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Wpkci, Encoding an Altered Form of *PKCI*, Is Conserved Widely on the Avian W Chromosome and Expressed in Early Female Embryos: Implication of Its Role in Female Sex Determination

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Two W chromosome–linked cDNA clones, p5fm2 and p5fm3, were obtained from a subtracted (female minus male) cDNA library prepared from a mixture of undifferentiated gonads and mesonephroi of male or female 5-d (stages 26–28) chicken embryos. These two clones were demonstrated to be derived from the mRNA encoding an altered form of PKC inhibitor/interacting protein (PKCI), and its gene was named *Wpkci*. The *Wpkci* gene reiterated ~40 times tandemly and located at the nonheterochromatic end of the chicken W chromosome. The W linkage and the moderate reiteration of *Wpkci* were conserved widely in Carinatae birds. The chicken *PKCI* gene, *chPKCI*, was shown to be a single-copy gene located near the centromere on the long arm of the Z chromosome. Deduced amino acid sequences of Wpkci and chPKCI showed ~65% identity. In the deduced sequence of Wpkci, the HIT motif, which is essential for PKCI function, was absent, but the α-helix region, which was conserved among the PKCI family, and a unique Leu- and Arg-rich region, were present. Transcripts from both *Wpkci* and *chPKCI* genes were present at significantly higher levels in 3- to 6-d (stages 20–29) embryos. These transcripts were detected in several embryonic tissues, including undifferentiated left and right gonads. When the green fluorescent protein–fused form of Wpkci was expressed in male chicken embryonic fibroblast, it was located almost exclusively in the nucleus. A model is presented suggesting that *Wpkci* may be involved in triggering the differentiation of ovary by interfering with PKCI function or by exhibiting its unique function in the nuclei of early female embryos.

INTRODUCTION

The constitution of sex chromosomes in vertebrates is either XX (female)/XY (male), as in mammals, or ZW (female)/ZZ (male), as in birds and some reptiles. The gene *SRY/Sry*, which triggers male sex determination, is present on the Y chromosome of mammals (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990), but its molecular function is still not fully understood. On the other hand, a gene triggering female sex determination has not been identified on the vertebrate W chromosome. Genes for a chromo-helicase-DNA–binding protein (*CHD1-W*) (Ellegren, 1996) and for the α-subunit of ATP synthase (*ATP5A1W*) (Fridolfsson *et al.*, 1998) were shown to be located on the avian W chromosome, but their expression into functional proteins has not been demonstrated. Furthermore, these two genes on the W chromosome have their counterparts on the Z chromosome (Griffiths and Korn, 1997; Fridolfsson *et al.*, 1998); thus, their unique functions in female sex determination are rather unlikely. Recently, conserved synteny for more than 10 genes was noticed between the chicken Z chromosome and human chromosome 9 (Nanda *et al.*, 1999). Among those conserved genes, *DMRT1* at 9p23.3-p24.1 on human chromosome 9 seemed to be particularly interesting, because this region has been shown to be deleted frequently in human XY sex reversal (Bennett *et al.*, 1993; Veitia *et al.*, 1997; Raymond *et al.*, 1998). The *DMRT1* gene has also been implicated in male sex determination in birds, because if the dosage compensation mechanism does not operate on genes on the Z chromosome in birds, a double dosage of *DMRT1* in the male versus a single dosage in the female may lead to male determination

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(Nanda *et al.*, 1999). In fact, the *DMRT1* mRNA was detected in the left and right genital ridges of chicken embryos of both sexes at stages 25–31 by in situ hybridization. The level of expression was higher in the male embryo, probably reflecting its gene dosage (Raymond *et al.*, 1999). It has been suggested that high *DMRT1* expression is necessary for testicular differentiation, whereas lower expression is compatible with ovarian differentiation in birds (Smith *et al.*, 1999). However, the molecular function of DMRT1 in birds has not been elucidated; thus, it is still uncertain if the lower-level expression of *DMRT1* in the early embryo can trigger the cascade of gene expression toward female sex differentiation.

In the present study, we prepared a subtracted (female minus male) cDNA library from the pooled tissues of undifferentiated gonads and mesonephroi of 5-d male or female chicken embryos (corresponding to stages 26–28) (Hamburger and Hamilton, 1951), which is the stage before the onset of gonadal differentiation (Romanoff, 1960). Of ~200 clones examined in this library, two clones, p5fm2 and p5fm3, were shown to be derived from the mRNA encoding an altered form of PKC inhibitor/interacting protein (PKCI) (Brzoska *et al.*, 1995; Lima *et al.*, 1996). The gene was named *Wpkci*, because it was located at the nonheterochromatic end region of the chicken W chromosome and the cDNA sequence suggested that it encoded a functionally altered form of PKCI. Molecular and cytological characterization of the *Wpkci* gene revealed that it reiterated ~40 times tandemly on the chicken W chromosome. The chicken *PKCI* gene (*chPKCI*) was found as a single-copy gene on the Z chromosome. The *Wpkci* gene was demonstrated to be conserved widely on the avian W chromosome and was expressed actively in the female chicken embryo before the onset of gonadal differentiation, at about the same time when the *PKCI* gene was expressed. When the green fluorescent protein (GFP)-fused form of Wpkci was expressed in male chicken embryonic fibroblasts, it was localized in the nuclei. A model is presented in which Wpkci may play a role in the differentiation of the female gonad by interfering with the function of PKCI or by exhibiting its unique function in the nucleus.

