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Demonstration of W Chromosome-specific Repetitive DNA Sequences in the Domestic Fowl, *Gallus g. domesticus*

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Abstract. Evidence is presented to demonstrate the presence of W chromosome-specific repetitive DNA sequences in the female White Leghorn chicken, *Gallus g. domesticus,* based on two different experimental approaches. First, ^{3}H-labelled, female chicken DNA was hybridized with excess, unlabelled, mercurated, male DNA, and unhybridized single-stranded ^{3}H-DNA (^{3}H-SHU-DNA) was recovered by SH-Sepharose and hydroxyapatite column chromatography. Approximately 24% of the hybridizable ^{3}H-SHU-DNA was female-specific and localized on the W chromosome. The second approach was to examine female-specific DNA fragments among the digests of chicken DNA with various restriction endonucleases. Among them, we found that digestion with XhoI produced two prominent female-specific bands of 0.60 kb (=kilobase pairs) and 1.1 kb. The 0.60 kb fragment was isolated and ^{3}H-labelled by nick-translation. Female-specificity of the ^{3}H-XhoI−0.60 kb DNA was judged to be at least 95% under the conditions of hybridization with membrane filter-bound DNA. Presence of amplified XhoI−0.60 kb DNA on the W chromosome seems to be limited to different lines of *G. g. domesticus* and no such repeat was detected in three species belonging to other genera in the order Galliformes and in three species belonging to other avian orders.

Introduction

The W chromosome, a female-specific sex chromosome in the female-heterogametic, ZW/ZZ sex-determining system, has certain common features with the Y chromosome of male mammals. In most female birds with some exceptions (Takagi and Sasaki, 1974), and female snakes except some primitive species (Singh et al., 1976), the W chromosome is small, essential in the determination of gonadal sex, but nearly totally heterochromatic in somatic cells. It can be identifiable as a C-banding-positive block in a set of mitotic chromosomes (Stefos and Arrighi, 1971; Singh et al., 1976, 1980).

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The heterochromatic nature of the Y or W chromosome presents an opportunity to study two fundamental questions: molecular mechanisms of heterochromatization and its biological significance in relation to evolution and function of sex-determining mechanisms. In many cases, highly repetitive satellite DNA sequences are associated with heterochromatin (John and Miklos, 1979), but it has not been clear if they are directly involved in the initiation or maintenance of heterochromatization of a wide range of chromatin. They occur often in blocks of highly repetitive, intersperced with single-copy DNA sequences. In this respect, it seems to be an important prerequisite to isolate and characterize the repetitive DNA sequence present in a particular heterochromatic locus.

Repetitive DNA sequences which are located on the long arm of human Y chromosome and represent at least 10% of the Y chromosomal DNA have been isolated by hybridizing highly labelled, male, repetitive DNA to a large excess of unlabelled, total DNA of females and isolating non-reassociated, labelled DNA by hydroxyapatite column chromatography (Kunkel et al., 1976, 1977). It has also been found that among the digests of human DNA with HaeIII restriction endonuclease, there are two kinds of DNA fragments, 1.6×10^6 daltons and 2.24×10^6 daltons, which are specific to the male cells (Cooke, 1976). The 2.24×10^6 daltons (3.4 kb) fragment has been shown to be co-purified with human satellite III DNA from male cells, and the sequence has been located, by in situ hybridization to human chromosome sets containing aberrant Y chromosomes, on the proximal end of band Yq12. Some additional loci on chromosome 9 and 15 have also been detected (Bostock et al., 1978).

Singh et al. (1976, 1980) have shown that satellite DNA III and IV from a snake, *Elaphe radiata*, and the DNA from a minor satellite of other snake species, *Bungarus fasciatus*, are all concentrated on the W chromosome and that these sequences have been conserved throughout the snake group. Complementary RNA of the *E. radiata* satellite III DNA even hybridized with DNA from males or females of japanese quail, although there was no significant in situ hybridization to any particular chromosomes (Singh et al., 1976). The satellite DNA sequences from the two snake species have also been found to hybridize with the DNA of males of various snake species. Relative levels of hybridization to the DNA of females were 1.19 to 3.59 times higher than those to the DNA of males in those species having morphologically differentiated sex chromosomes, but almost equal (1.03) in a primitive species where sex chromosomes were undifferentiated (Singh et al., 1976, 1980). Singh et al. (1976) have also noticed that when DNA from male or female chicken, *G.g. domesticus,* is centrifuged to equilibrium in Cs_2SO_4/Ag^+ density gradients, two satellite components are detectable, and one of them, satellite I, on the light side of the main band DNA is present in higher quantity in the female. Labelled, complementary RNA of the female, satellite I DNA hybridized in situ to the entire length of the W chromosome and to several microchromosomes, but female-specificity of the satellite DNA has not been documented.

In this work, we attempted to examine if there were any repetitive DNA sequences which were exclusively associated with the W chromosome of chicken, *G. g. domesticus*. We made two experimental approaches for this examination: first, highly ^{3}H-labelled, female repetitive DNA was hybridized with excess, mercurated, DNA of males and unhybridized ^{3}H-DNA was recovered by affinity chromatography on sulfhydryl Sepharose, and second, presence of female-specific DNA fragments were searched for among those produced with various restriction endonucleases. The first approach yielded a repetitive DNA fraction in which female-specific DNA sequences formed approximately 24% of the total hybridizable ^{3}H-DNA sequences. The second approach revealed that a restriction endonuclease XhoI could produce two major female-specific DNA fragments, about 0.60 kb and 1.1 kb. The 0.60 kb fragment was further purified and characterized with respect to its localization on the W chromosome and its species specificity.

Materials and Methods

Preparation of DNA

DNA was extracted from livers of 50 to 60 day-old, female or male, White Leghorn chickens. Freshly excised livers were washed in 0.005 M Tris-0.01 M EDTA (pH 8.4), stored at −80° C until they were used for the DNA extraction. Two different methods, as described below, were employed for extraction and purification of DNA.

Preparation of Sheared DNA

The livers were homogenized in 0.01 M Na-acetate (pH 6.0) containing 0.14 M LiCl, 0.001 M $MgSO_4$ and 6% (w/v) Na-p-aminosalicylate, and sodium laurylsulfate was added to the homogenate to give a final concentration of 0.5%, which was then extracted with phenol saturated with 0.01 M Na-acetate (pH 6.0) and containing 0.1% 8-hydroxyquinoline. DNA was spooled out from the aqueous phase after adding 2 vol of ethanol and dissolved in 0.1 × SSC. DNA was purified by repeating extraction with chloroform-isoamylalcohol (24:1, vol: vol), digestion with 50 μg/ml RNase A + 50 μg/ml α-amylase (Sigma, 4 × crystallized from *Bacillus subtilis*) at 37° C for 30 min and with 100 μg/ml self-digested pronase at 37° C for 30 min, and was precipitated with isopropanol according to Marmur (1961). DNA was dissolved in 1 × SSC and subjected to sonication (5 × 1 min-burst) using Branson Sonifier-200, and was further purified using previously described methods (Mizuno et al., 1978). The sheared DNA dissolved in 0.1 × SSC was adjusted to 0.3 N NaOH − 0.001 M EDTA, boiled for 3 min, neutralized with HCl, precipitated with ethanol, and was dissolved in distilled water. Single-strand molecular size of the sheared DNA was approximately 400 nucleotides.

