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Genus specificity and extensive methylation of the W chromosome-specific repetitive DNA sequences from the domestic fowl, *Gallus gallus domesticus*

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Abstract. Two female-specific repeating DNA units of 0.6 kilobase pairs (kb) and 1.1 kb, produced by digesting the genomic DNA of the White Leghorn chicken with Xho I, were cloned by inserting them into the Xho I site of an *Escherichia coli* plasmid vector pACYC177. Two such recombinant plasmids, pAGD0601 and pAGD1101, containing a single 0.6-kb and 1.1-kb sequence, respectively, were used as molecular probes. In situ hybridization of the ^{3}H-probes to the metaphase chromosomes from the female White Leghorn embryos revealed their localization in the W chromosome. Semiquantitative Southern blot hybridization with ^{32}P-probes in excess indicated that the 0.6-kb unit and 1.1-kb unit were repeated approximately 14,000 and 6,000 times, respectively, in the W chromosome. The two units comprised about 46% of the W chromosomal DNA. These two repeating units were found in the female genomes of every line of *Gallus g. domesticus* tested and in the female genomes of three jungle fowl species (*G. gallus*, *G. sonneratii*, and *G. varius*) but not in three species belonging to other genera in the suborder *Galli*. Hha I sites in the 0.6-kb and 1.1-kb repeating units were shown to be extensively methylated and a significant fraction of the Hpa II sites in the 0.6-kb repeating units were also shown to be methylated in the female genome of the White Leghorn. Methylation patterns of Hpa II sites in or around the 0.6-kb repeating units examined by the Msp I digestion were similar in the various lines of domestic fowls and the two species of jungle fowls, but *G. varius* (black or green jungle fowl) produced a different pattern of digestion with Msp I.

Introduction

In the female White Leghorn chicken about 20%–30% of the W chromosomal DNA consists of a repetitive DNA family in which a repeating unit of 0.6 kilobase pairs (kb) produced with Xho I is repeated 1.2×10^4–1.8×10^4 times (Tone et al. 1982). The same study suggested that at least one other repeating unit of 1.1 kb, produced also with Xho I, is present as multiple copies in the W chromosome. Thus, a substantial fraction of the total W chromosomal DNA of the chicken seems to consist of these two particular repetitive DNA families. In contrast to the location of some centromeric satellite DNA sequences (John and Miklos 1979) or middle repetitive sequences of the nomadic type (Young and Schwartz 1981), previous in situ hybridization experiments demonstrated that the presence of the Xho I 0.6-kb repetitive DNA family was largely confined to the W chromosome. Judging from the sensitivity of the filter hybridization technique using a ^{3}H-labelled probe, no more than 300 copies of the 0.6-kb related sequence were expected to be present in the male genome. The sex chromosomal localization of these repetitive families implied their possible role in the heterochromatization of the sex chromosome or in the maintenance and function of the sex determining system in the domestic fowl.

In this study, we cloned the above two repeating units by inserting them into the Xho I site of an *Escherichia coli* plasmid vector pACYC177 (Chang and Cohen 1978). Using these cloned repeating units as molecular probes, we examined more precisely, their female and W chromosomal specificity and their frequencies of repetition, as well as sequence homology between these two repeating units, their presence in other species, and the extent of methylation of these sequences in the female genome.

Materials and methods

Preparation of DNA. Blood or livers of male and female *G. gallus* (red jungle fowl), *G. sonneratii* (grey jungle fowl), and *G. varius* (black or green jungle fowl) were obtained from birds reared in the Laboratory of Animal Breeding, Faculty of Agriculture, Kagoshima University. Additional blood and liver samples were taken from a pair of *G. gallus* supplied by the Tama Zoological Park, Tokyo. Other species for the preparation of DNA were obtained as described previously (Tone et al. 1982). High molecular weight DNA was prepared from the blood or livers according to the method described by Tone et al. (1982).

Molecular cloning of the Xho I 0.6-kb and 1.1-kb repeating units. Two to 4 mg DNA from the female White Leghorn was digested with Xho I (2 units/μg DNA) in 0.01 M Tris

(pH 7.5) 0.007 M $MgCl_2$ 0.1 M NaCl 0.007 M 2-mercaptoethanol at 37° C for 3 h and subjected to electrophoresis on 1% agarose gels in 0.008 M Tris 0.0004 M EDTA (ethylenediaminetetraacetate) 0.001 M sodium acetate (pH 8.0) containing 1 μg/ml ethidium bromide. The 0.6-kb and 1.1-kb bands were detected by their fluorescence, cut out, and eluted electrophoretically according to McDonell et al. (1977). Each eluate was extracted with phenol saturated with TE (0.01 M Tris 0.001 M EDTA, pH 7.5) and with ether. The DNA fragments were precipitated with ethanol and dissolved in 0.1 × TE. The Xho I 0.6-kb or 1.1-kb fragment was ligated into the Xho I site of pACYC177 and transformed *E. coli* K-12 strain LE-392 using the RbCl–$CaCl_2$ method for the 0.6-kb fragment or the $CaCl_2$ method for the 1.1-kb fragment as described by Bolivar and Backman (1979). In the cloning of the 1.1-kb fragment, pACYC177 DNA was digested with Xho I and further treated with alkaline phosphatase. Ampicillin-resistant and kanamycin-sensitive transformants were subjected to colony hybridization with ^{32}P-labelled (by nick translation) Xho I 0.6-kb or 1.1-kb fragment. DNA was extracted from each positive clone by the rapid alkaline extraction method (Birnboim and Doly 1979) and digested with Xho I. The presence of the 0.6-kb or 1.1-kb unit was confirmed by agarose gel electrophoresis and staining with ethidium bromide and by Southern blot hybridization with ^{32}P-labelled Xho I 0.6-kb or 1.1-kb fragments. Two clones, pAGD0601 carrying a 0.6-kb unit and pAGD1101 carrying a 1.1-kb unit, were used throughout this study.