MATERIALS AND METHODS

DNA Preparations

High-molecular-weight DNAs were prepared according to Ogawa *et al.* (1997) from blood samples of the following male and female species of birds: ostrich and emu belonging to the order Struthioniformes of Ratitae, and 24 species belonging to the following 12 orders of Carinatae: Galliformes (white leghorn chicken, red jungle fowl, chukar partridge, green pheasant, common turkey, Japanese quail, Guinea fowl, common peafowl), Anseriformes (domestic duck), Psittaciformes (scarlet macaw), Musophagiformes (violet plantain-eater), Strigiformes (snowy owl), Columbiformes (rock dove), Gruiformes (Japanese crane), Falconiformes (secretary bird), Ciconiformes (Oriental white stork), Sphenisciformes (king penguin, bearded penguin, Humboldt penguin, macaroni penguin), Procellariiformes (streaked shearwater), and Passeriformes (common finch, white Java sparrow, Rothschild's starling).

Identification of the Sexes of Chicken Embryos

Fertilized eggs of white leghorn chickens were incubated at 38°C. The sexes of 2- to 7-d embryos (stages 12–31) were determined by Southern blot hybridization with the W chromosome–specific, 300-base pair (bp) EE0.6 genomic probe (Ogawa *et al.*, 1997) and the Z chromosome–specific *ZOV3* probe (a 2-kilobase [kb] *Hin*dIII–*Sac*I fragment of the cDNA clone) (Saitoh *et al.*, 1993; Kunita *et al.*, 1997) to the *Eco*RI-digested genomic DNA prepared from the extraembryonic membrane of an individual embryo. The sexes of later-stage embryos were identified by the morphology of gonads.

cDNA Cloning of Female-specific Transcripts by the PCR-Select cDNA Subtraction Method

Total RNA was prepared with Trizol reagent (GIBCO-BRL, Gaithersburg, MD) from a mixture of undifferentiated gonads and mesonephroi isolated from 37 male and 37 female embryos at about d 5 of incubation (stages 26–28), and poly(A)$^+$ RNA was obtained with the use of Oligotex-dT30 "Super" (Roche Diagnostics, Mannheim, Germany). The following procedure was adopted essentially according to the PCR-Select cDNA Subtraction Kit user manual (Clontech, Palo Alto, CA). Double-stranded and blunt-ended cDNA was synthesized, digested with *Rsa*I, and ligated with *Rsa*I adaptors (Gurskaya *et al.*, 1996). The adaptor-ligated cDNA was amplified by PCR and redigested with *Rsa*I. The *Rsa*I-digested cDNA from the female (tester) was ligated with either adaptor-1 or adaptor-2R. The first hybridization was performed between the excess of *Rsa*I-digested cDNA from the male (driver) and each one of the adaptor-ligated tester cDNAs. The two reaction mixtures were then mixed together, and the second hybridization was performed by adding freshly denatured driver cDNA. The hybrids between the adaptor-1–ligated and the adaptor-2R–ligated tester cDNAs were amplified by PCR, digested with *Rsa*I, and cloned into the *Sma*I site of pBluescriptII KS(+) (Stratagene, La Jolla, CA).

The subtracted cDNA library was screened by reverse Northern blot hybridization (von Stein *et al.*, 1997). Two hundred individual colonies were picked up, and each cDNA insert was amplified by PCR with the use of M13(-20) primer, M13 reverse primer (Stratagene), and *Taq* polymerase (Takara, Tokyo, Japan). PCR products were subjected twice to agarose gel electrophoresis, capillary transferred in 0.4 M NaOH onto Biodyne B membranes (Pall Specialty Materials, Port Washington, NY), hybridized with the driver or the tester cDNA, which was labeled with [α-^{32}P]dCTP by the random priming method (Feinberg and Vogelstein, 1983), in CG buffer (0.5 M Na-phosphate, pH 7.2, 1 mM EDTA, 7% SDS) (Church and Gilbert, 1984) at 65°C for 12 h. Twenty-nine clones that gave the tester (female)-specific signals were selected and further analyzed by Southern blot and Northern blot hybridization. Two clones, p5fm2 and p5fm3, exhibited female-specific patterns of hybridization in Southern blotting and female-specific expression patterns in Northern blotting for RNA preparations from 3- to 6-d (stages 20–29) chicken embryos.

