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Molecular characterization and cytological mapping of a non-repetitive DNA sequence region from the W chromosome of chicken and its use as a universal probe for sexing Carinatae birds

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A non-repetitive genomic DNA region of about 25 kb was cloned from the W chromosome of chicken using a genomic library prepared from a single W chromosome of the chicken. This region was mapped by fluorescence *in situ* hybridization (FISH) with mitotic and lampbrush chromosomes to a position between the major *Eco*RI family and the pericentromeric *Xho*I family on the W chromosome. A 0.6-kb *Eco*RI fragment (EE0.6) subcloned from this region consists of a sequence that can be obtained by the exon-trapping procedure and flanking sequences. Sequences, which are closely similar to that of EE0.6, are widely conserved on the W chromosomes of Carinatae birds, as revealed by Southern blot hybridization to *Hin*dIII-digested female and male genomic DNAs from 18 species of birds belonging to eight different taxonomic orders. The female sex of those birds can be determined by the presence of an unambiguous female-specific band. For many species of birds, the female sex can also be determined by polymerase chain reaction (PCR) using a set of primers from the flanking sequences in the chicken EE0.6.

Key words: Carinatae birds, EE0.6 sequence, *Gallus gallus domesticus*, universal sexing probe, W chromosome

Introduction

About 65% of DNA in the W chromosome of chicken consists of the *Xho*I and *Eco*RI families of repetitive sequences, both of which are specific to this chromosome and form conspicuous heterochromatin (Suka *et al*. 1993). Most of the non-repetitive DNA sequences in the W chromosome seem to be distributed towards one end of the chromosome and they form diffuse chromatin in interphase nuclei (Suka *et al*. 1993). This non-heterochromatic end region has been shown to contain an end-to-end pairing site with the non-heterochromatic end region of the Z chromosome in premeiotic pachytene and diplotene stages (Solovei *et al*. 1993, Solari & Dresser, 1995, Hori *et al*. 1996). Molecular characterization of DNA sequences in this end region has not yet been carried out.

With respect to the role of the W chromosome in female sex determination, the observations on the gonadal development in 3A + ZZW triploid chickens are informative. In these chickens, the right gonad develops into a testis and the left gonad develops into an ovotestis at hatching (Lin *et al*. 1995). However, recent observations by Sheldon & Thorne (1995) demonstrated that the initial differentiation of gonads in the 3A + ZZW embryos was similar to that in the normal female embryos. These results may imply that a gene concerning the early differentiation of the female left gonad is present on the W chromosome but its determinant role should not be as strong as that of the mammalian sex-determining gene on the Y chromosome. However, the existence of such a gene on the W chromosome has not been demonstrated.

In this study, we have attempted, by screening a W chromosome-specific genomic library, to isolate, clone and characterize non-repetitive DNA sequences that show biologically significant features in relation to the functions of avian W chromosome.

Materials and methods

Cloning of genomic fragments from the chicken W chromosome

All the chromosomes except for a single W chromosome in a Giemsa-stained metaphase set from the female chicken embryonic fibroblast were destroyed by irradiation with an argon-ion laser microbeam used in conjunction with the chromosome dissection microscope system (C3144, Hama-

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matsu Photonics). The DNA extracted from the remaining single W chromosome was subjected to the single unique primer PCR (SUP-PCR) according to Hadano *et al.* (1991). Those preparations that showed DNA fragment sizes ranging from 0.2 to 0.5 kb and hybridized with the W chromosome-specific *Xho*I and *Eco*RI repetitive family sequences (Kodama *et al.* 1987, Saitoh *et al.* 1991) were selected and subjected to ligation with the arms of λgt10 vector followed by *in vitro* packaging.

One of the W chromosome-derived genomic libraries, W-17, was subjected to plaque hybridization with a mixture of ^{32}P-labelled *Xho*I and *Eco*RI family probes, and 296 clones that did not give signals of hybridization were selected randomly. Inserts of these clones were individually amplified by PCR using the sequences flanking the *Eco*RI site of λgt10 as primers (Hori *et al.* 1996).

Each PCR product was ^{32}P-labelled by the random priming method (Feinberg & Vogelstein, 1983) and hybridized to the slot blot [5 μg/slot on Hybond N$^+$ membrane (Amersham)] of the male or female chicken DNA in 6 × SSPE (60 mM NaH_2PO_4, 1.08 M NaCl, 6 mM EDTA, pH 7.4) containing 1% sodium dodecyl sulphate (SDS), 1% blocking reagent (Boehringer Mannheim) and 250 mg/ml sheared, heat-denatured salmon sperm DNA at 65°C for 16 h. The stringency of hybridization under these conditions was calculated to allow about 14% basepair mismatches according to Sambrook *et al.* (1989). One clone, CW-01, which gave a relatively weak, female-specific signal, was selected assuming that it represented a non-repetitive sequence on the W chromosome. The λGEM12 genomic library of a female chicken (Hori *et al.* 1996) was screened with the ^{32}P-labelled CW-01 as a probe. One clone, λ7AGW, containing about 15-kb insert, was selected and its insert, recovered by digestion with *Not*I, was recloned into pBluescript SK$^+$ to yield p7AGW. A 1.8-kb *Eco*RI fragment (EE1.8) of p7AGW containing the CW-01 sequence was subcloned and used to screen further the λGEM12 genomic library and four clones, λ7A1GW, λ7A3GW, λ7A4GW and λ7A5GW, were obtained. Several restriction fragments of p7AGW were subcloned using pUC119 or pBluescript SK$^+$ as cloning vectors. One of those clones containing a 0.6-kb *Eco*RI fragment, EE0.6, was characterized in this study.