Digestion with restriction endonucleases and Southern blot hybridization. Digestion of genomic DNA or DNA from a recombinant plasmid with a single restriction enzyme or with a combination of two enzymes was carried out at 37° C (30° C for Bam HI) for 12 h with an individual enzyme/DNA ratio of 5:1 (units/μg) unless otherwise noted. The reaction mixture for each digestion was as follows: Xho I, 0.01 M Tris (pH 7.5) 0.007 M $MgCl_2$ 0.1 M NaCl 0.007 M 2-mercaptoethanol; Hpa II, 0.01 M Tris (pH 7.5) 0.01 M $MgCl_2$ 0.007 M 2-mercaptoethanol; Msp I, 0.01 M Tris (pH 7.5) 0.01 M $MgCl_2$ 0.001 M 2-mercaptoethanol 0.02% Triton X-100; Bam HI, 0.01 M Tris (pH 7.5) 0.007 M $MgCl_2$ 0.1 M NaCl 0.002 M 2-mercaptoethanol 0.01% bovine serum albumin; Hae II + Pst I, the two enzymes were added together in 0.01 M Tris (pH 7.5) 0.007 M $MgCl_2$ 0.06 M NaCl 0.007 M 2-mercaptoethanol; Hpa II + Xho I, Msp I + Xho I, Hha I + Xho I, digestion with Hpa II, Msp I, or Hha I was performed first in 0.01 M Tris (pH 7.5) 0.01 M $MgCl_2$ 0.1 M NaCl 0.007 M 2-mercaptoethanol, then Xho I was added and the mixture incubated at 37° C for another 12 h. At the end of each single or double digestion the reaction mixture was heated at 65° C for 10 min. The mixture was subjected to electrophoresis on a 1% agarose gel as described in the previous paper (Tone et al. 1982). A modified method of Southern blot hybridization was employed. After the electrophoresis, an agarose gel was placed in 0.5 N NaOH 1.5 M NaCl for 30 min, neutralized in 1 M Tris (pH 7.5) 0.6 M NaCl for 15 min twice, immersed in 20 × SSC (sodium chloride/sodium citrate) for 3 min, all at room temperature. DNA fragments were transferred from the gel to a sheet of nitrocellulose membrane filter (Sartorius-11306, pore size 0.45 μm) in 20 × SSC according to Southern (1975). After the transfer, the membrane filter was washed briefly in 2 × SSC twice at room temperature, baked at 80° C for 4 h, immersed in 5 × Denhardt's solution (Denhardt 1966) at room temperature for 2 h, dried at room temperature overnight, and baked again at 80° C for 2 h. Hybridization with an appropriate ^{32}P-probe labelled by nick translation was carried out in 6 × SSC 1 × Denhardt's solution containing 50–100 μg sheared, denatured *E. coli* DNA/ml at 65° C for 15 h in a sealed plastic bag. Unlabelled, sheared, denatured pACYC177 DNA at a concentration of 100 μg/ml was added to the reaction mixture when the ^{32}P-labelled 0.6-kb unit, isolated from pAGD0601, was used as a probe. The membrane filter was washed successively in 6 × SSC once and in 2 × SSC twice at 65° C for 30 min each. Additional washings in different salt concentrations in the presence of 0.1% sodium dodecyl sulfate (SDS) as specified in the figure legends were applied at 65° C for 1 h each. Autoradiography was performed using an Ortho G film (Kodak) and a Lanex regular screen (Kodak) at −70° C. Densitometry of exposed film was carried out using a Chromato-Scanner (Shimadzu, CS-900).

Dot-blot hybridization. A sheet of nitrocellulose membrane filter (Sartorius-11306) was soaked in 20 × SSC at room temperature for 15 min and baked at 80° C for 1 h. Ten μg DNA (DNA sample to be analyzed was mixed with DNA from the human female) dissolved in 5 μl of 0.1 × TE was sealed in a glass capillary tube, heat-denatured in boiling water for 10 min, and rapidly chilled. The content in the capillary was spotted on the above filter, and the filter was baked at 80° C for 4 h. The filter was immersed in 10 × Denhardt's solution at room temperature for 2 h, dried at room temperature overnight, and baked again at 80° C for 2 h. Hybridization with the ^{32}P-labelled Xho I 0.6-kb unit diluted properly with an unlabelled Xho I 0.6-kb unit was performed as described above for 48 h. The filter was washed as described above and further washed in 2 × SSC in the presence of 0.1% SDS at 65° C for 1 h, three times. Autoradiography and densitometry were carried out as described above.

In situ hybridization. Metaphase chromosome preparations were obtained from the 3-day-old embryos of White Leghorns as described previously (Tone et al. 1982) and preparations from the female embryos were selected by C-banding. Preparations that had not been C-banded were used for hybridization. Two recombinant plasmids, pAGD0601 and pAGD1101, were ^{3}H-labelled by nick translation to a specific activity of approximately 2.6×10^6 cpm/μg DNA, and 1.2×10^5 cpm of each probe in 30 μl of the reaction mixture was applied to each slide. Hybridization was carried out as described in the previous paper (Tone et al. 1982). Autoradiographic exposure time was 26 days.

Results

Molecular cloning of the Xho I 0.6-kb and 1.1-kb DNA from the female chicken

The 0.6-kb or 1.1-kb DNA fragment produced from the DNA of the female White Leghorn chicken with the restriction endonuclease Xho I was inserted into the Xho I site of the *E. coli* plasmid vector pACYC177 (Chang and Cohen 1978), and recombinant plasmids carrying either of these repeating units were isolated. Two of these recombinant

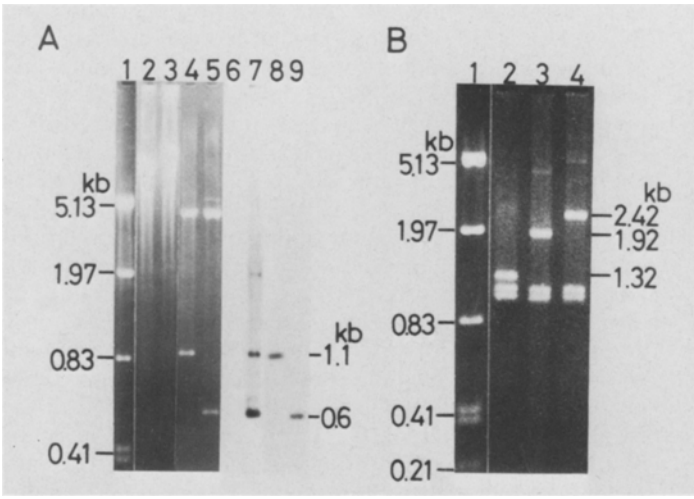

Fig. 1 A, B. Characterization of recombinant plasmid clones carrying the Xho I 0.6-kb and 1.1-kb repeating units from the female White Leghorn. **A** 1% agarose gel electrophoresis after digestion with Xho I (lanes 2–9). Ethidium bromide fluorescence (lanes 1–5) and autoradiographs after Southern blot hybridization with ^{32}P-labelled Xho I 0.6-kb unit and additional washing in 0.3 × SSC (lanes 6–9). Lane 1: PM2 DNA digested with Hind III (size markers), lanes 2 and 6: male White Leghorn DNA (5 μg each), lanes 3 and 7: female White Leghorn DNA (5 μg each), lanes 4 (0.5 μg) and 8 (0.05 μg): pAGD1101, lanes 5 (0.5 μg) and 9 (0.05 μg): pAGD0601. **B** Ethidium bromide fluorescence after 1% agarose gel electrophoresis. Lane 1: PM2 DNA digested with Hind III. 0.5 μg each of pACYC177 (lane 2), pAGD0601 (lane 3), pAGD1101 (lane 4), digested with Hae II + Pst I, with an individual enzyme/DNA ratio of 10:1 (units/μg)

plasmids, pAGD0601 carrying the 0.6-kb unit and pAGD1101 carrying the 1.1-kb unit, were used throughout this study.

When these recombinant plasmids were digested with Xho I, subjected to electrophoresis on agarose gels, and stained with ethidium bromide, pAGD1101 gave a 1.1-kb band and pAGD0601 gave a 0.6-kb band in addition to the band of linearized pACYC177 DNA (Fig. 1 A, lanes 4, 5). These gels as well as gels on which Xho I digests of the total DNA from either female or male White Leghorns had been subjected to electrophoresis (Fig. 1 A, lanes 2, 3) were subjected to Southern blot hybridization with ^{32}P-labelled pAGD0601. As shown in lanes 6 and 7 of Figure 1 A, the ^{32}P-probe hybridized at 0.6 kb and 1.1 kb plus some minor but multiple sites with the Xho I-digested DNA of the female but no such hybridization was obtained with the DNA of the male chicken under the same conditions of blotting, hybridization, and autoradiography. As shown in Figure 1 A lanes 8 and 9, the ^{32}P-probe hybridized with both the 1.1-kb band produced from pAGD1101 and the 0.6-kb band produced from pAGD0601, indicating that these two repeating units had some common sequences.