Isolation of cDNA Clones for Wpkci and PKCI from Chicken, Quail, and Duck

Poly(A)$^+$ RNAs were prepared from the mixed tissues of undifferentiated gonads and mesonephroi of female chicken embryos at stages 26–28 and from the sex-undetermined 3-d whole embryos of Japanese quail and domestic duck. The double-stranded and blunt-ended cDNA was synthesized as described above, except that the first-strand cDNA was synthesized with the use of pd(T)12-18 (Amersham Pharmacia Biotech, Uppsala, Sweden) as a primer. The cDNA was ligated with *Eco*RI–*Not*I–*Bam*HI adaptor (Takara), and its 5′ ends were phosphorylated and size-selected for 0.3- to 2-kb fragments by 1.2% agarose gel electrophoresis. The cDNA library was constructed by ligating the cDNA fragments with Lambda ZAPII/*Eco*RI, CIAP (Stratagene), followed by in vitro packaging with the use of Max Plax Packaging Extract (Epicentre Technologies, Madison, WI). The chicken cDNA library was screened with the insert of p5fm2, which had been ^{32}P-labeled by the random priming method described above, and pWpkci-7, pWpkci-8, and pfst5.2-5

(carrying a sequence region containing exon III of *Wpkci*) clones were obtained. The N-terminal 214-bp cDNA fragments of human and mouse PKCI were obtained by reverse transcription PCR from the total RNAs of HeLa cells and murine C127 cells, respectively, with the use of the following primers: 5′-TGGC(A/T)GA(T/C)GAGATTGCCAAGG-3′ (forward) and 5′-CTTTCATCATCATC(T/A)TCTGC-3′ (reverse). This chicken cDNA library was screened with a mixture of the PCR-amplified human and mouse PKCI cDNA probes, which were ^{32}P-labeled as described above, and the pchPKCI-3 clone was obtained. The Japanese quail and the domestic duck cDNA libraries were screened with the insert of pWpkci-8 or the *chPKCI* cDNA fragment (nucleotide positions 30–410) as a ^{32}P-labeled probe, and pquWpkci-16, pquPKCI-2, pduWpkci-20, and pduPKCI-8 clones were obtained.

Construction and Screening of a Bacterial Artificial Chromosome Library from Female Chicken Genomic DNA

Cultured fibroblasts established from an 8-d female embryo of white leghorn chicken were suspended in PBS (8.1 mM NaH_2PO_4, 1.47 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl) at a concentration of 1×10^8 cells/ml. The cell suspension and an equal volume of 1.2% solution of SeaPlaque GTG agarose (FMC Bioproducts, Rockland, ME) in PBS were incubated at 42°C, mixed, and poured into a sample mold of GeneLine System (Beckman, Fullerton, CA) and solidified at 4°C. The gel plug was soaked successively in ES solution (0.5 M EDTA, 1% *N*-lauroylsarcosine Na salt) at room temperature for 30 min, twice in ESP solution (1 mg/ml proteinase K in ES solution) at 50°C for 24 h with gentle shaking, and five times in TE50 (10 mM Tris-HCl, pH 8, 50 mM EDTA) at room temperature for 30 min. The extremely high-molecular-weight genomic DNA in the gel plug was digested partially with *Hin*dIII and subjected to pulsed-field gel electrophoresis in CHEF mapper (Bio-Rad, Richmond, CA), and DNA fragments of 150–200 kb were recovered. These DNA fragments were ligated with pBAC-Lac vector (Asakawa *et al.*, 1997), which had been digested with *Hin*dIII and treated with alkaline phosphatase (Roche Diagnostics), and subjected to electroporation into *Escherichia coli* DH10B electrocompetent cells (Hanahan *et al.*, 1991) by Gene Pulser (Bio-Rad) according to Asakawa *et al.* (1997). Individual recombinant clones were picked up and transferred into individual wells of 96-well microtiter plates, each well containing 150 μl of LB (1% bacto-tryptone, 0.5% bacto-yeast extract, 1% NaCl) containing 7.5% glycerol and 12.5 μg/ml chloramphenicol. Altogether, 512 plates containing 4.9×10^4 clones were made, and those plates were incubated at 37°C for 12 h. High-density replica filters (eight 22 × 7 cm filters per set) were prepared from these plates with the use of Bio-Grid (BioRobotics, Cambridge, UK), spotting 6144 clones per filter. The mean insert size of these clones was ~150 kb. The whole bacterial artificial chromosome (BAC) library contained ~3.2 times as much chicken genomic DNA as the diploid genome equivalent, assuming that the diploid genome corresponded to ~2300 megabase (Mizuno *et al.*, 1978).

Two sets of filters (16 high-density replica filters) were hybridized with the ^{32}P-labeled insert of p5fm2 or p5fm3 (*Wpkci* probes), and 10 BAC clones that gave hybridization signals on both filters were picked up. Similarly, three BAC clones were picked up after hybridization with the ^{32}P-labeled insert of pchPKCI-3 as a probe.

DNA Sequencing and Data Analysis

Sequencing was carried out with the use of the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI 377 DNA sequencer. Sequence data were analyzed by DNASIS-Mac version 3.0 (Hitachi Software Engineering, Tokyo, Japan). Deduced amino acid sequences were aligned with the use of Clustal W1.7 (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html), and common residues were shown with the use of Boxshade 3.21 (http://www.isrec.isb-sib.ch:8080/software/BOX_form.html).

Southern Blot Hybridization and Determination of Reiteration Frequencies of the Wpkci and chPKCI Genes

*Eco*RI-digested genomic DNAs from various species of birds were electrophoresed (5 μg/lane) on a 1% agarose gel, transferred to a Biodyne B nylon membrane (Pall Specialty Materials), hybridized with the ^{32}P-labeled StHi-0.37 genomic fragment (exon III probe for *Wpkci*; see Figure 2A) in CG buffer at 63°C for 12 h, and washed in 0.3× SSC, 0.1% SDS at 63°C for 30 min.