Preparation of DNA for Digestion with Restriction Endonucleases

The livers were homogenized in 0.1 M EDTA-0.15 M NaCl (pH 8.0) and 0.75 vol (of the homogenate) of a lysis mixture (2% sodium laurylsulfate/8% Na-triisopropylnaphthalene sulfonate-12% (vol/vol) sec. butanol) was added. After swirling at room temperature for 5 min, the mixture was adjusted to 1 M Na-perchlorate and was extracted with 0.57 vol of phenol-chloroform (1:1, vol:vol) for 5 min. Two volumes of ethanol were added to the aqueous phase obtained after centrifugation and DNA was precipitated as a clump, which was washed in 70% ethanol and was dissolved in 0.005 M Tris (pH 7.5) − 0.0005 M EDTA-0.01 M NaCl. DNA was purified by digestion with 100 μg/ml RNase A at 37° C for 30 min, and with 100 μg/

ml self-digested pronase at 37° C for another 30 min in the presence of 0.5% Na-N-lauroylsarcosine, and by repeating extraction with the phenol-chloroform. Contaminating polysaccharides were removed by extraction with 2-methoxyethanol in the presence of potassium phosphate and phosphoric acid according to Kirby (1956). DNA was precipitated with ethanol, dissolved in 0.002 M Tris (pH 7.5) and was dialyzed against the same buffer.

Preparation and Labelling of Repetitive DNA from Females

The sheared female DNA was dissolved in a hybridization buffer (0.01 M Pipes (pH 6.8) – 0.6 M NaCl-40% formamide), heat-denatured, and incubated to C_0t 2 at 44° C. A double-stranded DNA fraction was obtained by hydroxyapatite column chromatography under the previously described conditions (Mizuno and Macgregor, 1974). The C_0t 2-double-stranded DNA was heat-denatured and the 0-time-binding fraction (Davidson et al., 1973) was removed by hydroxyapatite chromatography, and the remaining single-stranded DNA was reassociated again to C_0t 2. A double-stranded DNA fraction was obtained as above and was used as a repetitive DNA fraction. The repetitive DNA was ^{3}H-labelled by nick-translation. The reaction mixture contained in a total volume of 2.0 ml, 40 μg repetitive DNA, 0.5 mCi 5-^{3}H-dCTP (RCC, TRK352, 19 Ci/mmol), 0.5 μmole each of dATP, dGTP and dTTP, 100 μg bovine serum albumin, 0.45 ng DNase I, 200 units DNA polymerase I (Worthington Biochem. Corp.) in 0.05 M Tris(pH 7.9) – 0.005 M $MgCl_2$ – 0.01 M 2-mercaptoethanol. The reaction was carried out for 90 min at 16° C. Two-tenth ml of 0.1 M EDTA (pH 7.0) and 0.1 ml of 10% sodium laurylsulfate were added and the mixture was shaken with an equal volume of water-saturated phenol containing 0.1% 8-hydroxyquinoline. The labelled DNA was purified by passing through a column of Sephadex G-50 (fine) and was concentrated by lyophilization.

Mercuration of Sheared DNA from Males

Mercuric acetate was dissolved in 0.005 M Na-acetate(pH 6.5) at a concentration of 10 mg/ml and 78.5 ml of this solution was added to 321.5 ml of 0.005 M Na-acetate (pH 6.5), pre-warmed at 50° C and containing 80 mg of the sheared, male DNA. The mixture, in which the molar ratio of $Hg(CH_3COO)_2$/DNA nucleotide was 10/1, was incubated for 8 min at 50° C. The reaction was terminated by chilling in ice and adding approximately 40 ml of Chelex-100(200–400 mesh, BioRad), which had been washed extensively with 0.05 M Na-acetate(pH 6.5), and the suspension was poured into a column (2.2 × about 10 cm) and an eluate was collected. The column was washed with 100 ml of 0.05 M Na-acetate(pH 6.5) and the eluate and the wash were pooled, adjusted to 0.10 M Na-acetate (pH 6.5), and 2.5 volumes of ethanol were added. The mercurated DNA(Hg-DNA) was precipitated by centrifugation after overnight storage at −20° C, dissolved in 0.05 M Na-acetae(pH 6.5), passed through a column of Sephadex G-50(fine) in the same buffer, precipitated again with ethanol, and dissolved in 10 ml of distilled water. Under these conditions, approximately 40% of the dCMP residues in the DNA was mercurated (determined by an atomic absorption analysis after oxidation with $KMnO_4$-H_2SO_4). The Hg-DNA thus prepared could hybridize with the female ^{3}H-repetitive DNA in the absence of mercaptan with a rate of approximately 80% of that obtained with non-mercurated DNA (Naomi Watanabe and S. Mizuno: presented at the Annual Meeting of the Agricultural Chemical Society of Japan, April, 1980).

Enrichment of Female-specific ^{3}H-Repetitive DNA

Approximately 35 μg (2.6×10^7 cpm) of the ^{3}H-repetitive DNA of females and 62 mg of the unlabelled Hg-DNA of males were dissolved in 40 ml of the hydration buffer, head-denatured, and incubated to C_0t 10 at 44° C. The mixture was diluted 10-fold with TN buffer (0.01 M Tris(pH 7.5) – 0.1 M NaCl) and passed through a column (3.2 × 13 cm) of SH-Sepharose CL-6B (4.1 μmoles SH-residues/ml of settled volume). The unbound fraction, after dialysis against distilled water and lyophilization, was hybridized again with 62 mg of the male Hg-DNA to C_0t 10 and passed through a SH-Sepharose column (3.2 × 16 cm) under the same conditions as in the first hybridization. The unbound fraction was dialyzed, lyophilized, dissolved in 0.05 M PB (0.05 M NaH_2PO_4 – 0.05 M Na_2HPO_4, pH 6.8), and applied on a column

(3.6×7 cm) of hydroxyapatite at 60° C. The column was washed with 0.05 M PB at 60° C, then single-stranded DNA was eluted with 0.14 M PB at 60° C. The single-stranded DNA fraction was dialyzed, lyophilized, and dissolved in 2 ml of distilled water. This SH-Sepharose-unbound, single-stranded DNA (^{3}H-SHU-DNA) fraction contained 1.1×10^6 cpm of the ^{3}H-DNA and 5.76 mg of the unlabelled DNA from males.