Preparation of genomic DNA and Southern blot hybridization

High molecular weight genomic DNA was prepared from a heparinized blood sample essentially as described in Saitoh *et al.* (1993) except that nuclei were isolated first for the extraction of genomic DNA as follows. All the procedures were carried out at 0–4°C. Blood (1–3 ml) was diluted to 20 ml with 1 × phosphate-buffered saline (PBS) (10 mM Na_2HPO_4, 1.4mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 10 mM EDTA and centrifuged at 300 ***g*** for 10 min. The pellet was resuspended in 5 ml of 1 × PBS by homogenizing in the Dounce homogenizer (Wheaton) and centrifuged. The pellet was resuspended gently in 10 ml of TMS [10 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.25 M sucrose] containing 0.5% Triton X-100 (Sigma) and centrifuged at 700 ***g*** for 10 min. The pellet (crude nuclei) was resuspended in TMS and subjected to the extraction of DNA.

The high molecular weight genomic DNA was digested with appropriate restriction enzyme(s), electrophoresed on 0.8% agarose gel (10 μg/lane), transferred to Hybond-N$^+$ membrane in 0.4 N NaOH, and hybridized with a ^{32}P-labelled CW-01 probe at 68°C (allowing 11% basepair mismatches) or ^{32}P-labelled EE0.6 probe at 60°C (allowing 19% basepair mismatches) in the same reaction mixture as for the slot blot hybridization. It was essential to label the EE0.6 probe to a sufficiently high specific activity. In practice, 10 ng of EE0.6 was labelled by the random priming method with at least 3.7 MBq [α-^{32}P]dCTP (approximately 110 TBq/mmol; Amersham). The membrane was washed in 2 × SSC, 0.1% SDS at room temperature for 15 min twice and at 60°C (for the EE0.6 probe) or 68°C (for the CW-01 probe) for 20 min twice, and subjected to autoradiography. The exposure time was 7 days with Kodak X-OMAT film when the EE0.6 probe was used.

Exon trapping and DNA sequencing

The exon-trapping procedure was applied to the two genomic clones in λGEM12 vectors, λ7AGW and λ7A4GW, using the Exon Trapping System (Gibco BRL) according to the manufacturer's instruction manual. Briefly, *Bam*HI-digested clones were ligated into the *Bam*HI site in the intron sequence of the HIV *tat* gene present in the pSPL1 splicing vector and transformed *E. coli*. The recombinant plasmid DNA was isolated and transfected COS-7 cells. The cytoplasmic RNA extracted from the COS-7 cells was subjected to reverse transcription followed by polymerase chain reaction (RT–PCR) using primers corresponding to the sequences in the flanking exons in the splicing vector. Putative exon sequences flanked by functional splicing signals, if present, could be detected as PCR products the fragment sizes of which were longer than the size produced from the splicing vector alone. The putative exon sequence was further amplified by PCR using a set of primers whose sequences were located closer to the insert in the splicing vector, and the products were recloned into pAMP10 vector (Gibco BRL). Nucleotide sequences of the inserts in this vector or other DNA fragments subcloned in pBluescript SK$^+$ or pUC119 were determined by the dideoxy chain termination method using the *Taq* Dye Primer Cycle Sequencing Kit (Perkin Elmer Cetus), the AutoSequencer Core Kit (Toyobo) or the Thermo Sequenase Fluorescent-labelled Primer Cycle Sequencing Kit (Amersham) and the model 373A DNA sequencer (Applied Biosystems).

Cloning of EE0.6 homologues of duck and rock dove

Lambda GEM12 genomic libraries of a female duck (*Anas plathynchos domestica*) and a female rock dove (=domestic pigeon) (*Columba livia*) were screened with ^{32}P-labelled ET15 probe, the putative exon sequence in EE0.6, under the same stringency of hybridization as in the Southern blot hybridization with the EE0.6 probe. Three overlapping sequences; 0.2-kb *Eco*RI–*Hin*dIII, 0.4-kb *Eco*RI–*Eco*RI and 0.5-kb *Hin*dIII–*Hin*dIII fragments, in a genomic clone from the duck and one 1.6-kb *Xba*I–*Hin*dIII fragment in a genomic clone from the rock dove were hybridized with the EE0.6 probe, and these fragments were subcloned using pBluescript SK$^+$ or pUC119 as a vector, and their nucleotide sequences were determined.

Conditions of PCR and detection of PCR products

A 50-μl reaction mixture containing 1.5 mM $MgCl_2$, 200 mM each of dATP, dGTP, dTTP and dCTP, 1 mM each of USP1 primer [5′-CTATGCCTACCAC(A/C)TTCCTATTTGC-3′] and USP3 primer [5′-AGCTGGA(T/C)TTCAG(A/T)(C/G)CATCTTCT-3′], 1.5 units Ampli*Taq* DNA polymerase (Perkin Elmer Cetus), 100 ng of high molecular weight genomic DNA and 1 × PCR buffer (Perkin Elmer Cetus) was prepared according to the instruction manual for Ampli*Taq* DNA polymerase. Each reaction mixture in a 500-μl Eppendorf tube

was inserted into a heating block of a PCR Thermal Cycler model TP2000 (TaKaRa Biomedicals) at 95°C and held at 95°C for 3 min before the cycling reactions. Conditions of reactions were: 80 s at 95°C, 90 s at 60°C (for species belonging to the order Galliformes) or 58°C (for species belonging to other orders), and 60 s at 72°C per cycle, and 35 cycles were applied, which was then followed by single cycle reactions at 72°C for 9 min and at 4°C for 5 min. A 20-μl portion of the reaction mixture was subjected to 2% agarose gel electrophoresis and staining with ethidium bromide.