The BAC clone 216G1, selected with the p5fm2 cDNA probe for *Wpkci*, was first digested with *Not*I, followed by partial digestion with *Pst*I with the use of six different enzyme concentrations. Each digest was separated by electrophoresis on a 0.4% Seakem Gold agarose (FMC) gel in 0.5× TBE (1× TBE is 0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA), treated with 0.25 M HCl for 10 min, and transferred onto a Biodyne B membrane in 0.4 N NaOH. The blot was hybridized with the ^{32}P-labeled insert of the cDNA clone pfst5.2-5 (for *Wpkci*) in CG buffer at 65°C for 12 h, followed by washing in 0.3× SSC, 0.1% SDS at 65°C for 30 min, and subjected to autoradiography.

The BAC clone 224D8 was digested with *Not*I, followed by partial digestion with *Bam*HI, with the use of eight different enzyme concentrations. Each digest was separated by pulsed-field gel electrophoresis in GeneLine System (Beckman) and subjected to Southern blot hybridization as described above, but with ^{32}P-labeled fragment (nucleotide positions 30–410) of the *chPKCI-3* cDNA sequence as a probe.

Different amounts (0.25–3.0 μg) of *Eco*RV-digested genomic DNA of female white leghorn chicken and 3.0 μg each of *Eco*RV-digested genomic DNA of male white leghorn chicken mixed with different amounts (10–90 pg) of *Eco*RI-cut p5fm2 DNA (3.83 kb) were electrophoresed on a 1% LO3 agarose (Takara) gel in 0.5× TBE and transferred onto a Biodyne B membrane in 0.4 N NaOH. The DNA blot was hybridized with the ^{32}P-labeled insert of p5fm2 and washed under the same conditions described above. Hybrids were detected and intensities of signals were quantified with a FLA-2000 image analyzer (Fuji Film, Tokyo, Japan). The reiteration frequency of the sequence detected with the p5fm2 probe in the diploid genome was calculated with the use of the following equations: $Ng \times X/Nc = Sg/Sc$, and $X = Nc \times Sg/Ng \times Sc$, where X = reiteration frequency, Ng = genomic DNA (pg) fixed on the membrane/diploid genome size of chicken (2.54 pg), Nc = molecular numbers of p5fm2 clone fixed on the membrane, calculated from the size of the clone (3.83 kb) with 650 as the mean molecular weight of a base pair, Sg = intensity value of the hybridization signal to the genomic DNA, and Sc = intensity value of the hybridization signal to p5fm2 DNA.

A fixed amount (3 μg) of the *Bam*HI-digested genomic DNA from male chicken and different amounts (5–100 pg) of *Hin*dIII-digested DNA of the cDNA clone pchPKCI-3 (3.589 kb) were subjected to Southern blot hybridization as described above, with a ^{32}P-labeled subfragment (nucleotide positions 30–410, generated by PCR) of pchPKCI-3 as a probe. By comparing the former signal intensity with the slope of the latter signal intensities, the reiteration frequency of the *chPKCI* sequence in the haploid male genome was calculated.

Northern Blot Hybridization

Poly(A)$^+$ RNAs were prepared as described above from sex-identified whole embryos: 6 each for 3-d (stage 20), 4 each for 4-d (stage 24), and one each for 5- to 14.5-d (stages 27–40) embryos; from undifferentiated gonads plus mesonephroi of sex-identified 5- to 16-d (stages 27–42) embryos: 37 each for 5-d, 30 each for 6-d, 29 male and 18 female for 7-d, 22 male and 18 female for 8-d, and 3 each for 16-d embryos; and also from different tissues of an 80-d male or female chicken. Poly(A)$^+$ RNA (1 μg each) was electrophoresed on an agarose/formaldehyde gel (Sambrook *et al.*, 1989) and capillary

transferred onto a Biodyne B membrane in 7.5 mM NaOH. The RNA blot was hybridized with a ^{32}P-labeled probe in CG buffer at 65°C for 12 h, followed by washing in 2× SSC, 0.1% SDS at 65°C for 15 min and autoradiography. The 63-bp *Wpkci*-specific probe (nucleotide positions 365–427 in Figure 1A) was prepared by PCR with the use of 5′-ATGGCTGTGAGATACC-3′ (forward) and 5′-ACCCAGAATACAGAATATGG-3′ (reverse) primers, and similarly, the 72-bp *chPKCI*-specific probe (nucleotide positions 312–383 in Figure 1B) was prepared with the use of 5′-CGGATGGTTTTGAATG-3′ (forward) and 5′-ACCTCCCAGAATATGG-3′ (reverse) primers. The mRNA of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was probed with the cloned chicken *GAPDH* cDNA sequence (Dugaiczyk *et al.*, 1983). Intensities of hybridization signals with the *Wpkci* or *chPKCI* probe were divided by the intensities of *GAPDH* signals, and their relative levels were compared through embryonic development.