DNA-Filter Hybridization

High molecular weight DNA was heat-denatured, immobilized on a membrane-filter (Sartorius, pore size 0.45 μm, diameter 45 mm), treated with the Denhardt's solution, and cut into discs of 5 mm diameter as described previously (Hennen et al., 1975). Appropriate numbers of the DNA-filter disc were mounted on a stainless steel insect pin and immersed in 0.4 ml of the hybridization buffer containing heat-denatured ^{3}H-DNA, and the reaction was incubated at 44° C. After the reaction, the DNA filter-discs with a pin were taken out, blotted, and washed successively in 10 ml each of $2 \times$ SSC, twice at room temperature and twice at 60° C. The filter-discs were dried at 80° C for 30 min and the radioactivity was determined in a toluene-PPO-dimethylPOPOP scintillator. Melting temperature (Tm) of the hybrid (approximately 1,500 cpm for each determination) was determined according to Whiteley et al. (1970) in $1 \times$ SSC after washing the filter-discs as above. The eluted ^{3}H-DNA at each temperature was precipitated in the presence of 50 μg of bovine serum albumin with cold 10% trichloroacetic acid. The precipitate was collected on a glassfiber filter (GF/A, Whatman), washed with cold 5% trichloroacetic acid-1% Na-pyrophosphate, dried and counted as above.

Digestion of DNA with Restriction Endonucleases and Isolation of a Particular DNA Fragment

Twenty five μg chicken DNA of males or females was digested with 50 units of one of restriction endonucleases (Takara Shuzo Co., Ltd.) in 30 μl of 0.0066 M Tris (pH 7.5) − 0.06 M NaCl − 0.007 M $MgCl_2$ − 0.007 M 2-mercaptoethanol at 37° C (30° C for SmaI, PstI and BamHI) for 8 h, and the reaction was stopped by heating at 65° C for 5 min. The mixture was made to 7% glycerol − 0.03 M EDTA − 0.007% bromophenol-blue and electrophoresed on 1% agarose-gel in an electrophoresis buffer (0.008 M Tris − 0.0004 M EDTA − 0.001 M Na-acetate, pH 8.0) containing 1 μg/ml ethidium bromide. Molecular weight markers were digests of PM2-phage DNA with HindIII. Ethidium bromide fluorescence of the gel was photographed and the negative was scanned by a soft laser scanning densitometer (MDA/BIOMED). The 0.60 kb DNA fragment produced with XhoI from the DNA of females was recovered from the gel by adsorption to hydroxyapatite and elution with 0.40 M PB (Tabak and Flavell, 1978). The eluate was extracted with isoamylalcohol, then with chloroform-isoamylalcohol (24:1, vol:vol), and dialyzed against distilled water. The dialysate was made to 0.05 M PB and applied on a hydroxyapatite column. A fraction containing double-stranded DNA was eluted with 0.40 M PB at 60° C after eluting single-stranded DNA with 0.18 M PB. The double-stranded DNA fraction was dialyzed, lyophilized and dissolved in distilled water.

Labelling and Further Purification of the XhoI 0.60 kb Fragment from the DNA of Females by Exhaustive Hybridization with the Filters with Male DNA

Five μg of the 0.60 kb DNA fragment isolated from the gel was ^{3}H-labelled by nick-translation, dissolved in 8 ml of the hybridization buffer, heat-denatured, and divided into ten 0.8 ml reactions. Each reaction mixture received 17 membrane filter-discs each of which carrying approximately 10 μg of the denatured DNA from males and was incubated for 2 h at 44° C. The DNA-filter discs were removed, the reaction-mixture heat-denatured again, and a new set of male DNA-filter discs added and the reaction repeated. After three such reactions; i.e. exposing 5 μg of the ^{3}H-DNA to 5.1 mg of the DNA of males, ^{3}H-DNA remained in the reaction mixture was dialyzed against distilled water, lyophilized and dissolved in 0.12 M PB. During the exhaustive hybridization, a part of the male DNA was released from the filter discs. To remove this DNA, the ^{3}H-DNA fraction in 0.12 M PB was applied on a 5 to 20% linear sucrose gradient in 0.12 M PB − 0.001 M EDTA and centrifuged at 64,000 g

for 25 h at 4° C in a 25 ml × 3 swinging-bucket rotor (MSE). The ^{3}H-DNA which was separated from the heavier, unlabelled DNA, was dialyzed, lyophilized and dissolved in distilled water.

C-Banding of the Chicken Embryo Chromosomes

One-tenth ml of 10 μg/ml colcemid solution (Gibco) was applied on a chorio-allantoic membrane over a 3 day-old chicken embryo for 1 h at 37° C, then a whole embryo with the membrane was taken out and fixed in 50% acetic acid. A part of the fixed embryonic tissues and the membrane was squashed under a siliconized coverslip. The coverslip was removed by the dry-ice method and the specimen dehydrated immediately in 95% ethanol, air-dried, and stored in a desiccator for a few days at 4° C. C-banding was carried out according to Sumner et al. (1971) with some modifications. The slide was dipped in a series of solutions at room temperature (unless otherwise noted) with the following order: 1 h in 0.2 N HCl, a few sec in distilled water, 2 min in saturated solution of $Ba(OH)_2$ at 30° C, a few sec in distilled water, 5 min in 2 × SSC, a few sec in distilled water, and two 5 min in 2 × SSC. The slide was then incubated in 2 × SSC for 1 h at 60° C, rinsed in distilled water for a few sec, dehydrated through 70% and 95% ethanol, and air-dried. It was stained with 3% Giemsa for 5 min.

In situ Hybridization and Autoradiography

The procedures described by Hennen et al. (1975) were followed with following modifications: (1) slides were placed in 2 × SSC for 30 min at 70° C (Bonner and Pardue, 1976) before the ribonuclease treatment, (2) ribonuclease Tl was not included, (3) the ^{3}H-DNA was heat-denatured at 100° C for 5 min in 4 × SSC – 50% formamide and the reaction was carried out in this solution for 24 h at 37° C, (4) slides were coated with Sakura NR-M2 liquid emulsion (diluted 1:1 with distilled water), and (5) the emulsion-coated slides were air-dried and placed in the atmosphere of 6% H_2O_2 for 1–2 h at room temperature before starting the exposure at 10° C. When C-banded specimens were used for hybridization, the stained specimens were first photographed, destained in methanol-acetic acid (3:1, vol:vol), dehydrated through 70% and 95% ethanol, then above procedures were applied except that the reaction time was prolonged to 45 h.