To determine how many of these repeating units are carried in the recombinant plasmids, each of these recombinant plasmids and the original pACYC177 were digested with Hae II plus Pst I, subjected to electrophoresis on an agarose gel, and stained with ethidium bromide. It has been shown that pACYC177 has two Hae II sites and one Pst I site and that the Xho I site is located on the opposite side of the Pst I site with respect to the two Hae II sites (Chang and Cohen 1978). Figure 1 B shows that pACYC177 (lane 2) produced three bands (1.32 kb, 1.20 kb, and 1.08 kb) corresponding to the Hae II–Hae II, Hae II–Pst I, and Pst I–Hae II fragments, respectively. On the other hand, pAGD0601 (Fig. 1 B, lane 3) and pAGD1101 (Fig. 1 B, lane 4) produced the same 1.20-kb (Hae II–Pst I) and 1.08-kb (Pst I + Hae II) fragments plus a unique band of either 1.92 kb (pAGD0601, lane 3) or 2.42 kb (pAGD1101, lane 4). When the length (1.32 kb) of the original Hae II–Hae II fragment was subtracted from the length of each unique band, 0.6 kb (1.92 − 1.32) for pAGD0601 and 1.1 kb (2.42 − 1.32) for pAGD1101 were obtained. It is concluded from these results that pAGD0601 contains one 0.6-kb repeating unit and pAGD1101 contains one 1.1-kb repeating unit.

The relationship of the 0.6-kb and 1.1-kb sequences in the two recombinant plasmids was further studied by Southern blot hybridization of the electrophoresed Xho I digests of the female White Leghorn DNA with either ^{32}P-pAGD0601 or ^{32}P-pAGD1101. DNA from the male White Leghorn and female human leucocytes were also digested with Xho I, subjected to electrophoresis, transferred to the nitrocellulose membrane filter, and hybridized with the same ^{32}P-probes. After the reaction, these nitrocellulose filters were washed at 65° C under different salt concentrations, i.e., in 2 × SSC, 0.3 × SSC, or 0.1 × SSC, and autoradiographed. As shown in Figure 2, hybridization of each ^{32}P-probe with both 0.6 kb and 1.1-kb bands was observed when the filter was washed with 2 × SSC or 0.3 × SSC (sets 1, 2, 4, 5), whereas preferential hybridization of ^{32}P-pAGD0601 to the 0.6-kb band and ^{32}P-pAGD1101 to the 1.1-kb band was observed when the filter was washed with 0.1 × SSC (sets 3, 6). Hybridization of these ^{32}P-probes to the DNA of the female human was undetectable under any conditions of washing. Hybridization to the DNA from the male White Leghorn chicken was barely detectable when the filters were washed with 2 × SSC or 0.3 × SSC (Fig. 2, sets 1, 2, 4, 5 and Fig. 1 A, lane 6). In each of these cases, a smearlike hybridization was detectable in the higher molecular size region but no hybridization was demonstrated with the fragment of 0.6 kb or 1.1 kb. Although the level is low, we think this hybridization with the DNA of male chicken is significant, compared with the level of hybridization with the DNA from the human female. More quantitative estimation of the sequences that are related to the 0.6-kb repeating unit in the genome of the male chicken is presented in the following section. The results shown in Figure 2 indicate that although the cloned 0.6-kb and 1.1-kb repeating units contain some common sequences, they are not identical and the hybrids between them should have sufficient base pair mismatches so that they are dissociated in 0.1 × SSC at 65° C.

In situ hybridization of ^{3}H-pAGD0601 or ^{3}H-pAGD1101 to the metaphase chromosome sets from the female White Leghorn embryos demonstrated a localized binding to a small chromosome interpreted as the W chromosome (Fig. 3 A, B). No hybridization of either probe to the male chromosome sets was detected under the same conditions of hybridization and autoradiography (data not shown). Hybridization of these probes to the interphase

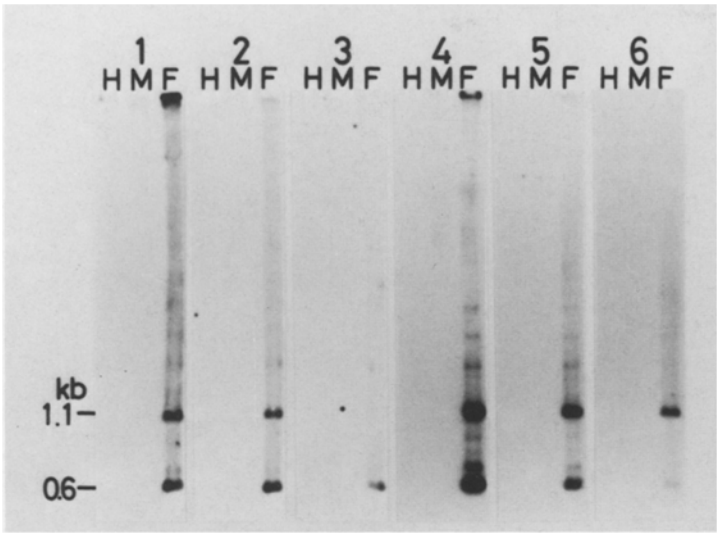

Fig. 2. Southern blot hybridization of 2.5 μg DNA each of female human (*H*) and male (*M*) and female (*F*) White Leghorn, digested with Xho I and subjected to electrophoresis on a 1% agarose gel. The labelled probe was ^{32}P-pAGD0601 (sets 1–3) or ^{32}P-pAGD1101 (sets 4–6). After hybridization, membrane filters were washed as described in Materials and methods and then washed again in 2 × SSC (sets 1 and 4), 0.3 × SSC (sets 2 and 5) or 0.1 × SSC (sets 3 and 6) and autoradiographed