When the mRNA molar ratio of *Wpkci* and *chPKCI* was determined, different amounts (0.25–2 μg) of poly(A)$^+$ RNA preparations from female 6-d (stage 29) whole embryo or from a mixture of undifferentiated gonads and mesonephroi of female 6-d embryo were electrophoresed and transferred to the membrane as described above. The cDNA clones pWpkci-8 and pchPKCI-3 were linearized by digestion with *Bam*HI, and different amounts (10–200 pg) were electrophoresed on an agarose gel and transferred to the membrane as for the RNA samples. The RNA and the DNA blots were hybridized together with the *Wpkci* or *chPKCI*-specific probe described above, washed as described above, and the radioactivity of the hybrids was converted to the fluorescence intensity values by FLA-2000 image analyzer. The intensity of the Northern blot signal was corrected with the signal intensity for *GAPDH* mRNA and also with the ratio of signal intensity (pWpkci-8/pchPKCI-3) by Southern blot hybridization.

In Situ Hybridization to Mitotic Chromosome Preparations

Fluorescence in situ hybridization (FISH) to mitotic chromosomes prepared from chicken embryonic fibroblasts was carried out as described (Hori *et al.*, 1996). Probes used were pCZTH5–8 (pFN-1–type macrosatellite sequence on the chicken Z chromosome; Hori *et al.*, 1996), pUGD1201 (*Eco*RI family sequence on the chicken W chromosome; Saitoh *et al.*, 1991), pGP-3 (5.6-kb *Pst*I genomic fragment containing a *Wpkci* gene; this study), and 224D8 (BAC clone containing the *chPKCI* gene; this study), which were labeled by nick translation with either digoxigenin (DIG)-11-dUTP (Boehringer Mannheim, Indianapolis, IN) or biotin-16-dUTP (Boehringer Mannheim) according to Saitoh and Mizuno (1992).

In Situ Detection of Wpkci and chPKCI Transcripts in the Whole Mount Preparation or Paraffin Sections of Early Chicken Embryos

For the whole mount hybridization, a part of the 4.5-d (stage 25) embryo containing undifferentiated gonads and mesonephroi was dissected out and fixed in 4% paraformaldehyde in PBT (0.1% Tween 20 in PBS) at 4°C overnight, and the preparation was hybridized with DIG-labeled antisense or sense riboprobe for *Wpkci* or *chPKCI*, as described below, according to Wilkinson and Nieto (1993). For hybridization to paraffin sections, stages 26 to 27 chicken embryos were fixed in 4% paraformaldehyde and 0.2% glutaraldehyde in PBS at 4°C for 8 h and embedded in paraffin. Sections 4 μm thick were cut with a microtome (Sledge Microtome IVS-400, Sakura, Tokyo, Japan) and attached to the surface of 3-aminopropyltriethoxysidane-coated slide glasses. DIG-labeled antisense or sense riboprobe was prepared by transcribing *Xba*I- or *Hin*dIII-digested pBluescriptII KS(+) containing the *Wpkci* cDNA fragment (nucleotide positions 63–534) or the *chPKCI* cDNA fragment (nucleotide positions 30–410) with T3- or T7-RNA polymerase, respectively, with the use of DIG-RNA labeling mix (Boehringer Mannheim). The DIG-labeled hybrids were detected with the use of anti-DIG antibody–coupled alkaline phosphatase (Boehringer Mannheim) and the AP-Conjugate Substrate Kit (Bio-Rad).

Construction and Expression of GFP-fused Forms of Wpkci and chPKCI

The sequence of the Wpkci-encoding region was amplified by PCR with the use of the cDNA clone pWpkci-8 as a template, the following primers: 5′-GAAGATCTATGGCCGGCGGGATCGT-3′ (forward) and 5′-GGCTGCAGCTAGGCTGACGGGCAAC-3′ (reverse), and *Pfu* DNA polymerase (Toyobo, Osaka, Japan). The sequence of the chPKCI-encoding region was amplified as described above but with the use of pchPKCI-3 and the following primers: 5′-GAAGATCTATGGCTGACGAGATCC-3′ (forward) and 5′-TTCTGCAGTTAGCCAGGAGGCCAGCCCA-3′ (reverse). The amplified fragments were digested with *Bgl*II (the site is present in the forward primer) and *Pst*I (the site is present in the reverse primer) and ligated to pS65T-C1 vector (Clontech), which had been digested with *Bgl*II and *Pst*I. The recombinant plasmids and the plasmid vector were purified with the use of the Concert High-Purity Plasmid Purification System (GIBCO-BRL). Fibroblasts established from an 8-d (stage 34) male chicken embryo (2×10^5 cells/24 × 24 mm coverglass) were cultured in DMEM (Sigma Chemical, St. Louis, MO) containing 0.03% L-glutamine, 0.16% Na-bicarbonate, 8% FBS (JRH Biosciences, Lenexa, KS), 2% chicken serum (JRH Biosciences), 50 U/ml penicillin G, and 50 μg/ml streptomycin at 37°C in 5% CO_2/95% air and subjected to lipofection with 1 μg of the recombinant plasmid, or the plasmid vector as a control, and 5 μl of Lipofectin Reagent (GIBCO-BRL), according to the manufacturer's protocol. After incubation in the serum-free medium for 6 h, the medium was changed to one containing sera, and the culture was continued for 12 h at 37°C for cells transfected with *Wpkci*-containing vector or for 24 h for cells transfected with *chPKCI*-containing vector or the control vector. The temperature of each culture was then shifted to 30°C for 8 h. Cells were washed in PBS, fixed in PBS containing 4% paraformaldehyde for 30 min, stained for 1 min with 1 μg/ml DAPI (Sigma) in PBS, covered with Vectashield antifade (Vector Laboratories, Burlingame, CA), and observed under a Leica (Bensheim, Germany) DMRB fluorescence microscope with the Cytovision (Applied Imaging, Santa Clara, CA) image-processing system.