Results

Enrichment of Female-Specific Repetitive DNA Sequences by Hybridization Involving Mercurated DNA of Males

As described in detail in the Methods, ^{3}H-labelled repetitive DNA from the female chicken was hybridized with excess, mercurated, sheared DNA of males to C_0t 10, and unhybridized ^{3}H-DNA was recovered as an unbound fraction from the SH-Sepharose affinity column. The unbound ^{3}H-DNA was subjected to the second cycle of hybridization with the mercurated DNA of males and the SH-Sepharose chromatography was repeated. The unbound ^{3}H-DNA was applied on a hydroxyapatite column and a ^{3}H-DNA fraction remaining as a single-stranded form was separated from a ^{3}H-DNA fraction containing double-stranded regions, due to possible presence of foldback sequences or extremely highly repetitive sequences. The single-stranded ^{3}H-DNA fraction, that was about 4% of the input ^{3}H-repetitive DNA, should contain female-specific DNA sequences in a high proportion, if excessiveness of the mercurated DNA of males in the

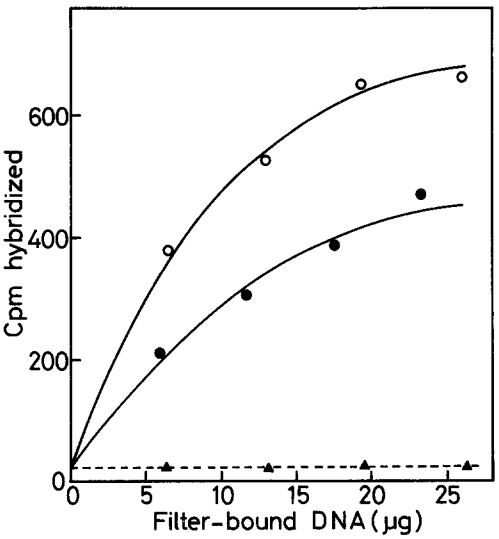

Fig. 1. Hybridization of ^{3}H-SHU-DNA (1,000 cpm) with increasing amount of filter-bound DNA from the female chicken (○), the male chicken (●), or *H. retardatus* (▲). Reactions were carried out for 24 h at 44° C

hybridization reactions and capacity of the SH-Sepharose column were sufficiently high.

The single-stranded, SH-Sepharose-unbound ^{3}H-DNA (^{3}H-SHU-DNA) thus obtained was hybridized with denatured chicken DNA from males or females or *Hynobius retardatus* DNA (a salamander DNA included as a control), each of which was immobilized on membrane-filters. Figure 1 shows levels of hybridization with increasing amount of each filter-bound DNA. The levels of hybridization with 20 to 25 μg of the DNA of males and *H. retardatus* DNA were 65–70% and approximately 4%, respectively, of the level obtained with the DNA of females, indicating that at most 30% of the hybridizable ^{3}H-SHU-DNA consisted of female-specific sequences or sequences that were abundant in the female genome but were present more infrequently in the male genome. The evidence for female-specific repetitive DNA sequences was further supported by the competition-hybridization experiment shown in Figure 2. When ^{3}H-SHU-DNA was hybridized with a fixed amount (28 μg each) of the filter-bound DNA of males or females in the presence of increasing amount (up to 500 μg) of unlabelled, sheared, DNA of males, hybridization of the ^{3}H-DNA with the DNA of females was reduced by competition but to a level which was distinctly higher than that obtained with the DNA of males. These results are expressed in the double-reciprocal form (Hansen et al., 1970) in Figure 2B, which suggests that approximately 24% (see the legend of the figure) of the hybridizable ^{3}H-SHU-DNA would be uncompetable in the presence of an infinite amount of the unlabelled DNA of males. It is most likely that such uncompetable fraction consists of female-specific repetitive DNA sequences.

The results of in situ hybridization of ^{3}H-SHU-DNA with metaphase chromosomes from the male or female chicken embryos are shown in Fig-

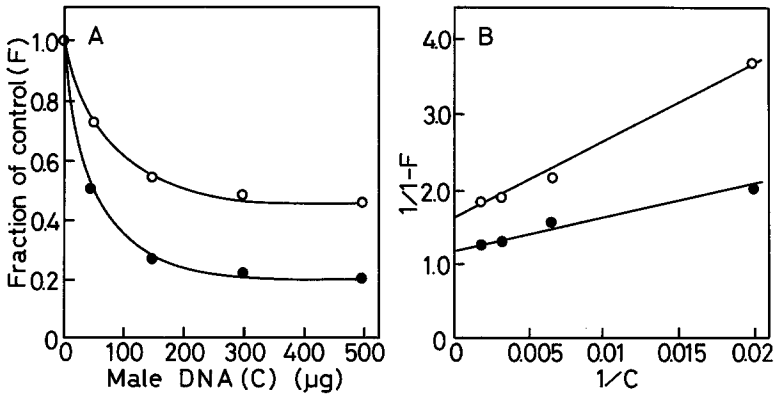

Fig. 2. A Competition by unlabelled, male chicken DNA in the hybridization of ^{3}H-SHU-DNA (1,000 cpm) with filter-bound DNA (28 μg) from the female chicken (○) or the male chicken (●). The level of hybridization in the absence of competitor was 620 cpm with the DNA-filter from females and 400 cpm with the DNA-filter from males. **B** Double reciprocal plots of the results shown in A. Intercept on the ordinate was 1.16 with the DNA-filter from males and 1.62 with the DNA-filter from females, which gave a fraction of 0.24 that hybridized with the DNA-filter from females in the presence of an infinite amount of the unlabelled DNA of males

ure 3. Although only about 24% of the hybridizable ^{3}H-SHU-DNA appears to be female-specific, it hybridized predominantly with one chromosome in the female set (Fig. 3A) after 15 days of exposure but no meaningful hybridization was obtained with any chromosome in the male set under the same conditions (Fig. 3B). The chromosome in the female set on which grains were localized is most certainly the W chromosome judging from its size and the specificity of the hybridization. It is unlikely that the apparent specificity of the hybridization to the W chromosome shown in Figure 3A was caused by competition with the unlabelled DNA of males present in the ^{3}H-SHU-DNA preparation, because ^{3}H-total repetitive DNA of females (no DNA of males included) of the same input radioactivity (4×10^4 cpm) as ^{3}H-SHU-DNA did not show significant hybridization to any chromosomes (Fig. 3C) under the same conditions of hybridization and autoradiographic exposure time, on the other hand when the input radioactivity was increased to 8×10^5 cpm, it hybridized to various parts of the chromosome set with the heavy labelling of the W chromosome and a telomeric region of the Z-like chromosome after the same exposure time (Fig. 3D). Thus, the preferential labelling of the W chromosome by ^{3}H-SHU-DNA is likely to be caused primarily by enrichment of the female-specific repetitive DNA sequences relative to other sequences and perhaps the labelling was more exaggerated by possibilities that the female-specific sequences might be more highly repetitive than average repetitive sequences or more highly concentrated on the W chromosome (or both) than other sequences would be on other chromosome regions.