Fluorescence *in situ* hybridization (FISH)

Mitotic metaphase chromosome spreads from female chicken embryonic fibroblasts and lampbrush chromosome spreads from chicken oocytes were prepared for FISH as described in Saitoh & Mizuno (1992) and Solovei *et al*. (1993) respectively. pUGD1201 (*Eco*RI family; Saitoh *et al*. 1991), pUGD0600 (*Xho*I family; Kodama *et al*. 1987) and p7AGW were used as probes. These recombinant plasmids were labelled by nick translation using digoxigenin (DIG)-11-dUTP (Boehringer Mannheim) or biotin-16-dUTP (Boehringer Mannheim) as described (Saitoh & Mizuno, 1992). FISH was carried out as described in Saitoh & Mizuno (1992) except that the concentration of formamide in the reaction mixture was reduced to 45% and slides were washed after the hybridization in three changes of 45% formamide, 2 × SSC at 37°C for 3 min each. Hybrids were detected by a series of reactions with (1) sheep anti-DIG antibody (IgG) (Boehringer Mannheim); and (2) tetramethylrhodamine isothiocyanate (TRITC)-labelled rabbit anti-sheep IgG (EY Laboratory) for the DIG-labelled probe; or with (3) fluorescein isothiocyanate (FITC)-labelled avidin DCS (Vector Laboratories); (4) biotinylated goat anti-avidin antibody (Vector Laboratories); and (5) FITC-labelled avidin DCS for biotinylated probe. When the above two probes were used together, reactions for the detection of hybrids were combined as follows: (1) + (3), (2) + (4), then (5). Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI) and observed under an Olympus BH2-RFL microscope or a Leica DRMB microscope coupled with a CytoVision system (Applied Imaging Corporation).

Results

Cloning and cytological mapping of the 25-kb non-repetitive sequence region on the W chromosome of chicken

From one of the λgt10 genomic libraries prepared from single chicken W chromosome libraries, about 300 phage clones that did not hybridize with the W chromosome-specific *Xho*I and *Eco*RI family repetitive DNA probes were selected randomly. Further selection by the slot-blot hybridization to male and female chicken genomic DNAs with PCR-amplified inserts of individual clones as probes yielded a few clones that hybridized only to the female genomic DNA. One such clone, CW-01, hybridized to a single band only in the digests of female chicken genomic DNA with different restriction enzymes as shown in Figure 1, suggesting that the CW-01 sequence is a non-repetitive sequence on the W chromosome.

As the size of the CW-01 sequence was only about 250 bp, this clone was then used as a probe to screen a λGEM12 total genomic library of a female chicken, and altogether five λ clones covering a 25-kb region were obtained as shown in Figure 2.

Localization of this cloned region on the chicken W chromosome was shown by FISH to the mitotic chromosome set with mixed probes consisting of the biotin-labelled p7AGW, a plasmid clone containing the 15-kb insert of λ7AGW (Figure 2), and DIG-labelled *Xho*I and *Eco*RI family clones. As shown in Figure 3A and its inset, a pair of signals of p7AGW (white, arrowhead) was found in the area containing the repetitive sequence families (pink, arrow) but not in the DAPI-stained repetitive sequence-free end region on the W chromosome. The localization was further examined by FISH to the lampbrush ZW bivalent with the biotin-labelled p7AGW probe (Figure 3B). The location of a pair of signals (yellow, arrowhead) on the PI-counterstained chromosome is interpreted to be in between chromomere 1, containing the major *Eco*RI family, plus chromomere 2 and chromomere 3 containing the *Xho*I family on the non-pairing arm of the W chromosome

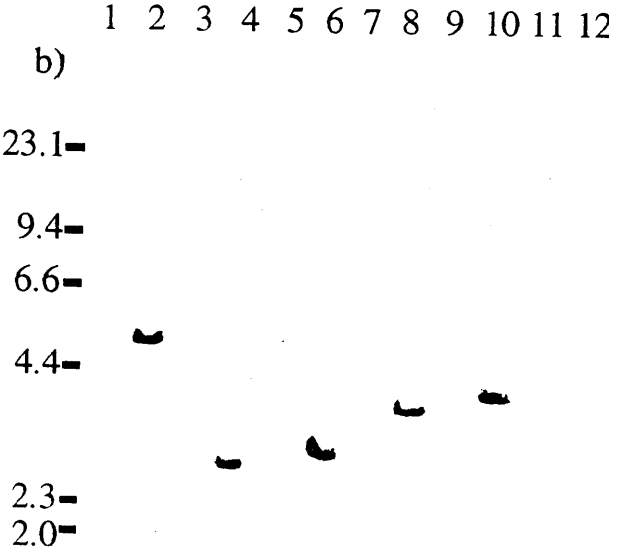

Figure 1. Southern blot hybridization with the CW-01 probe isolated from the chicken W chromosome-specific genomic library. High molecular weight genomic DNA preparations from the male (odd-numbered lanes) or female (even-numbered lanes) chicken digested with *Bgl*II + *Bam*HI (lanes 1 and 2), *Eco*RI (lanes 3 and 4), *Hin*dIII (lanes 5 and 6), *Pst*I (lanes 7 and 8), *Pvu*II (lanes 9 and 10) or *Apa*I + *Hap*II (lanes 11 and 12) were subjected to agarose gel electrophoresis and Southern blot hybridization with the ^{32}P-labelled insert of CW-01. Size markers are λDNA digested with *Hin*dIII.