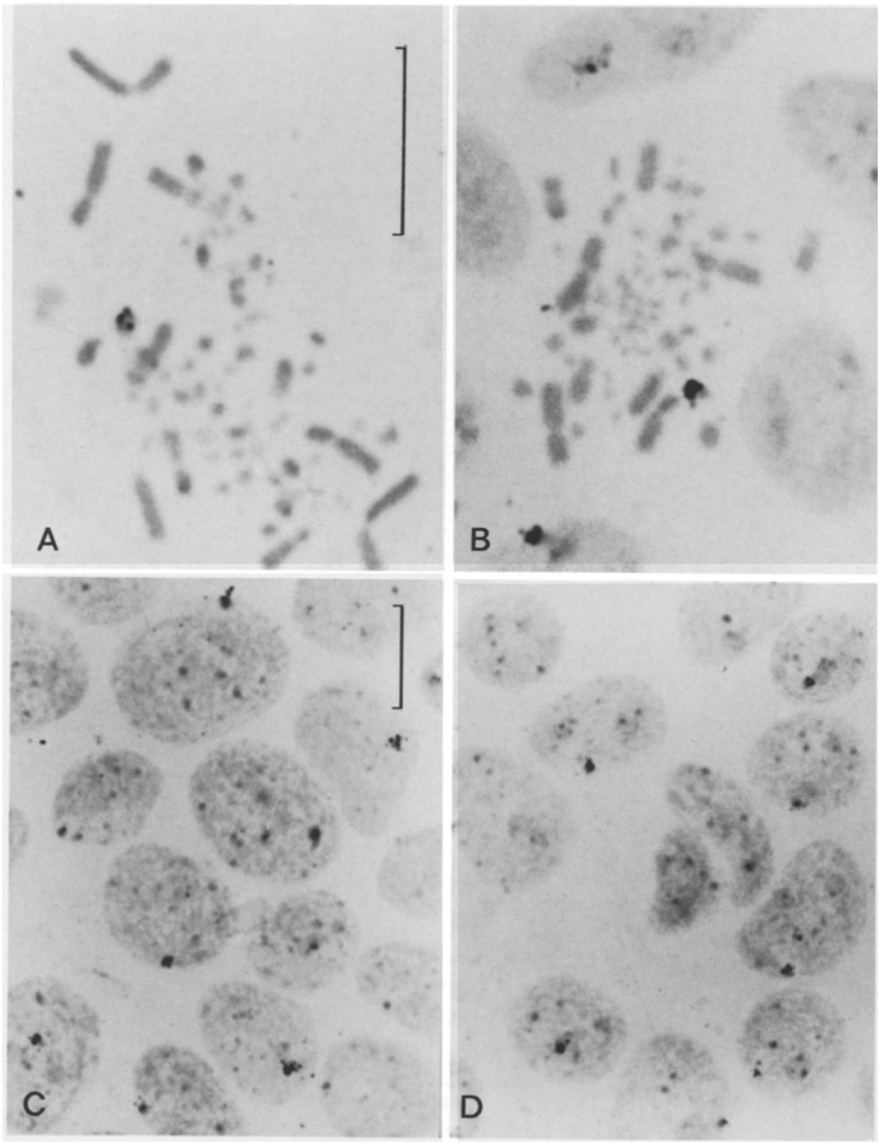

Fig. 3A–D. In situ hybridization of ^{3}H-pAGD0601 (**A**, **C**) and ^{3}H-pAGD1101 (**B**, **D**) to the metaphase chromosome (**A**, **B**) and interphase nuclei (**C**, **D**) from female White Leghorn embryos. Bars represent 10 μm (magnification: **A** = **B**, **C** = **D**)

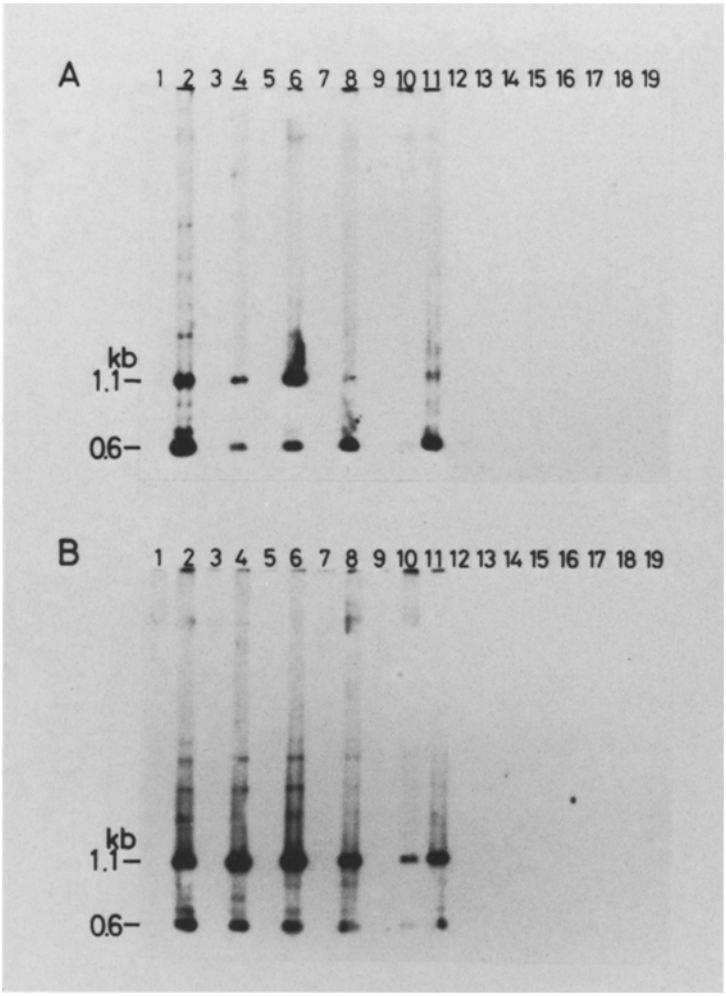

Fig. 4A, B. Southern blot hybridization of Xho I-digested genomic DNA with ^{32}P-pAGD0601 (**A**) or ^{32}P-pAGD1101 (**B**). DNA samples (2.5 μg each) were from White Leghorn (lanes 1 and 2), Japanese Game (lanes 3 and 4), Nagoya Cochin (lanes 5 and 6), Barred Plymouth Rock (lanes 7 and 8), Fayomi (lanes 9 and 10), MSB-1 cells (lane 11), Japanese common pheasant (lanes 12 and 13), guinea fowl (lanes 14 and 15), Japanese quail (lanes 16 and 17), and rock dove (lanes 18 and 19). DNA samples in lanes 1, 3, 5, 7, 9, 12, 14, 16, 18 were from the males of the species and in lanes 2, 4, 6, 8, 10, 11, 13, 15, 17, 19 were from the females. After hybridization and standard washing, membrane filters were washed additionally in 0.3 × SSC and autoradiographed

nuclei from the female chicken embryos produced single grains with localized distribution (Fig. 3C, D).

Presence of the Xho I 0.6-kb and 1.1-kb sequences as repeating units is confined to the female species of the genus Gallus

In the previous paper (Tone et al. 1982), we demonstrated in the hybridization of the ^{3}H-labelled, genomic Xho I 0.6-kb DNA to the membrane-filter-bound DNA from various species that levels of hybridization comparable to those with the DNA of the female White Leghorn were attained only with DNA from different female lines of domestic fowl, *Gallus g. domesticus*. We repeated this experiment with the Southern blot hybridization technique using ^{32}P-labelled pAGD0601 or pAGD1101 as a probe, expecting to examine the species specificity and the sex specificity of the hybridization with much higher sensitivity. DNA from White Leghorns, four other lines of *G. g. domesticus* (Japanese Game, Nagoya Cochin, Barred Plymouth Rock, and Fayomi), MSB-1 cells (a lymphoblastoid cell line established from the spleen of the Marek virus-infected female White Leghorn: Akiyama and Kato 1974), Japanese common pheasant (*Phasianus versicolor*), guinea fowl (*Numida meleagris*), Japanese quail (*Coturnix c. japonica*) or rock dove (*Columba livia*) was digested to completion with Xho I, subjected to electrophoresis on an agarose gel, subjected to Southern blot hybridization, washed in 0.3 × SSC, and autoradiographed. DNA from the male and female were compared for all the species tested. Figure 4A shows the results with ^{32}P-pAGD0601 and Figure 4B, ^{32}P-pAGD1101. Both probes hybridized mainly with the DNA fragments of 0.6 kb and 1.1 kb and with several other fragments produced from the DNA of the female White Leghorn (lane 2). Similar patterns of hybridization were obtained with the DNA from all the female lines of *G. g. domesticus* tested (lanes 4, 6, 8 and 10) and with the DNA from MSB-1 cells (lane 11). Weak and smearlike hybridization to the region of higher fragment sizes was barely detectable with the DNA from each of the male lines of *G. g. domesticus* (lanes 1, 3, 5, 7 and 9), however no hybridization to the 0.6-kb or 1.1-kb fragment was observed in these cases. Even using these ^{32}P-labelled, cloned probes no hybridization could be demonstrated with the DNA from the three species belonging to the suborder Galli (Japanese common pheasant, guinea fowl, Japanese quail) or from one species belonging to the order Columbiformes (rock dove) (lanes 12 to 19). Hybridization of both ^{32}P-probes to the multiple bands (lanes 2, 4, 6, 8, 11) suggests that besides the major repeating units of 0.6 kb and 1.1 kb several other types of related repeating units are present in the female genome, presumably including base changes or methylation