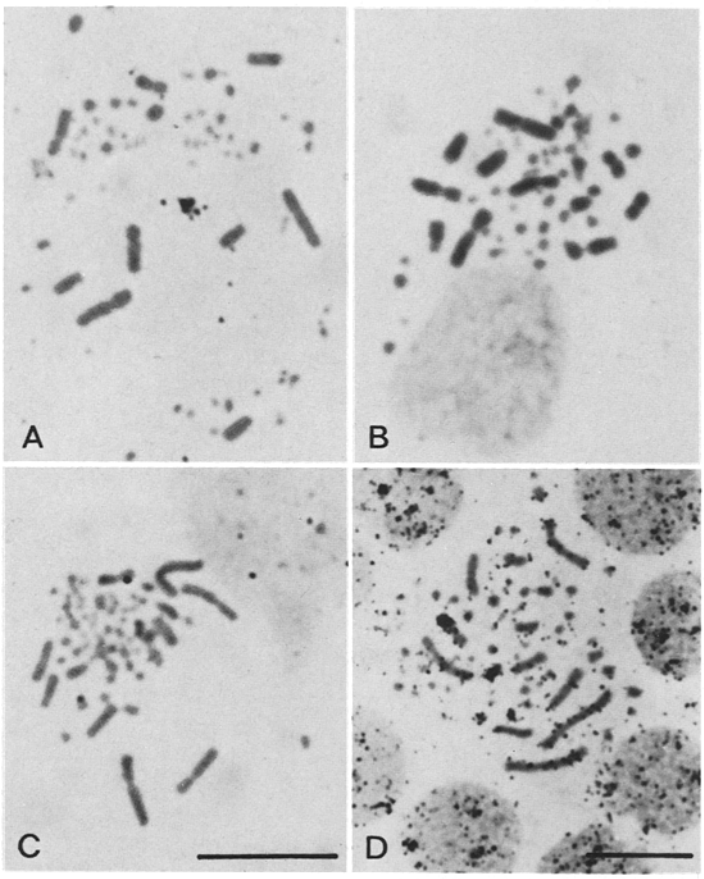

Fig. 3A–D. In situ hybridization of ^{3}H-SHU-DNA to the metaphase chromosomes from **A** the female or **B** male chicken embryo, and of ^{3}H-total female repetitive DNA to the metaphase chromosomes from the female chicken embryos (**C** and **D**). Input radioactivity was 4×10^{4} cpm in A, B, and C, and 8×10^{5} cpm in D. Autoradiographic exposure time was 15 days in A and B, and 10 days in C and D. Bar = 10 μm

Demonstration that the 0.60 kb DNA Fragment Produced by a Restriction Endonuclease XhoI is a Female-Specific Sequence

As above results suggested strongly that a distinct class(es) of repetitive DNA sequence is present in the W chromosome of the female chicken, we examined a female-specific DNA fragment(s) in the digestion products of chicken DNA with various restriction endonucleases. Among fifteen kinds of enzymes tested (AccI, AluI, Bam HI, Eco RI, HaeIII, HapII, HhaI, HincII, HindIII, HinfI, KpnI, PstI, SalI, SmaI, XhoI), XhoI produced two well-recognizable, female-specific DNA fragments, 0.60 kb and 1.1 kb, as shown in Figure 4A. It is estimated from the densitometric tracing of negatives such as shown in Figure 4B that the 0.60 kb DNA fragment may

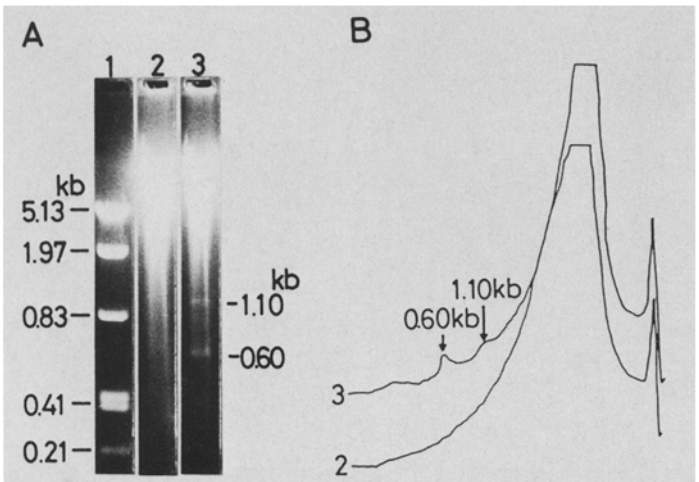

Fig. 4. A Ethidium bromide-stained 1.0% agarose-gel after electrophoresis. Lane 1: PM2 DNA digested with HindIII (molecular size markers), lane 2: chicken DNA from males digested with XhoI, lane 3: chicken DNA from females digested with XhoI. **B** Densitometric tracing of the lane 2 (male) and the lane 3 (female) in A

account for 0.31 to 0.46% of the total genomic DNA. If the 0.60 kb fragment is assumed to consist of a homogeneous repeating unit, these values would imply that the sequence is repeated 1.2×10^4 to 1.8×10^4 times in the diploid genome (taking 2.54 pg for the diploid genome size according to Mizuno et al., 1978).

As the 0.60 kb band is separated rather well from the bulk DNA fragments, we decided to isolate and characterize this DNA fragment. The 0.60 kb DNA fragment was extracted from the gel and ^{3}H-labelled by nick-translation. Most of contaminating repetitive sequences which are common to both female and male genomes were removed by the exhaustive hybridization with the filter-bound DNA from males as described in Methods. Female-specificity of the purified ^{3}H-0.60 kb DNA was tested by hybridizing it with the filter-bound chicken DNA of females or males. Filters carrying *H. retardatus* DNA were also included to monitor blank levels of hybridization. As shown in Figure 5A, about 95% of the hybridization of ^{3}H-0.60 kb DNA was attained with the DNA of females after 24 h reaction, when the reaction reached a level of apparent saturation with respect to the amount of unlabelled DNA in the reaction mixture. The difference in the levels of hybridization with the DNA of males or females was maintained when the time of reaction was extended to 4 days (Fig. 5B), indicating that the difference observed is most likely due to a female-specific nature of the sequence, but a possibility that the sequence is present in the male genome with much lower frequency of repetition is still not eliminated under these experimental conditions. About 5% of the hybridization observed with the DNA of males (Fig. 5A, B) seems to be caused by the presence of sequences in the ^{3}H-DNA preparation which are common to both the