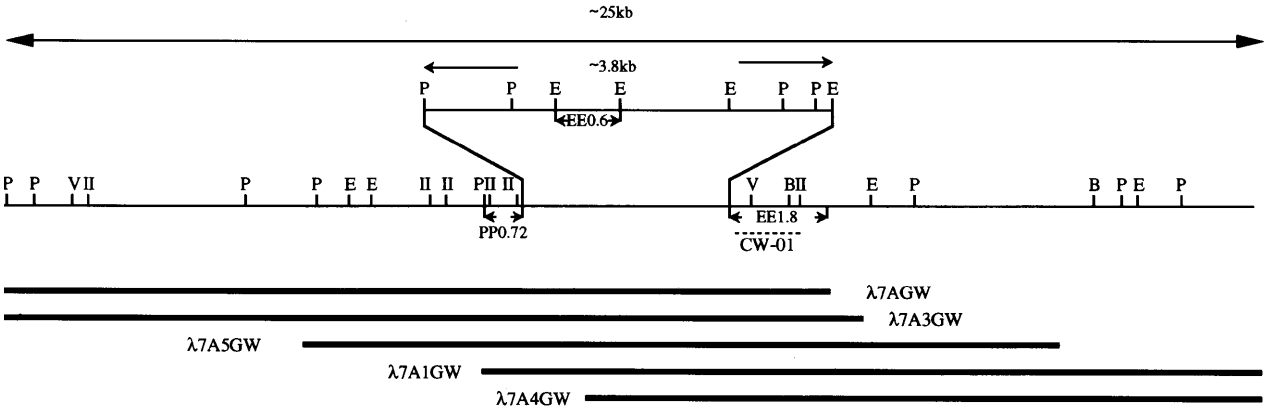

Figure 2. A 25-kb non-repetitive sequence region cloned from the W chromosome of chicken. Recognition sites of several restriction enzymes, positions of five genomic clones obtained from the λGEM12 genomic library of a female chicken, positions of the CW-01 sequence, EE0.6 and other restriction fragments are indicated. The about 3.8-kb region is widely conserved among Carinatae birds. Restriction enzymes are abbreviated as follows: P, *Pst*I; V, *Eco*RV; II, *Bgl*II; E, *Eco*RI; B, *Bam*HI.