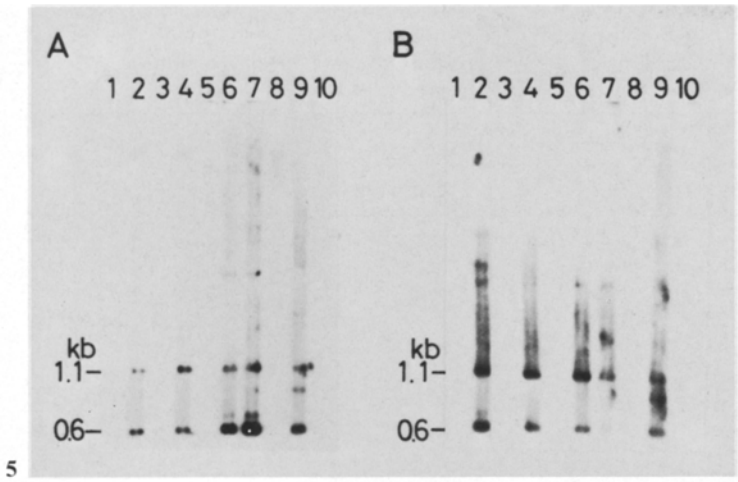

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Fig. 5A, B. Southern blot hybridization of Xho I-digested genomic DNA from domestic and jungle fowl with ^{32}P-pAGD0601 (**A**) and ^{32}P-pAGD1101 (**B**). DNA samples (2.5 μg each) were from the livers of White Leghorn (lanes 1 and 2), the livers of red jungle fowl (lanes 3 and 4), the blood of red jungle fowl (lanes 5 and 6), the blood of grey jungle fowl (lane 7), the livers of black or green jungle fowl (lanes 8 and 9), and the blood of black or green jungle fowl (lane 10). DNA samples in lanes 1, 3, 5, 8, 10 were from the males of the species and in lanes 2, 4, 6, 7, 9 were from the females. After hybridization, membrane filters were washed additionally in 0.3 × SSC and autoradiographed

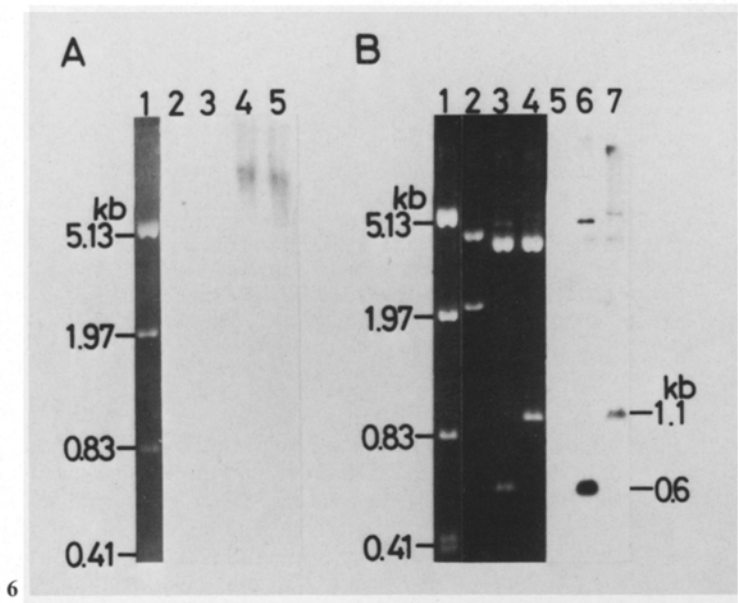

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Fig. 6A, B. Demonstration that the Xho I 0.6-kb and 1.1-kb sequences and a conserved Bkm-related sequence are not cross-hybridized. **A** Southern blot hybridization of 2.5 μg Xho I digested DNA each of male (lane 2) and female (lane 3) White Leghorns and male (lane 4) and female (lane 5) humans with ^{32}P-labelled subclone 316-8A. **B** Subclone 316-8A digested with Bam HI (lanes 2 and 5), pAGD0601 digested with Xho I (lanes 3 and 6) and pAGD1101 digested with Xho I (lanes 4 and 7) were subjected to electrophoresis on a 1% agarose gel and detected with ethidium bromide fluorescence (lanes 2–4) or subjected to Southern blot hybridization with ^{32}P-labelled Xho I 0.6-kb unit (lanes 5–7). After hybridization, membrane filters were washed additionally in 2 × SSC and autoradiographed in both **A** and **B**. Lane 1 (**A** and **B**): PM2 DNA digested with Hind III (size markers)

at the Xho I site or insertion of other sequence elements. We think it rather unlikely that these multiple bands were produced due to partial digestion with Xho I, because the same pattern of hybridization was obtained even when the digestion was carried out at 37° C for 12 h with the Xho I/DNA ratio of 10:1 (units/μg) (data not shown).

We then extended our survey to the DNA of jungle fowls. Among the existing four species of jungle fowls, both male and female *G. gallus* (red jungle fowl) and *G. varius* (black or green jungle fowl), and female *G. sonneratii* (grey jungle fowl) were available for the extraction of DNA. The DNA prepared from livers or blood cells was digested with Xho I and subjected to Southern blot hybridization with ^{32}P-labelled pAGD0601 or pAGD1101. Figure 5 shows that every DNA from the female species of jungle fowls tested (lanes 4, 6, 7, 9) produced 0.6 kb- and 1.1-kb bands of hybridization with ^{32}P-pAGD0601 (panel A) or ^{32}P-pAGD1101 (panel B). Again, DNA from the male materials (lanes 3, 5, 8, 10) did not show any detectable band of hybridization in the region of these fragment sizes. Thus, the presence of 0.6-kb and 1.1-kb repeating units produced with Xho I is confined to the genomes of various female species of both domestic and jungle fowls belonging to the genus *Gallus*. These repeating units were undetectable in the DNA of other avian species tested including the three species (*P. versicolor*, *N. meleagris*, *C. c. japonica*) representing three other genera in the suborder Galli.