RESULTS

cDNA and Deduced Amino Acid Sequences of Wpkci and chPKCI

Poly(A)$^+$ RNA was prepared from pooled tissues of undifferentiated gonads and mesonephroi of male or female chicken embryos at about d 5 of incubation (stages 26–28), and those RNA preparations were subjected to cDNA synthesis and PCR-Select cDNA subtraction. In this process, the female-derived tester cDNA molecules ligated with one of the two different adaptors were hybridized with the excess male-derived driver cDNA without those adaptors, the cDNA duplexes formed between the two different adaptor-ligated tester cDNA molecules were amplified by PCR with the use of primers complementary to a part of each adaptor sequence, and the PCR products were cloned with the use of pBluescriptII KS(+). Two hundred colonies were picked up randomly, and their inserts were amplified by PCR and subjected to reverse Northern blot hybridization (von Stein *et al.*, 1997) with ^{32}P-labeled PCR-amplified cDNA fragments from male or female embryos that had been used for the PCR-Select cDNA subtraction. Twenty-nine clones were confirmed to be derived from mRNA species expressed in a

female-specific manner. Of these clones, two (5fm2 and 5fm3) were suggested to be derived from genes on the W chromosome because of their female-specific patterns in Southern blot hybridization. A cDNA library, which was prepared from mixed tissues of undifferentiated gonads and mesonephroi of 5-d (stages 26–28) female embryos and size-selected for 0.3- to 2-kb cDNA molecules, was screened with 5fm2 or 5fm3 as a probe. A clone selected with the 5fm2 probe and containing an ~660-bp insert was sequenced (Figure 1A) and designated pWpkci-8, because the 130 amino acid residues deduced from the sequence of its ORF showed 61% identity to PKCI of human (Brzoska *et al.*, 1995; Lima *et al.*, 1996). The 5fm3 clone was later found to be derived from the same gene.

Next, a cDNA clone for the chicken PKCI was obtained from the same cDNA library described above but by probing with the mixed sequences of human and mouse *PKCI* cDNAs (Lima *et al.*, 1996; Klein *et al.*, 1998). This clone, pchPKCI-3, was sequenced (Figure 1B), revealing the presence of an ORF encoding 126 amino acid residues and showing ~87% identity with the deduced sequences of human and mouse PKCI (Lima *et al.*, 1996; Klein *et al.*, 1998).

Molecular mass values of Wpkci and chPKCI, calculated from the deduced amino acid sequences, were almost identical, 13.9 and 13.8 kDa, respectively. On the other hand, the deduced isoelectric point values were significantly different: 10.93 for Wpkci and 6.33 for chPKCI. When the deduced sequences of Wpkci and chPKCI were compared with respect to their different regions (Figure 1C), the HIT motif, a characteristic motif of PKCI containing the conserved triad of histidine residues (Seraphin, 1992; Brenner *et al.*, 1997), was absent in Wpkci, whereas adjacent regions on both sides of the HIT motif were highly conserved between Wpkci and chPKCI. Wpkci contained a Leu- and Arg-rich region, which was not present in chPKCI, next to the N-terminal region.