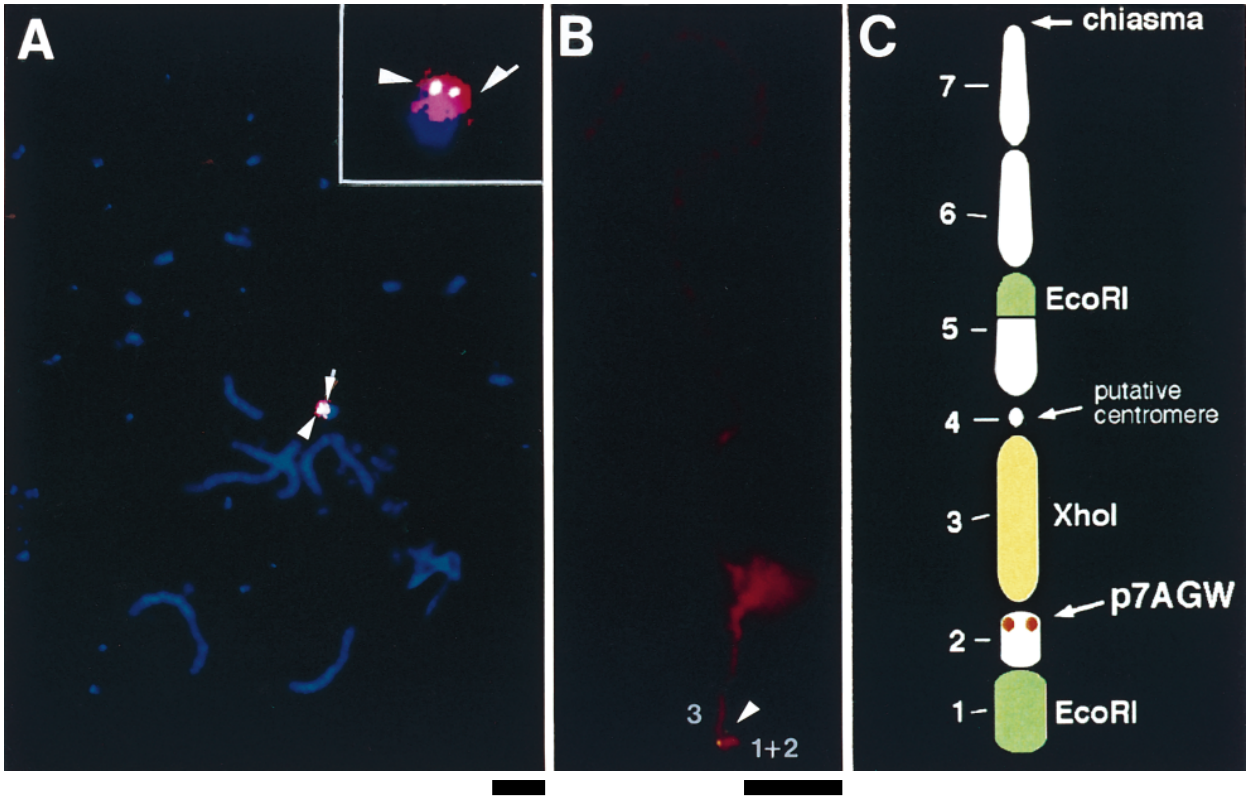

Figure 3. Localization of the p7AGW sequence on the W chromosome of chicken by FISH. **A** A mitotic metaphase set from a female chicken embryonic fibroblast was hybridized with DIG-labelled pUGD1201 (*Eco*RI family), DIG-labelled pUGD0600 (*Xho*I family) and biotinylated p7AGW. Hybridization of the DIG-labelled probes was detected with TRITC fluorescence (pink, arrow) and that of the biotinylated probe was detected with FITC fluorescence (white, arrowhead). Chromosomes were counterstained with DAPI. The inset shows an enlarged W chromosome. Bar = 10 μm. **B** A lampbrush ZW bivalent from the oocyte of chicken was hybridized with biotinylated p7AGW and detected with FITC fluorescence (yellow, arrowhead). The chromosomes were counterstained with PI. Bar = 10 μm. **C** A schematic illustration of the chicken lampbrush W chromosome, indicating chromomeres (numbered) and distributions of *Xho*I and *Eco*RI family and p7AGW sequences. Chromomeres are numbered according to Solovei *et al.* (1993).

(Solovei *et al*. 1993), or at the centromere-proximal edge of chromomere 2 as illustrated in Figure 3C.

A subregion, EE0.6, is widely conserved on the W chromosome of Carinatae birds

When several restriction fragments derived from the clones covering the 25-kb region on the W chromosome (Figure 2) were hybridized to a panel of male and female genomic DNA restriction fragments from a variety of avian species, EE1.8, which contains the original CW-01 sequence, and PP0.72 did not hybridize with genomic DNAs from the species outside the order Galliformes. Fragments within the 3.8-kb PstI–*Eco*RI region flanked by PP0.72 and EE1.8 hybridized with genomic DNAs from a wide variety of birds (data not shown). Among them, the 0.6-kb *Eco*RI fragment (EE0.6) was particularly notable in that it hybridized to a single major female-specific band in the hybridization to *Hin*dIII-digested genomic DNAs from 18 species representing eight different orders (Figure 4). In some species, it also hybridized to a band (or bands) common to both sexes. The intensities of such bands from the male and female are about 2:1 (Figure 4), suggesting that the EE0.6-related sequences are also present on the Z chromosome in some species.

When putative exon sequences were searched for in the 25-kb genomic region cloned in λ7AGW and λ7A4GW (Figure 2) by applying the exon-trapping procedure (Buckler *et al*. 1991), a few candidate sequences were obtained only from the 3.8-kb *Pst*I–*Eco*RI region and the flanking region containing EE1.8. The EE0.6 sequence contains one putative exon (Figure 5) that was obtained from both λ7AGW and λ7A4GW. Sequences having significant similarity to that of the putative exon in EE0.6 have not been identified by homology search against the GSDB, DDBJ, EMBL and NCBI DNA databases.

Although the putative exon in EE0.6 is flanked by functional splicing signals, its protein coding function seems to have been lost, because each one of its reading frames contains at least one stop codon.

The levels of similarity in nucleotide sequences between chicken EE0.6 and its homologues from duck and rock dove (Figure 5) are 82% and 78% respectively. The high level of conservation of EE0.6 sequence