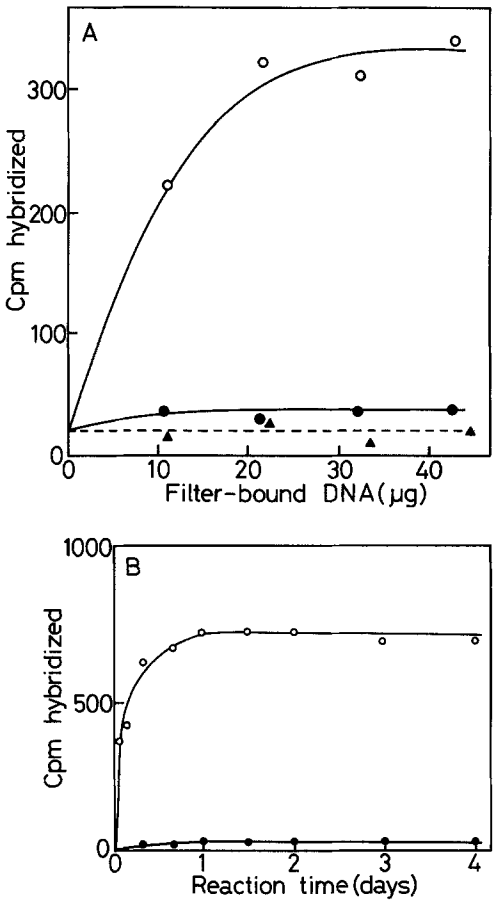

Fig. 5. A Hybridization of ^{3}H-XhoI-0.60 kb DNA (1,300 cpm) with increasing amount of filter-bound DNA from the female chicken (o), the male chicken (●), or *H. retardatus* (▲). Reactions were carried out for 24 h. **B** Hybridization of ^{3}H-XhoI-0.60 kb DNA (3,000 cpm) with filter-bound DNA (42 μg) from the female chicken (o) or the male chicken (●) after different time of incubation. Blank levels of hybridization with *H. retardatus* DNA-filters were subtracted

female and the male genomes, because addition of increasing amount of the unlabelled DNA of males in the hybridization of the ^{3}H-DNA with the filter-bound DNA of females reduced the level of hybridization to a similar extent as shown in Figure 6. It is not known at present if the common sequences are present as impurities or as a part of the repeating unit.

Localization of the XhoI 0.60 kb DNA on the W Chromosome

In situ hybridization of the ^{3}H-0.60 kb DNA to the female or male chromosomes prepared from the chicken embryos showed a single site of hybridization on the female chromosome set (Fig. 7A) but no significant hybridization on the male set (Fig. 7B). The chromosome in the female set on which grains were localized was confirmed to be the W chromosome by hybridizing the ^{3}H-DNA to the female chromosome set that had previously been subjected to the C-banding. The W chromosome exists as a prominent C-banding-positive unit in the female chromosome set as indicated by an arrow in Figure 7C. The same set was destained and hybridized with the ^{3}H-0.60 kb DNA. The autoradiograph (Fig. 7D) showed localization of

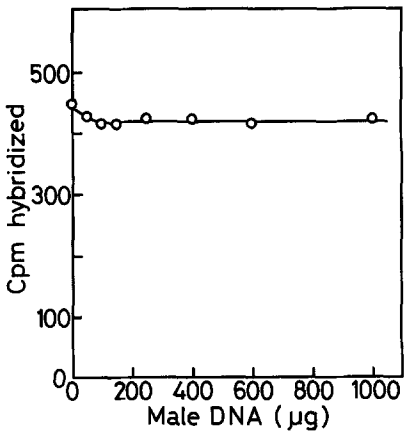

Fig. 6. Competition by unlabelled, sheared chicken DNA from males in the hybridization of ^{3}H-XhoI-0.60 kb DNA (2,000 cpm) with the filter-bound chicken DNA from females (42 μg). Reactions were carried out for 24 h

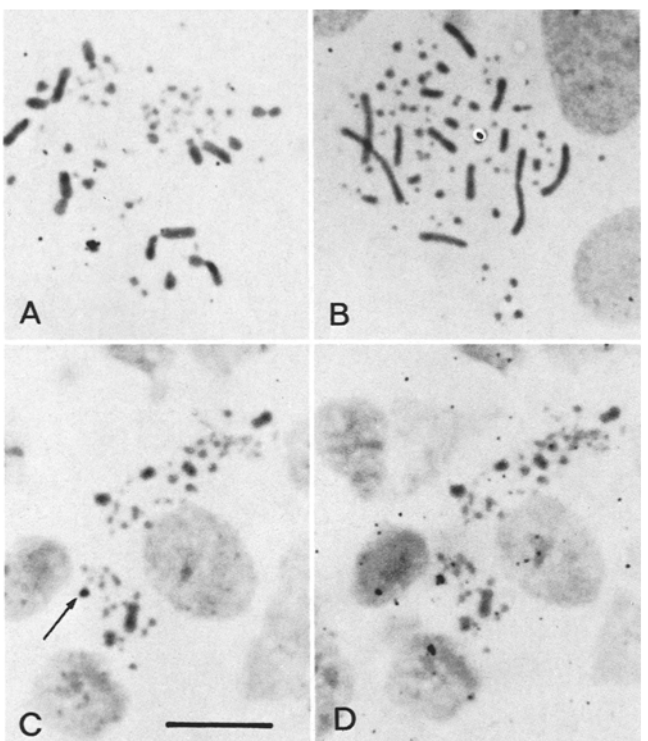

Fig. 7A–D. In situ hybridization of ^{3}H-XhoI-0.60 kb DNA to the metaphase chromosomes from **A** the female or **B** the male chicken embryo. **C** A set of female chicken metaphase chromosomes stained with the C-banding technique. **D** In situ hybridization of ^{3}H-XhoI-0.60 kb DNA to the female metaphase chromosome set shown in C. Input radioactivity was 5×10^{4} cpm in A and B, and 1.4×10^{5} cpm in D. Autoradiographic exposure time was 15 days in A and B, and 65 days in D. Bar = 10 μm