Singh et al. (1981) reported that a minor satellite DNA sequence (Bkm DNA) from the Indian banded krait, *Bungarus fasciatus*, was relatively concentrated in the W chromosome of various species of snakes and that related DNA sequences to the Bkm DNA could be found in the DNA from various vertebrate and invertebrate species. They isolated six clones (CS314–CS319) from the *Drosophila melanogaster* DNA library that showed positive hybridization with ^{32}P-labelled Bkm DNA (Singh et al. 1981). We tested a subclone in pBR322, 316-8A, from one of those clones for its hybridizability with the Xho I 0.6-kb and 1.1-kb repeating units in the DNA from the female White Leghorn. As shown in Figure 6A, ^{32}P-labelled 316-8A did not show any hybridization with the Xho I digests of the DNA from either the male or female White Leghorn (lanes 2, 3), although it gave positive hybridization with the Xho I digests of the DNA from male or female human leucocytes (lanes

Table 1. Repetition frequencies of the Xho I 0.6-kb and 1.1-kb units and the Xho-I 0.6-kb-related sequences in the diploid genomes of the female and male White Leghorn and the female Fayomi. Methods of determination were: densitometry of ethidium bromide fluorescence (EtBr), semiquantitative Southern blot hybridization under the condition of probe excess, and dot-blot hybridization. UD means undetectable

Genome	Xho I 0.6-kb unit		Xho I 1.1-kb unit		Xho I 0.6-kb related sequences
	EtBr	Southern blot	EtBr	Southern blot	Dot-blot
Female White Leghorn	12,000–18,000	11,000–14,000	5,500–6,600	4,300–6,300	25,000–34,000
Male White Leghorn	UD	UD	UD	UD	500–1,200
Female Fayomi	UD	300	UD	400	5,000–5,500

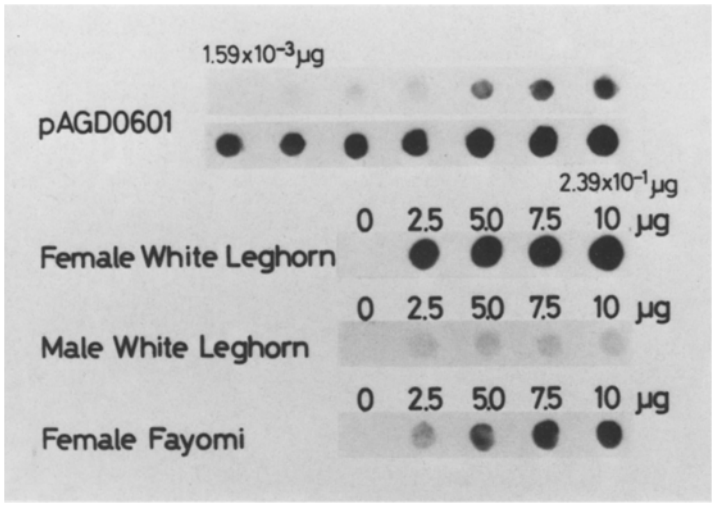

Fig. 7. Semiquantification of the Xho I 0.6-kb-related sequences by dot-blot hybridization. Different amounts of DNA of pAGD0601, male and female White Leghorns, and female Fayomi as indicated were spotted and fixed on the membrane filter and subjected to hybridization with ^{32}P-labelled Xho I 0.6-kb unit. The amount of pAGD0601 DNA per dot was increased in the following order: 1.59, 7.95, 11.9, 15.9, 23.9, 31.8, 39.7, 47.8, 59.5, 79.5, 120, 159, 199, 239 ($\times 10^{-3}$ µg)

4, 5). Conversely, the ^{32}P-labelled Xho I 0.6-kb unit did not hybridize with the Bam HI digests of 316-8A (Fig. 6B, lane 5) under the conditions where the ^{32}P-probe hybridized with both 0.6-kb and 1.1-kb units (Fig. 6B, lanes 6, 7). We can thus conclude that the sequences of Xho I 0.6-kb and 1.1-kb repeating units are unrelated to the evolutionary conserved sequence found in the Bkm DNA.

Repetition frequency of the Xho I 0.6-kb and 1.1-kb units and related sequences in the genomes of female and male White Leghorns and the Fayomi female

Copy numbers of the Xho I 0.6-kb and 1.1-kb repeating units in the diploid genome of the female White Leghorn were estimated by two methods. First, the genomic DNA of females was digested to completion with Xho I, subjected to electrophoresis on an agarose gel in the presence of ethidium bromide, and the ethidium bromide fluorescence was photographed. The percentage of the repetitive DNA family consisting of the 0.6-kb or 1.1-kb repeating units in the total DNA was estimated from the densitometric tracing of negatives as shown in Tone et al. (1982). The 0.6-kb family accounted for 0.31%–0.46% (Tone et al. 1982) and the 1.1-kb family, for 0.26%–0.31% of the total genomic DNA. Assuming that each repetitive family consists of homogeneous repeating units and taking 2.54 pg for the diploid genome size (Mizuno et al. 1978), these percentage values were converted to the repetition frequency (copies/diploid genome) as summarized in Table 1.

The second method was based on Southern blot hybridization under probe excess. Different amounts of unlabelled pAGD0601, pAGD1101, or genomic DNA of the female White Leghorn were digested to completion with Xho I and subjected to Southern blot hybridization with a two- to threefold excess of ^{32}P-pAGD0601 or ^{32}P-pAGD1101. After autoradiography, the film was traced densitometrically, and the area corresponding to the autoradiographic exposure at 0.6 kb or 1.1 kb was cut out and weighed. Repetition frequency of the 0.6-kb or the 1.1-kb unit in the female genome was calculated in proportion to the values obtained for the recombinant plasmid DNA, for which the content of the repeating unit is known. As shown in Table 1, values obtained by these two methods are close. The 0.6-kb and the 1.1-kb units are repeated approximately 14,000 and 6,000 times, respectively, in the diploid genome of the female White Leghorn.

As noted in the previous section, both ^{32}P-pAGD0601 and ^{32}P-pAGD1101 gave weak, smearlike hybridization to the region of longer fragments for the DNA from the male White Leghorn (Figs. 1, 2, 4, 5). We also noticed that the levels of hybridization of both ^{32}P-probes to the 0.6-kb and the 1.1-kb fragments produced from the DNA of female Fayomi (an Egyptian line of domestic fowl) were exceptionally lower than those from other female lines of domestic fowls tested (Fig. 4). We applied a semiquantitative dot-blot hybridization procedure to estimate the amount of sequences related to the Xho I 0.6-kb sequence in these genomes. An autoradiograph showing the results of such an assay is shown in Figure 7. Various amounts of