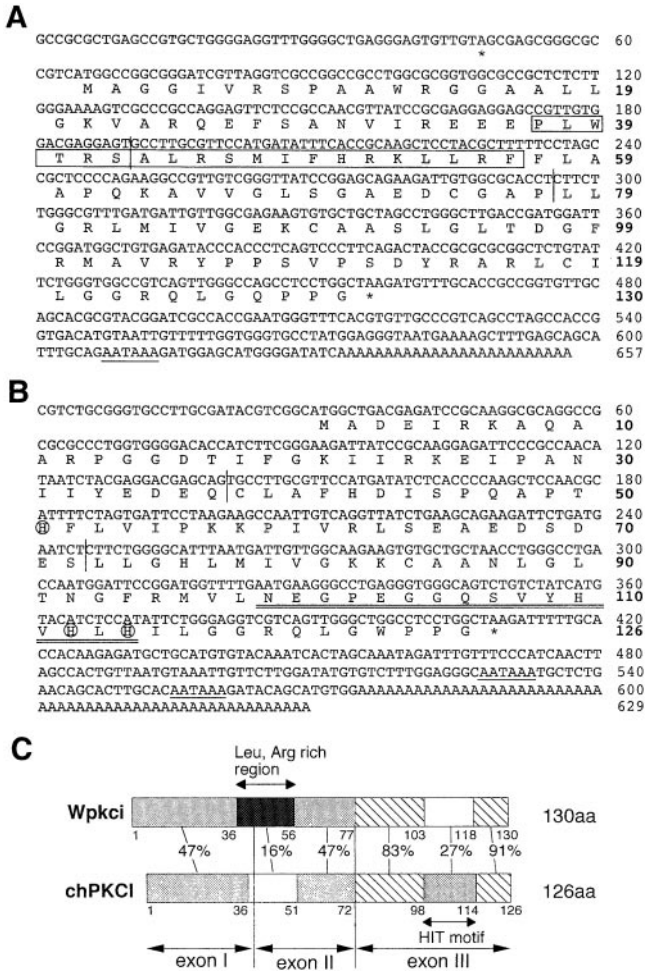

Figure 1. The cDNA and deduced amino acid sequences of Wpkci (A) and chPKCI (B) and comparison of the levels of sequence identity of their protein subregions (C). The cDNA sequences of pWpkci-8 (A) and pchPKCI-3 (B) were determined. The N-terminal Met residue in A was assigned because of its location nearest to the termination codon in the 5′ untranslated region, and that in B was assigned by comparison with the mammalian PKCI sequences. In A and B, termination codons (asterisks), polyadenylation signals (underlines), and boundaries of exons (vertical lines) are indicated. The Leu- and Arg-rich region of Wpkci is boxed in A. The HIT motif containing the conserved His triad (HVHLH) is double-underlined, and the three His residues involved in the binding of zinc are circled in B. In C, the residue numbers for the N terminus, the C terminus, and the last residue of each region, correspondence to exons I–III, and levels (%) of identity of the deduced sequences between corresponding regions of Wpkci and chPKCI are indicated. The sequences of the inserts of pWpkci-8 and pchPKCI-3 are deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with accession numbers AB026677 and AB026675, respectively.

Structure of Wpkci and chPKCI Genes

The BAC genomic library of the female chicken was screened with the 5fm2 or 5fm3 cDNA clone (Figure 2A) as a probe. One clone, 216G1, which was hybridized with both probes, contained tandem repeats of a 5-kb *Bam*HI fragment and a 5.6-kb *Pst*I fragment, both of which contained the *Wpkci* sequence. These two repeating units were then subcloned to yield pGB-1 (5-kb *Bam*HI fragment) and pGP-3 (5.6-kb *Pst*I fragment) (Figure 2A). Sequencing of the pGB-1 and pGP-3 clones revealed that the *Wpkci* gene consisted of three exons and suggested that the gene was reiterated tandemly (Figure 2A).

A BAC clone, 224D8, containing the *chPKCI* gene sequence was obtained by screening the female chicken BAC library with the pchPKCI cDNA clone. Subregions (1.3- and 3.3-kb *Hin*dIII fragments) of this BAC clone, each containing a part of the *chPKCI* sequence, were subcloned (pGH1.3-1 and pGH3.3-3 in Figure 2C). Sequences of these subcloned regions and a part of the 224D8 BAC clone, flanked with these subcloned sequences, were determined, which indicated that the *chPKCI* gene also consisted of three exons (Figure 2C).

Intron-1 of the *chPKCI* gene was 2682 bp long and that of the *Wpkci* gene was 985 bp long, and no significant homology was found between these sequences. On the other hand, intron-2 of the *chPKCI* gene was 762 bp long and that of the *Wpkci* gene was 831 bp long, and their sequence identity was 57%. The relatively high similarity of exon sequences (Figure 1C) and the intron-2 sequence between *chPKCI* and *Wpkci* genes suggested that these two genes had evolved from the same origin.