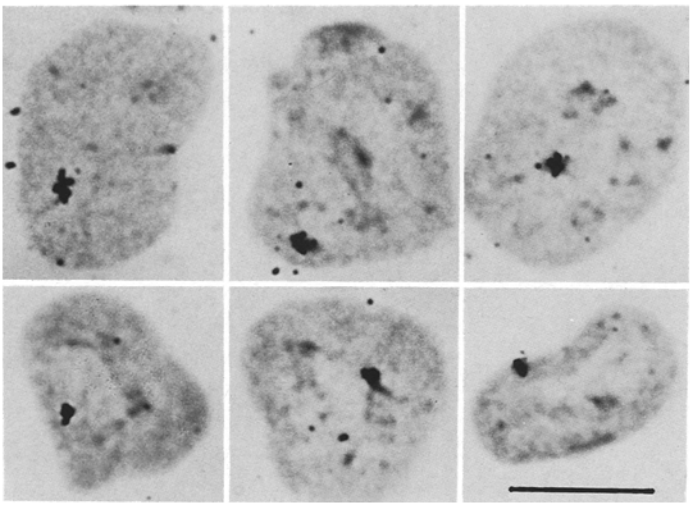

Fig. 8. In situ hybridization of ^{3}H-XhoI-0.60 kb DNA to the female chicken interphase nuclei. Input radioactivity was 5×10^4 cpm and autoradiographic exposure time was 15 days. Bar = 10 μm

grains on the chromosome which had been identified as W in Figure 7C. It is also evident from the results of in situ hybridization shown in Figure 8 that the ^{3}H-0.60 kb DNA is associated with a heterochromatic area in the interphase nucleus of the female chicken embryo.

Species Specificity of the XhoI 0.60 kb DNA Sequence

To know evolutionary origin and stability of the XhoI 0.60 kb DNA, the ^{3}H-0.60 kb DNA from the female White Leghorn chicken was hybridized with filter-bound DNAs of females or males from four other lines of domestic fowl (*G. g. domesticus*), a transformed lymphoblastoid cell line (MSB-1) from a female chicken (Akiyama and Kato, 1974), three species belonging to the order Galliformes, and one species each from the order Columbiformes, Psittaciformes, and Passeriformes, and their levels of hybridization were compared (Table 1). Hybridization of the ^{3}H-total repetitive DNA from the female White Leghorn to those DNAs was also carried out to give a measure of relatedness of general repetitive DNA sequences among those species. As shown in Table 1, the ^{3}H-0.60 kb DNA hybridized with the DNA of females, but not with the DNA of males, from the four other lines of *G. g. domesticus* and with the DNA from the MSB-1 cells to the similar extent as with the female White Leghorn DNA, but no significant levels of hybridization were obtained with the DNA from other avian species tested. In contrast, the ^{3}H-total repetitive DNA from the female White Leghorn hybridized with the DNA from the three species (*P. versicolor, N. meleagris*, and *C. c. japonica*) belonging to the genus Galliformes to the levels that were 20 to 40% of the level of hybridization obtained with the

Table 1. Hybridization of ^{3}H-XhoI-0.60 kb DNA and ^{3}H-total repetitive DNA from the female White Leghorn with filter-bound DNAs from other species. ^{3}H-XhoI-0.60 kb DNA (1,300 cpm) or ^{3}H-total repetitive DNA (2,000 cpm) was hybridized with 42 ± 6 μg of filter-bound DNA from each species for 4 days. The 100% level of hybridization with the ^{3}H-total repetitive DNA was 600 cpm

Species	Male (M) or Female (F)	Hybridization of ^{3}H-XhoI-0.60 kb DNA		Hybridization of ^{3}H-total repetitive DNA (%)
		(cpm)	(%)	
Gallus g. domesticus (domestic fowl)				
White Leghorn	M	17	5.8	80
	F	295	100	100
Japanese Game	M	19	6.4	98
	F	282	96	104
Nagoya Cochin	M	26	8.8	97
	F	299	101	96
Barred Plymouth Rock	M	34	11.5	91
	F	289	98	101
Fayomi	M	20	6.8	99
	F	308	104	110
MSB-1 cell line	F	274	93	102
Phasianus versicolor (japanese common pheasant)	M	8	2.7	36
	F	0	0	38
Numida meleagris (guinea fowl)	M	0	0	33
	F	2	0.7	35
Coturnix c. japonica (japanese quail)	M	0	0	24
	F	8	2.7	18
Columba livia (rock dove)	M	1	0.3	6
	F	0	0	6
Melopsittacus undulatus (shell parrakeet)	M	26	8.8	5
	F	9	3.1	4
Zosterops palpebrosa (japanese white-eye)	M	25	8.5	2
	F	27	9.1	2

White Leghorn DNA. It is clear from these results that the XhoI 0.60 kb DNA sequence found in the female White Leghorn genome is only represented as a repetitive component among the female genomes within the same *genus* but not beyond.

Thermal stability of the hybrids between the ^{3}H-0.60 kb DNA and DNAs from other lines of domestic fowl or from MSB-1 cells was very similar to that of the homologous hybrid; i.e. differences in the Tm values were within 1.5° C, as shown in Figure 9. These results indicated that no particularly higher rate of base sequence changes had occurred within this sequence either during the establishment of any one of the present lines of domestic fowl tested or during passages of the MSB-1 cell line.

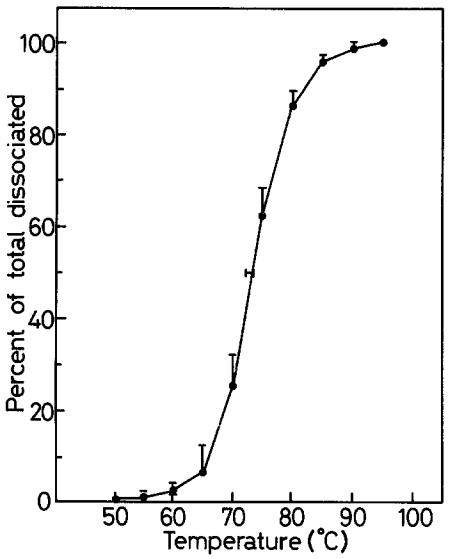

Fig. 9. Thermal dissociation profiles of the hybrids between ^{3}H-XhoI-0.60 kb DNA and filter-bound DNAs of female from different lines of *G. g. domesticus* or from the MSB-1 cells. The lines tested were White Leghorn, Japanese Game, Nagoya Cochin, Barred Plymouth Rock, and Fayomi. All the experimental points were found within the range of the vertical bar at each temperature. The line in the figure connects the points obtained with the female White Leghorn DNA