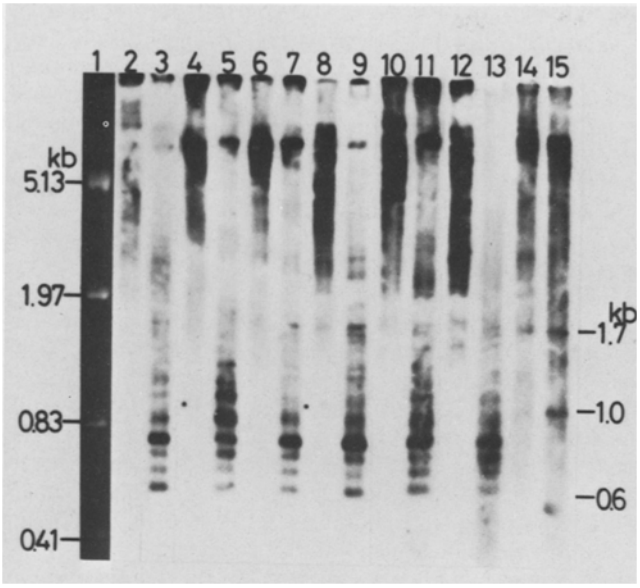

Fig. 8. Southern blot hybridization of Hpa II- or Msp I-digested DNA from the female species of domestic and jungle fowl with ^{32}P-pAGD0601. DNA samples (2.5 μg each) were from White Leghorn (lanes 2 and 3), Japanese Game (lanes 4 and 5), Nagoya Cochin (lanes 6 and 7), Barred Plymouth Rock (lanes 8 and 9), red jungle fowl (lanes 10 and 11), grey jungle fowl (lanes 12 and 13), and black or green jungle fowl (lanes 14 and 15). DNA samples in lanes 2, 4, 6, 8, 10, 12, 14 were digested with Hpa II and in lanes 3, 5, 7, 9, 11, 13, 15 were digested with Msp I. Lane 1: PM2 DNA digested with Hind III (size markers). After hybridization, the membrane filter was washed additionally in 0.3 × SSC and autoradiographed

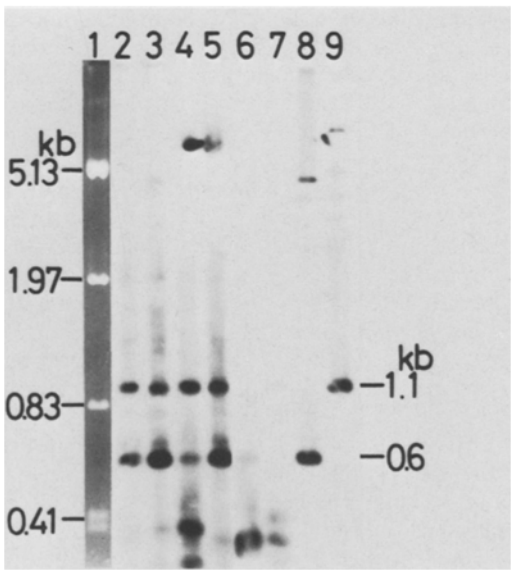

Fig. 9. Search for the site of methylation in the Xho I 0.6-kb and 1.1-kb repeating units in 2.5 μg DNA from female White Leghorn digested with Xho I (lane 2), Hpa II + Xho I (lane 3), Msp I + Xho I (lane 4) or Hha I + Xho I (lane 5), pAGD0601 (2.5 μg each) digested with Xho I (lane 8) or Hha I + Xho I (lane 6), pAGD1101 (2.5 μg each) digested with Xho I (lane 9) or Hha I + Xho I (lane 7) were subjected to electrophoresis on a 1% agarose gel and Southern blot hybridization with ^{32}P-labelled Xho I 0.6-kb unit. After hybridization, the membrane filter was washed additionally in 0.3 × SSC and autoradiographed. Lane 1: PM2 DNA digested with Hind III (size markers)

pAGD0601 that were linearized by digestion with Hind III were spotted on the filter and hybridized with the ^{32}P-labelled Xho I 0.6-kb unit to obtain a quantitative relation between the amount of 0.6-kb sequence in a dot and the darkness of the dot on the autoradiograph. Using this relationship, the amount of sequences hybridizable with the ^{32}P-labelled Xho I 0.6-kb unit in the DNA of the male White Leghorn or the female Fayomi was estimated and converted to the repetition frequency (copies of the 0.6-kb equivalent unit/diploid genome; Table 1). Total copy numbers of the Xho I 0.6-kb related sequences in the diploid genome were estimated to be about 30,000 for the female White Leghorn, about 900 for the male White Leghorn, and about 5,000 for the female Fayomi.

These results revealed that sequences related to the Xho I 0.6-kb sequence are present in the male genome but their repetition frequency is much lower than in the female genome and they do not consist of either a 0.6-kb or 1.1-kb repeating unit. These related sequences in the male genome may not be localized to a particular site in the chromosome set, because we have not observed any significant in situ hybridization of the ^{3}H-pAGD0601 or ^{3}H-pAGD1101 probe to the metaphase chromosomes from the male White Leghorn. The result for the female Fayomi suggested that the total copy numbers of the Xho I 0.6-kb repeating unit or its related sequences in the genomes of the present-day species of the genus *Gallus* could be widely variable in certain species.

Both Xho I 0.6-kb and 1.1-kb repeating units are extensively methylated in the female genomes of the genus Gallus

When DNA from the female White Leghorn was digested with Hpa II, subjected to electrophoresis, and Southern blot hybridized with ^{32}P-pAGD0601, smearlike hybridization to the region of greater fragment lengths was obtained (Fig. 8, lane 2). However, when the same DNA was digested with Msp I, an isoschizomer of Hpa II which can cleave CCGG even when the second cytosine residue is methylated (Waalwijk and Flavell 1978), the ^{32}P-probe hybridized with a series of DNA fragments ranging from 0.6 kb to about 1.5 kb (Fig. 8, lane 3). Similar patterns of hybridization to the Hpa II- vs Msp I-digested DNA were obtained for three other lines of *G. g. domesticus* (Japanese Game, Nagoya Cochin, Barred Plymouth Rock), *G. gallus* (red jungle fowl), and *G. sonneratii* (grey jungle fowl) as shown in Figure 8, lanes 4–13. DNA from the female *G. varius* (black or green jungle fowl) produced a different pattern (Fig. 8, lanes 14, 15). In the latter case, the Msp I digestion did not produce a series of discrete fragments hybridizing with ^{32}P-pAGD0601, instead it gave two bands at 1.7 kb and 1.0 kb. These results indicate that the Hpa II sites in or around the repeating units are extensively methylated and these sites are generally well conserved, except for *G. varius*, among the female species belonging to the genus *Gallus*.

To obtain further information on whether the repeating units contain sites of methylation, DNA from the female White Leghorn was first digested with Hpa II, Msp I, or Hha I, then with Xho I, and subjected to electrophoresis and Southern blot hybridization with the ^{32}P-labelled Xho I 0.6-kb unit. It is shown in an autoradiograph of Figure 9 that both DNA digested with Xho I (lane 2) and with Hpa II plus Xho I (lane 3) gave two major bands of hybrid-

ization at 1.1 kb and 0.6 kb, whereas the DNA digested with Msp I plus Xho I gave a 1.1-kb band of similar intensity as in lanes 2 and 3, a weak 0.6-kb band, and smaller fragments (lane 4). These results indicate that the 0.6-kb repeating unit contains Hpa II site(s) and the sites are methylated in a significant fraction of the repetitions in the female genome. These results also suggest that the 1.1-kb repeating unit does not contain a Hpa II site. This point was confirmed by the experiment showing that the isolated 1.1-kb fragment from pAGD1101 was not cleaved by Hpa II (data not shown). Some hybridizable fragments at 0.6 kb remained after digestion with Msp I plus Xho I (lane 4). This band did not disappear even after digestion with a higher concentration of Msp I (Msp I/DNA = 10/1 in units/μg).

