZOV3* (Z-linked ovary-specifc) gene is situated in the middle of the short arm of the chicken Z chromosome (Fig. 8). This gene was first identified as a Z-linked partial cDNA clone, pLON3901, representing a relatively abundant cDNA species prepared from early ovaries of chickens (Mizuno et al., 1993). ZOV3 is a novel member of the immunoglobulin (Ig) superfamily with two Ig-like loops in its extracellular region, a single membrane-spanning region and a short C-terminal cytoplasmic domain. ZOV3 is unique among the superfamily in that its expression is highly specific to embryonic gonads of both sexes and the ovarian follicles of hen. In the ovarian follicle, ZOV3 is present as membrane-bound glycoprotein in the granulosa cells and in islets of cells producing estradiol-17β in the outer theca layer (Kunita et al., 1997). It is of interest to note that two genes which seem likely to be concerned with the development of ovarian follicles are located on the Z chromosome: *ZOV3* whose function is probably related to the differentiation of sex steroid hormone-producing cells and the VLDL-receptor gene (*VLDLR*) whose function is required for vitellogenesis in the oocyte. A defect in the latter gene correlates with the restricted ovulator (RO) phenotype (Bujo et al., 1994). Although the Z-linkage of *VLDLR* has been suggested by Southern blotting and from its correlation to the RO phenotype, its exact location on the Z chromosome has not been reported. It has been shown that both *IREBP* and *ZOV3* are conserved on Z chromosomes of carinate species belonging to at least five different orders (Saitoh et al., 1993).

M. Teranishi and co-workers in the author's (SM) laboratory have recently found an MHM (male hypermethylated) region on the short arm of the chicken Z chromosome (Fig. 8) and its biological functions are under investigation. Cytosines of the CpG dinucleotide sequences in this region are highly methylated on both Z chromosomes in the male but the region on the single Z chromosome in the female is much less methylated. This region includes a site-specific repetitive sequence

that is transcribed into high molecular weight, heterogeneous nuclear RNA only in the female in various tissues and on a particular pair of loops on the Z-LBC. The demethylation in the female takes place after fertilization presumably under some influence from factors on the W chromosome.

A common evolutionary origin for avian sex chromosomes

The ratite birds (infraclass Eoaves) have remained flightless and possess no keel on the breast bone. They would seem to represent remnants of the large, ground living, flightless birds of the early Cenozoic era 70 MYr ago. The sex chromosomes of Ratitae are largely homomorphic and euchromatic. Although banding studies have suggested a slight morphological differentiation of the W chromosome in the emu (*Dromaius novaehollandiae*), Darwin's rhea (*Pterocnemia pennata*) and the American rhea (*Rhea americana*) (Benirschke et al., 1976; Ansari et al., 1988), such a difference has not been observed for the ostrich (*Struthio camelus*), the cassowary (*Casuarius casuarius*) and the kiwi (*Apteryx australis*) (Takagi et al., 1972; de Boer, 1980). Ogawa et al. in the author's (SM) laboratory have recently cloned genomic sequences of the ostrich and the emu homologues of *EE0.6, IREBP* and *ZOV3* and showed that those sequences are 70 to 90% identical to the corresponding sequences in the chicken. When those ratite sequences were used as probes in FISH, all three sequences colocalized to a single pair of chromosomes, chromosome 5 in the emu and chromosome 4 in the ostrich. Furthermore, the *IREBP* locus was found to be missing from one of the chromosomes of the pair in the female ostrich (Ogawa et al., 1998). These results would seem to support the idea, proposed by Ohno in 1967, that sex chromosomes of ratite and carinate species have evolved from a common pair of homologous chromosomes and that the process of morphological differentiation of sex chromosomes in Ratitae has been frozen at its early stages or has been happening extremely slowly.

Concluding remarks

In our experience, spanning the last 30 years of progress in the field of molecular cytogenetics, few systems have proved to be so rewarding and experimentally accessible as the avian genome and karyotype. It presents an opportunity to combine three powerful experimental approaches, the comparative approach, since there are many species, the cytological approach, since the karyotypes are interesting and varied and we can look at mitosis, meiosis and lampbrush chromosomes, and the molecular approach, since the genome is small and relatively easy to explore and map.

With the most skillful lampbrushology, led by our colleague Dr. Irina Solovei, extensive use of FISH and advanced light microscopy, delicate chromosome microdissection, led by Dr. J-E. Ikeda, and all the tricks of modern molecular biology, we have been able not only to explore the fine scale molecular make-up of the avian sex chromosome pair, but also to investigate their molecular evolution and some remarkable situations that are found elsewhere in the avian germinal vesicle (Macgregor et al., 1996; Solovei et al., 1996).

The field of avian molecular cytogenetics is wider and more challenging than ever. Perhaps in particular, we would like to use this opportunity to urge young scientists to learn about chromosomes, as well as spending their time manipulating DNA, and, best of all, to "have a go" at working with lampbrush chromosomes, for we believe that they, perhaps more than any other cytogenetic object, offer that very special blend technical challenge, aesthetic pleasure and experimental promise.

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