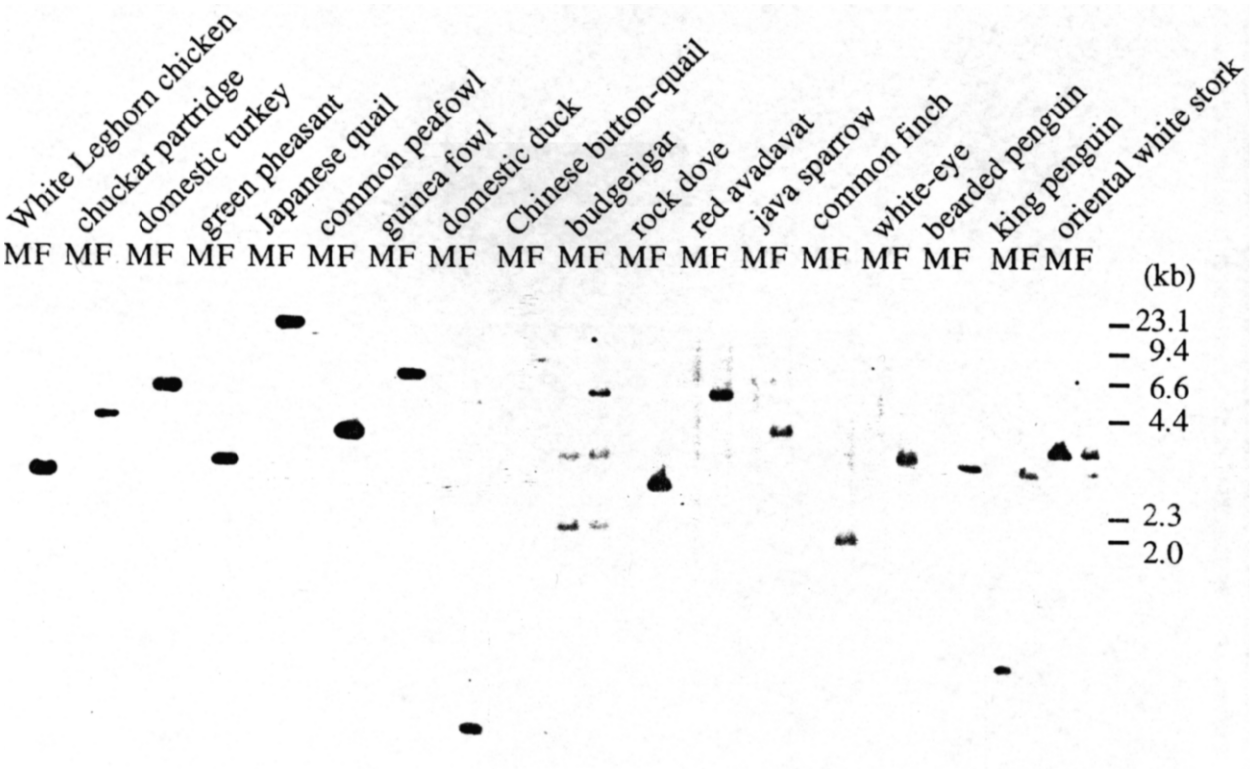

Figure 4. Detection of female-specific bands by Southern blot hybridization with ^{32}P-labelled EE0.6 probe. *Hin*dIII-digested genomic DNA samples (10 μg/lane) were from female (F) and male (M) species, as indicated, belonging to eight different orders: Galliformes (White Leghorn chicken, chukar partridge, domestic turkey, green pheasant, Japanese quail, common peafowl, guinea fowl); Anseriformes (domestic duck); Gruiformes (Chinese button-quail); Psittaciformes (budgerigar); Columbiformes (rock dove); Passeriformes (red avadavat, java sparrow, common finch, white-eye); Sphenisciformes (bearded penguin, king penguin) and Ciconiiformes (oriental white stork). DNA size markers are as in Figure 1.

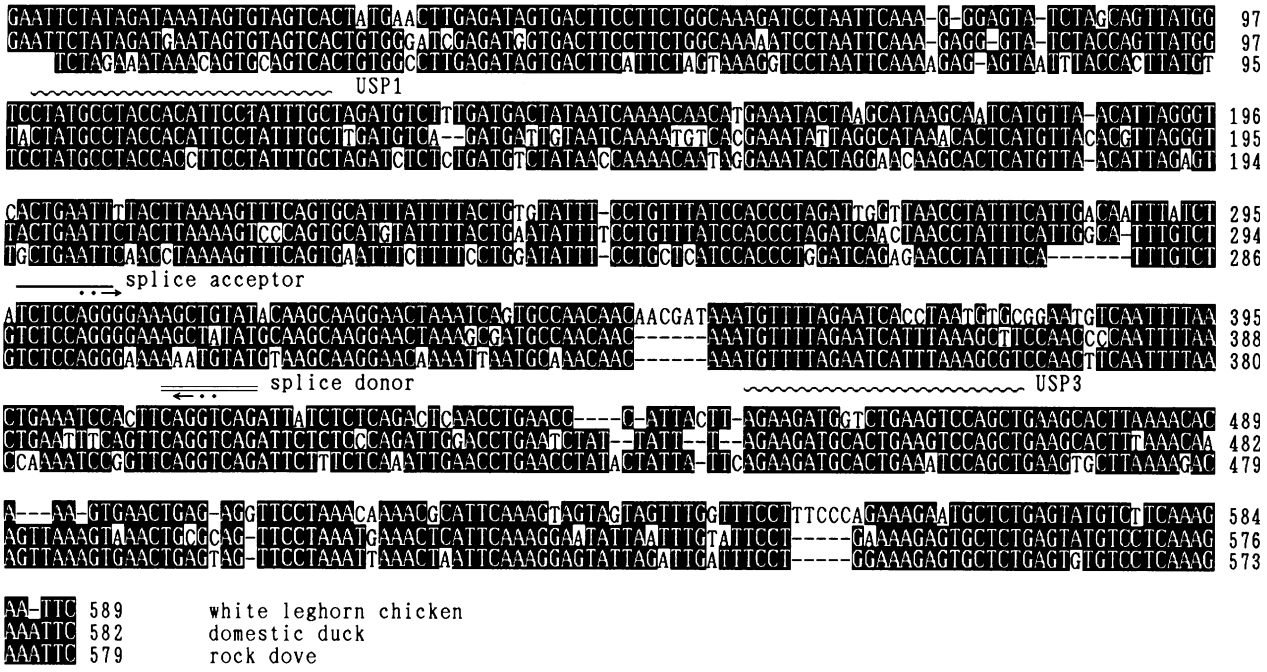

Figure 5. Comparison of nucleotide sequences of chicken EE0.6 and its homologues from the female duck and female rock dove. Identical nucleotides between two species or among three species are indicated with white letters in black boxes. Bars are included to align sequences. Numbers on the right indicate nucleotide positions. Putative acceptor and donor signals for pre-mRNA splicing (Shapiro & Senapathy 1987) are indicated with overline and double overline, respectively, with dots on AG and GT. The putative exon between two arrows is designated ET15 (clone no. 15 obtained by exon trapping). A set of PCR primers, USP1 (forward) and USP3 (reverse), are indicated with wavy overlines. These sequences will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers: D85614 for chicken, D85615 for domestic duck and D85616 for rock dove.

among the three species belonging to different orders is consistent with the presence of EE0.6-related sequences in the wide variety of avian species as demonstrated by Southern blot hybridization (Figure 4).