Discussion

When metaphase chromosomes of female chicken are stained with the C-banding method, an entire W chromosome and a telomeric region of the Z chromosome are stained heavily (Takagi et al., 1977; Carlenius et al., 1981), suggesting that those regions are heterochromatic and rich in highly repetitive DNA sequences (Inhorn and Meisner, 1975; John and Miklos, 1979). As the W chromosome is present only in the female genome, if any female-specific repetitive DNA sequences were present in the W chromosome, they should be recovered in the fraction that remained single-stranded after hybridizing a trace amount of labelled repetitive DNA of females with an excess, sheared DNA of males. Using a similar approach, Kunkel et al. (1976) demonstrated the presence of the Y chromosome-specific repetitive DNA sequences in the human male genome. One disadvantage of this method is that the final fraction also contains a large amount of unlabelled, non-repetitive DNA, which might be difficult to be separated from the labelled repetitive DNA by the conventional differential Cot fractionation, as the concentration of the labelled DNA is very low. In this study, we solved this problem partially by hybridizing the ^{3}H-labelled repetitive DNA of females with an excess of mercurated, sheared DNA of males. The extent of mercuration was limited to be less than 40% of the cytosine residues, so that a comparable rate of hybridization as with non-mercurated DNA was obtained without adding a mercaptan in the hybridization mixture. Addition of a mercaptan caused a lower efficiency of binding of the Hg-DNA to the SH-affinity column (Mizuno et al., 1978). After hybridization, the unhybridized ^{3}H-DNA was separated from most of the single- and double-stranded Hg-DNA and also from the hybridized ^{3}H-DNA with the Hg-DNA by the SH-Sepharose column chromatography. The SH-Sepharose-unbound, single-stranded ^{3}H-DNA (^{3}H-SHU-DNA) fraction, which

contained about 4% of the unlabelled male DNA, was shown by the competition-hybridization technique to contain female-specific repetitive DNA sequences as about 24% of the total hybridizable sequences. The partial enrichment is probably largely attributable to an insufficient excess of the Hg-DNA of males in the hybridization reaction. In spite of this partial enrichment, the ^{3}H-SHU-DNA showed preferential in situ hybridization to the W chromosome and we reasoned this because of the highly repetitive nature of the female-specific sequences and their high local concentrations on the W chromosome. Although this approach has a certain advantage that every family of female-specific repetitive sequences would be recovered in the final fraction, the relatively low enrichment made their further characterization difficult, and we decided to examine female-specific DNA fragment among the digests of chicken DNA with various restriction endonucleases.

The 0.60 kb DNA fragment produced from the female chicken DNA with XhoI was chosen as a main subject of the present study, because it was separated fairly well from other fragments by the agarose-gel electrophoresis and was purifiable relatively easily. The content of 0.60 kb repeats in the DNA of female was estimated from the densitometric tracing of the ethidium bromide staining to be 0.31–0.46%, i.e. 1.2×10^4 to 1.8×10^4 copies per diploid genome. Assuming that the W chromosomal DNA represents approximately 1.4% of the diploid amount of the female chicken DNA (calculated from Takagi and Sasaki (1974) with an assumption that the DNA of microchromosomes accounts for 26% of the total diploid DNA content (Ohno, 1967)), the family of DNA sequences consisting of the 0.60 kb repeating units would account for 22–33% of the W chromosomal DNA. These values might become even higher if some related sequences forming different repeating units were present and taken into account. A similar situation has been found for the organization of human Y chromosome, where a HaeIII 3.4 kb DNA sequence is repeated approximately 7,500 times and they account for about 0.5% of the total DNA of males (Cooke, 1976; Kunkel et al., 1979). As the Y chromosomal DNA is about 1.2% of the diploid amount of the male human DNA, as much as 40% of the Y chromosomal DNA consists of repeats of a particular type of sequence unit. In this case, the 3.4 kb DNA sequence has been shown to contain three 800 bp units of Y-specific sequences and four 250 bp units of non-Y-specific sequences and it has also been suggested that the 3.4 kb units are heterogeneous with respect to the kinds of Y-specific sequences included (Kunkel et al., 1979).

When properties of the chicken XhoI 0.60 kb DNA and the snake satellite DNA, which has been shown to be associated with the W chromosome (Singh et al., 1976, 1980), are compared, we can notice two clear differences. Firstly, female-specificity of the chicken sequence is much higher. As shown in Figure 5 and Table 1, the level of hybridization of the 0.60 kb DNA with the DNA of females was more than 10 times as high as with the DNA of males, while the snake satellite sequence hybridized with the DNA

of females 1.19 to 3.59 times higher than with the DNA of males (Singh et al., 1980). This might be caused by the fact that the satellite DNA (*B. fasciatus* minor satellite) is composed of complex sequences of different reassociation kinetic properties (Singh et al., 1981), as some of them may be non-W-specific as in the case of the above mentioned 3.4 kb sequence from the male human DNA. We can not conclude, however, from the present study that the female 0.60 kb sequence or its related sequences are absent in the male genome. In the competition-hybridization experiment shown in Figure 6, 1,000 μg of the unlabelled DNA of males added as competitor would contain 5.2×10^{-4} μg of the 0.60 kb sequence if the sequence is present only once in a haploid male genome, while 42 μg of the filter-bound DNA of females should contain about 0.17 μg of the repeats of the 0.60 kb DNA sequence assuming that this particular family of repetitive sequence accounts for about 0.4% of the diploid genome. Thus, if 327 ($0.17/5.2 \times 10^{-4}$) copies per haploid were present in a male genome, 1,000 μg of the DNA of males would contain a comparable amount of the 0.60 kb sequence as in the 42 μg of the DNA of females and we would start seeing definite competition with this amount of the unlabelled DNA of males. Thus, we can only conclude from the present results that if the 0.60 kb sequence or related sequences were present in the male genome, they should have less than about 330 copies per haploid genome.

The second difference is about the conservatism of the sequence. It has been shown that the *B. fasciatus* minor satellite DNA sequence can be detected not only throughout the snake species having W chromosome (Singh et al., 1980) but also in a wide range of eukaryotic genomes such as male mice and *Drosophila* (Singh et al., 1981). The latter observation was made by the Southern-blot hybridization using a ^{32}P-labelled probe, thus sensitivity of detection should be much greater than with the method employed in the present study. Although we must keep this difference in sensitivity in mind, the feature of species-specificity of the 0.60 kb DNA shown in Table 1 is striking in that the sequence was found as a repetitive component only in the female genomes of different lines of domestic fowl (*G. g. domesticus*) and no such component was found in the genomes of other members of the order Galliformes such as japanese quail (*C. c. japonica*), guinea fowl (*N. meleagris*) and japanese common pheasant (*P. versicolor*). Thus, it would be reasonable to assume that amplification of the 0.60 kb sequence in the female genome has occurred rather recently, either during the evolution of domestic fowl from jungle fowls or during the evolution of jungle fowls from their common ancestral species.

The present finding indicated that despite the common heterochromatic nature of the W chromosome among female birds, its major constituent repetitive DNA family can be widely variable with respect to its unit sequence. It is conceivable that size, organization and numbers of a repeating unit rather than the DNA sequence *per se* would be more important factors in causing heterochromatization of the W chromosome and in maintaining function and evolutionary stability of the female-determining system.

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