Figure 9 also shows that the genomic DNA of the female that had been digested with Hha I plus Xho I hybridized with the ^{32}P-probe at 1.1 kb and 0.6 kb (lane 5), as in the case of digestion with Xho I alone (lane 2) or Hpa II plus Xho I (lane 3). In contrast, 0.6-kb and 1.1-kb bands, revealed by hybridizing the Xho I-digested pAGD0601 and pAGD1101, respectively, with the ^{32}P-probe (lanes 8, 9), disappeared nearly completely after an additional digestion with Hha I (lanes 6, 7). These results indicate that both the 0.6-kb and 1.1-kb repeating units contain Hha I sites and these sites are methylated extensively in the female genome of the White Leghorn.

Discussion

Both the W chromosome in female birds and snakes and the Y chromosome in male mammals are largely heterochromatic in somatic cells. It has been shown that the W chromosome of the female domestic fowl is observed as a densely stained chromatin body in the somatic nucleus (Bloom 1974). There seems to be two types of repetitive sequences associated with these sex chromosomes: (1) sequences relatively enriched in the sex chromosome and widely distributed among vertebrates and invertebrates and (2) sequences highly localized to the sex chromosome and found within a limited taxonomic group. The first type is represented by Bkm satellite DNA from a female Indian banded krait, *Bungarus fasciatus*, or satellite III + IV from a female tree snake, *Elaphe radiata* (Singh et al. 1976, 1980). It has been shown that the Bkm satellite DNA hybridizes with DNA from various vertebrate and invertebrate species with a ratio of hybridization to the female DNA/male DNA of 3.99 for a species of snake (*Bungarus caeruleus*), 2.41 for Japanese quail (*Coturnix c. japonica*), and about 1.0 for sea urchin, newt, lizard, and humans (Jones and Singh 1982). One interesting observation was that the Bkm satellite DNA hybridized to a specific region, 19F–20AB, at the euchromatic base of the *X* chromosome of *Drosophila melanogaster*, and several clones which hybridized with the Bkm satellite DNA were isolated from the library of *D. melanogaster* genomic DNA (Singh et al. 1981; Jones and Singh 1982).

Epplen et al. (1982) constructed a recombinant plasmid pErs5 containing a 2.5-kb sequence derived from *E. radiata* satellite III + IV fractions and found that the sequence contained 44 consecutive amino acid-specifying codons at one end and two kinds of simple repeating sequences among others. When the whole 2.5-kb sequence was ^{32}P-labelled and hybridized to the Hae III + Alu I-digested DNA from a human male and female, it gave generally similar patterns of hybridization with both preparations and it did not hybridize with the male-specific 3.4-kb fragment produced with Hae III (Cooke 1976). When the same ^{32}P-labelled probe was hybridized with the Hae III-digested genomic DNA of female and male chickens, it produced multiple bands of hybridization with both male and female preparations but it gave additional smearlike hybridization to the region of higher fragment sizes for the DNA from the *male* chicken (Epplen et al. 1982).

The second type is best represented by the W chromosome-specific repetitive sequences in the domestic fowl described in the previous (Tone et al. 1982) and present papers. As summarized in Table 1, the Xho I 0.6-kb unit and the Xho I 1.1-kb unit are repeated about 14,000 times and 6,000 times, respectively, in the diploid genome of the female White Leghorn. When all the related sequences to the 0.6-kb unit are included, approximately 30,000 copies of the 0.6-kb equivalent unit are estimated to be present in the diploid female genome. If we assume that the W chromosomal DNA represents 1.4% of the diploid amount (2.54 pg) of the DNA of the female chicken (Tone et al. 1982) and these repeating units are localized in the W chromosome as suggested by the results of in situ hybridization (Fig. 3), total repetitions of the 0.6-kb and the 1.1-kb units account for 46% of the W chromosomal DNA. If all the 0.6-kb related sequences are assumed to be present in the W chromosome, they would account for as much as 55% of the W chromosomal DNA.

A similar case has been shown for the Y chromosome of the human male, in which 3.4-kb and 2.1-kb repeating units produced with Hae III are present as multiple copies in the heterochromatic region of the long arm of the Y chromosome (Bostock et al. 1978; Schmidtke and Schmid 1980). About 70% of the 3.4-kb repeating unit comprise Y chromosome-specific sequences and the unit is repeated approximately 7,500 times in the Y chromosome, accounting for about 40% of all the Y chromosomal DNA (Kunkel et al. 1979).

Neither the Y-specific 3.4-kb repeating unit (Epplen et al. 1982) nor the W-specific 0.6-kb or 1.1-kb repeating unit (this study) is hybridizable with the conserved sequence derived from the *E. radiata* satellite III + IV DNA. Although precise information about the taxonomic distribution has not been documented, some sequences related to the human Y-specific 3.4-kb and 2.1-kb repeating units seem to be present in the autosomes of the gorilla and the chimpanzee (Szabo et al. 1980; Cooke et al. 1982). The W-specific 0.6-kb and 1.1-kb repeating units are found only among the species belonging to the genus *Gallus*, so far as we have investigated. The significance of such repetitive families highly localized in the sex chromosome and shared only by closely related species is not clear at the moment. We speculated (Tone et al. 1982) that size, organization, and numbers of repeating units rather than the DNA sequence per se might be important factors in causing heterochromatization of the W chromosome. From this point of view, it would be interesting to look into the nature of repetitive families in the W chromosome of Fayomi, an Egyptian line of *G. g. domesticus*, as the repetition frequency of the Xho I 0.6-kb related sequence is significantly lower in this species (Table 1).

One of the features of the W-specific repetitive units is their extensive methylation. Most of the Hha I sites in

the 0.6-kb and 1.1-kb repeating units and a significant fraction of the Hpa II sites in the 0.6-kb units are methylated in the genomic DNA of the female White Leghorn. Some Hpa II sites were resistant to the digestion with Msp I and we suspect this is due to ^{m}CpC-type methylation at the Hpa II site as reported for the α-globin gene cluster from various chicken tissues (Haigh et al. 1982). As we have not yet established base sequences and sequence organization of the repetitive units in the W chromosome, we cannot interpret an individual band of hybridization that appeared after digestion of the DNA from the female White Leghorn with Msp I (Fig. 8). We can at least suggest that tandem arrangement of the 1.1-kb unit is unlikely in view of the absence of an Hpa II site in this repeating unit and the likelihood that most of the 0.6-kb units are interspersed with other repeating elements containing Hpa II sites.

Of the existing four species of jungle fowl, three species (*G. gallus*, *G. sonneratii*, *G. varius*) were available for this study. Both Xho I 0.6-kb and 1.1-kb repeating units are present in the female genome of these three species (Fig. 5). It is thus evident that these W-specific repeating units were present in the genome of a species ancestral to the present-day species. However, we could not detect these repeating units in the female genomes of *C. c. japonica* (Japanese quail) and *P. versicolor* (Japanese common pheasant) both of which belong to the family *Phasianidae*. It is conceivable that these W-specific repeating units were amplified in the course of speciation of an ancestral species of the genus *Gallus* in the family *Phasianidae*.

Of the three species of jungle fowl tested, *G. varius* (black or green jungle fowl) differed from the other two species with respect to the Msp I sites in or around the Xho I 0.6-kb-related sequences (Fig. 8). These results are consistent with the observation by Hashiguchi et al. (1981) in which they compared, electrophoretically, 18 loci of blood proteins from various species of domestic and jungle fowl and found that the four jungle fowl species showed the same range of phenotypes as the domestic ones. However, estimation of genetic distance based on the isozyme patterns of these loci indicated that *G. varius* is relatively remote from the other three species of jungle fowl.

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