PCR detection of the female-specific EE0.6-related sequences in the wide variety of avian species

Although EE0.6 was demonstrated to be useful as a sexing probe for a wide variety of avian species when used in Southern blot hybridization with the *Hin*dIII-digested genomic DNA preparations (Figure 4), it would be more convenient if a set of primers were selected from its sequence to produce a female-specific PCR product from a genomic DNA preparation of a given species. After preliminary trials, a combination of primers, USP1 (forward) and USP3 (reverse), which span the putative exon as shown in Figure 5, were adopted.

A female-specific PCR product of about 380 bp was produced for each of the seven species belonging to the order Galliformes (Figure 6, lanes 1–14). Similarly, about a 380-bp female-specific band was produced for duck (lane 16), rock dove (lane 20), red avadavat (*Estrilda amandava*) (lane 22) and common finch (*Lonchura striata*) (lane 26) belonging to the orders Anseriformes, Columbiformes and Passeriformes respectively. Two bands, one specific to the female and one common to both sexes, were formed in java sparrow (*Padda oryzivora*, order Passeriformes) (lanes 23 and 24). In budgerigar (*Melopsittacus undulatus*, order Psittaciformes) and king penguin (*Aptenodytes patagonica*, order Sphenisciformes), one common band that was much more intense for the female was produced (lanes 17 vs. 18 and lanes 27 vs. 28). These results indicate that the set of primers adopted is useful for unambiguous sexing by PCR in a wide variety of avian species with some exceptions.

Discussion

Non-repetitive DNA sequences specific to the avian W chromosome

It has been shown by C-banding that W chromosomes are largely heterochromatic in a wide variety of avian species (Raman *et al.* 1978, Kaul & Ansari 1978, Ansari *et al.* 1986). In chicken, about 65% of the DNA in the W chromosome consists of *Xho*I and *Eco*RI family repetitive sequences and these families form heterochro-

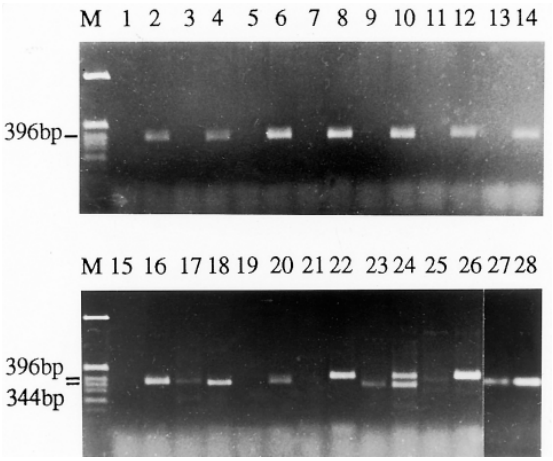

Figure 6. Amplification of EE0.6-related sequences by PCR from the genomic DNAs of species belonging to different orders of Carinatae birds. Genomic DNA samples from males (odd-numbered lanes) and females (even-numbered lanes); White Leghorn chicken (lanes 1 and 2), chukar partridge (lanes 3 and 4), domestic turkey (lanes 5 and 6), green pheasant (lanes 7 and 8), Japanese quail (lanes 9 and 10), common peafowl (lanes 11 and 12), guinea fowl (lanes 13 and 14), domestic duck (lanes 15 and 16), budgerigar (lanes 17 and 18), rock dove (lanes 19 and 20), red avadavat (lanes 21 and 22), java sparrow (lanes 23 and 24), common finch (lanes 25 and 26) and king penguin (lanes 27 and 28), were subjected to PCR using a set of primers, USP1 and USP3 (Figure 5), agarose gel electrophoresis and ethidium bromide staining as described in Materials and methods. Size markers (M) are *Hin*fI-digested pBR322 DNA. Only the sizes of markers close to the amplified band are indicated on the left.

matin (Saitoh & Mizuno 1992, Suka *et al*. 1993). There is a large terminal region in the chicken W chromosome where neither of the repetitive families are distributed (Saitoh & Mizuno 1992) and where the W chromosome pairs with the non-heterochromatic end of the Z chromosome during pachytene and diplotene of female meiosis (Solovei *et al*. 1993, Solari & Dresser 1995, Hori *et al*. 1996). On the analogy of the mammalian Y chromosome (Simmler *et al*. 1985, Sinclair *et al*. 1990), this repetitive family-free terminal region of the chicken W chromosome may contain pseudoautosomal genes and a female sex-determining gene. No genes, however, have yet been assigned to this region.

In the search for the avian W chromosome-specific non-repetitive sequences, four different approaches have been employed: (1) search for the female sex-linked polymorphic microsatellite, minisatellite or other restriction fragment length polymorphism (Quinn & White 1987, Longmire *et al*. 1991, Rabenold *et al*. 1991, T. Burke, personal communication); (2) differential hybridization of clones from the embryonic cDNA library to the male and female genomic DNAs (Dvorak *et al*. 1992, Halverson & Dvorak 1993); (3) detection of female-specific products after a low-stringency PCR with single or a set of primers against sets of male and female genomic DNAs (Griffiths & Tiwari 1993, 1995); and (4) selection of clones from the W chromosome-specific genomic library and application of the exon-trapping procedure (this study). So far, a definitively W chromosome-linked, functional gene has not been identified, except for a candidate, CHD-W, a homologue of mammalian gene encoding a chromodomain-helicase-DNA-binding protein, cloned from the great tit (*Parus major*) using the above approach (3) (Griffiths *et al*. 1996).

The EE0.6 sequence found in the 3.8-kb conserved region contains a putative exon that was obtained by the exon-trapping procedure. Although sequences that have significant similarities to this putative exon have not been identified by searching nucleotide sequence databases, we have noticed features of the Southern blotting patterns that suggest that similar sequences to the 3.8-kb region, including EE0.6, are present on the Z chromosome. The present results show that:

1. the EE0.6 probe gives signals only on the W chromosome by FISH in chicken;
2. Southern blotting with the EE0.6 probe shows two different patterns among the species tested; a single female-specific band or a single female-specific major band plus weaker bands common to both sexes having about 2:1 intensity ratio for male to female;
3. different profiles of amplification are noted among different species by PCR using a set of primers derived from the EE0.6 sequence: a single female-specific band, a single major female-specific band plus other band(s) common to both sexes, and a single band common to both sexes in which the intensity is higher for the female.

These results suggest that EE0.6-related sequences were present in the W/Z chromosomes or on a pair of autosomes, as predicted by Ohno (1967), at an early point in avian evolution. Since then, these sequences have changed independently in the W and Z chromosomes during the evolution of the Carinatae birds. The location of the EE0.6 sequence within the present-day heterochromatic arm of the W chromosome in chicken suggests that its genetic function has been lost during the evolution by the appearance and accumulation of W chromosome-specific repetitive families, which probably enabled the sequence to acquire further chromosome-specific changes. This notion is consistent with the fact that each of the reading frames in the putative exon in EE0.6 contains at least one stop codon. In this context, it would be of interest to see whether the EE0.6 counterpart on the Z chromosome shows any gene function. We have observed that EE0.6-related sequences are present in the genomic DNA of Ratitae birds (ostrich and emu) (unpublished observations), but whether these sequences are located on the sex chromosome is unknown because these species do not possess unambiguously distinguishable sex chromosome pairs.

The genomic clones for the repeating units of *Xho*I and *Eco*RI families, whose sequences are confined to the W chromosome of chicken, have been widely used for the identification of the genetic sex of chicken embryos or cells derived from them by Southern blot (Tone *et al*. 1984), dot blot (Uryu *et al*. 1989), slot blot (Mizuno *et al*. 1993), *in situ* hybridization (Kagami *et al*. 1995) and PCR (Clinton 1994). The related repetitive families confined to the W chromosome of turkey (*Pst*I family) and pheasant (*Taq*I family) have been cloned and used successfully for the identification of the sexes of these birds by Southern blot hybridization (Saitoh *et al*. 1989, Mizuno *et al*. 1993). Although these probes are useful in identifying the genetic sex within the same genus, they are not applicable for species belonging to other genera.

The ZOV3 (an immunoglobulin superfamily protein) and IREBP (iron-responsive element-binding protein) genes on the chicken Z chromosome are widely conserved on the avian Z chromosomes (Saitoh *et al*. 1993) and may be used for the identification of the sexes of a wide variety of birds. Although Southern blot hybridization with the latter probe successfully identified the genetic sex of zebra finch (*Taeniopygia castanotis*) (Wade & Arnold 1996), the definite assessment of the 2:1 (male–female) ratio of the intensities of Southern blot signals is not always easy.

Griffiths & Tiwari (1995) described a highly conserved W-linked gene (C-W) that could be identified in chicken by applying the low-stringency PCR using randomly selected primers, and they further isolated its homologue from the genomic library of hyacinth macaw (*Andorhynchus hyacinthinus*). They showed that the sex of Spix's macaw (*Cyanopsitta spixii*) could be identified by PCR against male and female genomic DNAs using primers derived from the sequence of hyacinth macaw followed by digestion with *Dde*I. The latter procedure was necessary to distinguish the PCR product from the closely related gene (C-2) located on the other chromosome. A similar method was applied to distinguish PCR products from the W and non-W sequence using a set of primers from the great tit CHD gene (Griffith *et al*. 1996).

The EE0.6 sequence is a reliable and widely applicable probe for the identification of the sex of a wide variety of Carinatae species when used in Southern blot hybridization. Because of the presence of an evolutionarily independently changed but still related sequence on the Z chromosome, identification of the sex by PCR using a set of primers from the chicken EE0.6 has not always been possible among a wide variety of avian species. One such example, oriental white stork (*Ciconia boyciana*), produced PCR products of the same size and about the same intensity between male and female when USP1 and USP3 primers were used. We cloned the EE0.6-related sequences from the male and female oriental white stork, sequenced and selected proper sequences as primers. When such primers were used in PCR, only the female-specific amplification product was produced (Y. Ito *et al*., in preparation). We consider that EE0.6-related sequences are potentially universal probes for the identification of the female sex. Sequence level studies can be carried out to select a suitable set of primers, if the simple application of the chicken primers proves unsuccessful for the particular species under study.

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