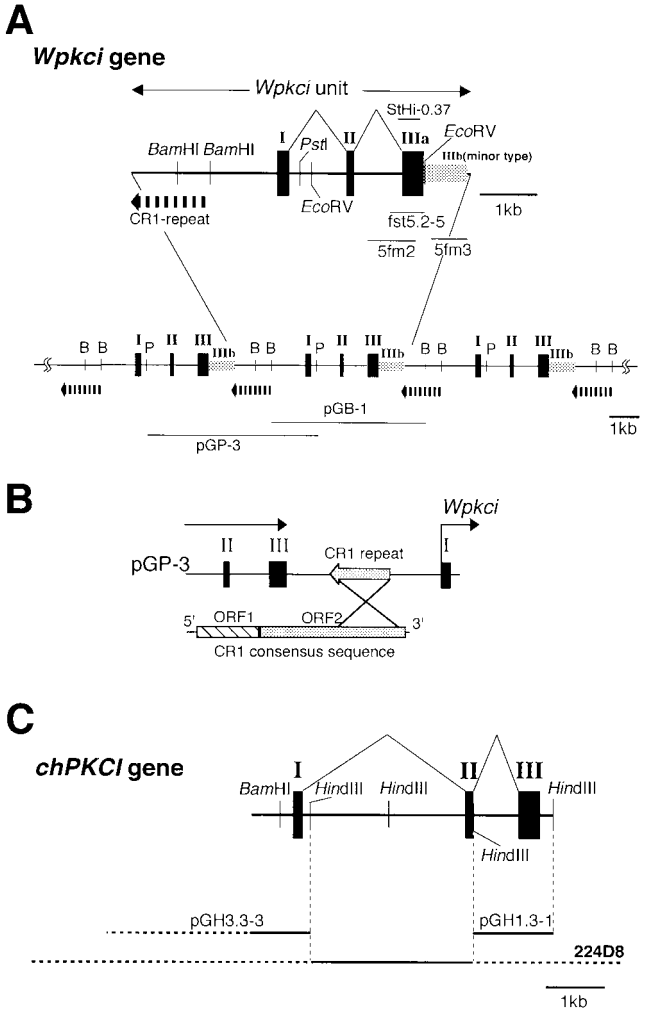

Figure 2. Genomic structure of the tandemly reiterated *Wpkci* (A and B) and the nonreiterated *chPKCI* (C) genes determined by restriction mapping and sequencing of BAC clones (216G1 for *Wpkci* and 224D8 for *chPKCI*) and their subclones. Exons are indicated with closed rectangular boxes. The location of the partial sequence of CR1 between *Wpkci* genes is indicated in A and B. A minor 1.4-kb transcript of *Wpkci* contains an additional region to exon III, indicated as IIIb in A. Positions of cDNA clones (5fm2, 5fm3, and fst5.2-5) and genomic subclones (StHi-0.37, pGP-3, pGB-1, pGH3.3-3, and pGH1.3-1) are indicated. Accession numbers for the DDBJ, EMBL, and GenBank nucleotide sequence databases are AB026678 for pGB-1 and AB026679 for pGP-3.

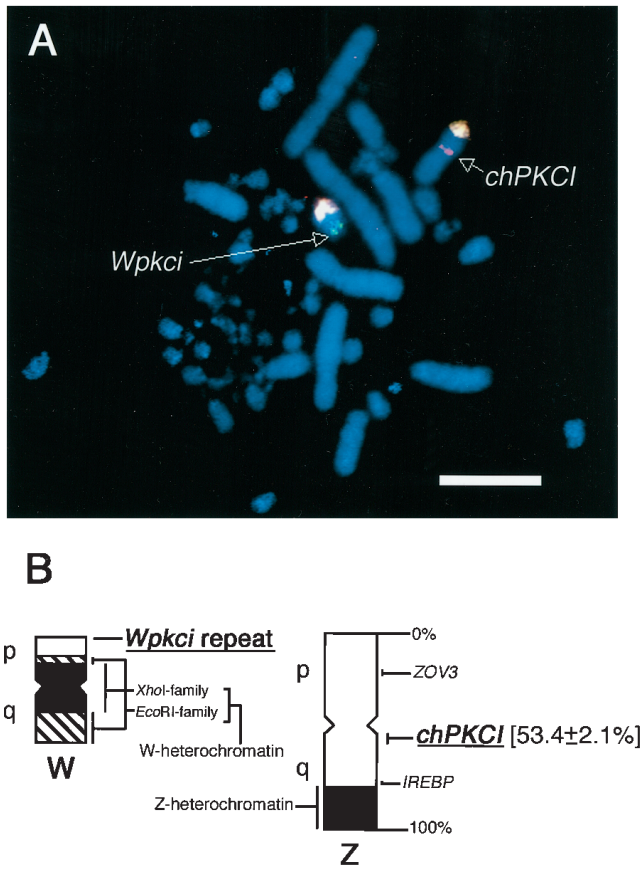

Figure 3. Chromosomal locations of *Wpkci* and *chPKCI* demonstrated by FISH with the mixed probes. (A) A metaphase set from the female chicken embryonic fibroblast was hybridized with biotinylated pGP-3 (for *Wpkci*), DIG-labeled BAC clone 224D8 (for *chPKCI*), a 1:1 mixture of biotinylated and DIG-labeled pUGD1201 (for the *Eco*RI family on the W chromosome), and a 1:1 mixture of biotinylated and DIG-labeled pCZTH5–8 (for the pFN-1 macrosatellite on the Z chromosome). The biotinylated probes were detected with FITC–avidin, and the DIG-labeled probes were detected with sheep anti-DIG Fab fragment followed by rhodamine-labeled anti-sheep Fab. Locations of *Wpkci* on the nonheterochromatic end of the W chromosome and *chPKCI* near the centromere on the long arm of the Z chromosome are illustrated in B. The *chPKCI* locus on the Z chromosome was assigned by measuring 122 FISH figures.

Sequencing of the pGB-1 and pGP-3 genomic clones disclosed that part of the CR1 repeat, corresponding to the 3′ half of ORF2 of the CR1 consensus sequence (Haas *et al.*, 1997), was present between exon III and exon I in the adjacent gene set (Figure 2B), suggesting that one copy of the partial CR1 sequence was present in every boundary sequence between sets of the tandemly reiterated *Wpkci* gene (Figure 2A). The presence of the CR1 sequence might have contributed to the formation of the tandem repeats of the *Wpkci* gene by the mechanism of unequal crossing over.

Chromosomal Locations of Wpkci and chPKCI Genes

Chromosomal locations of *Wpkci* and *chPKCI* genes were examined by FISH to mitotic chromosome sets from the female chicken embryonic fibroblasts with the use of the following probes: the 5.6-kb insert of pGP-3 (Figure 2A) for the *Wpkci* gene and the BAC clone 224D8, which contains the *chPKCI* gene sequence (Figure 2C). W and Z chromosomes were identified by cohybridization with the chromosome-specific repetitive sequence probes: pUGD1201 for the *Eco*RI

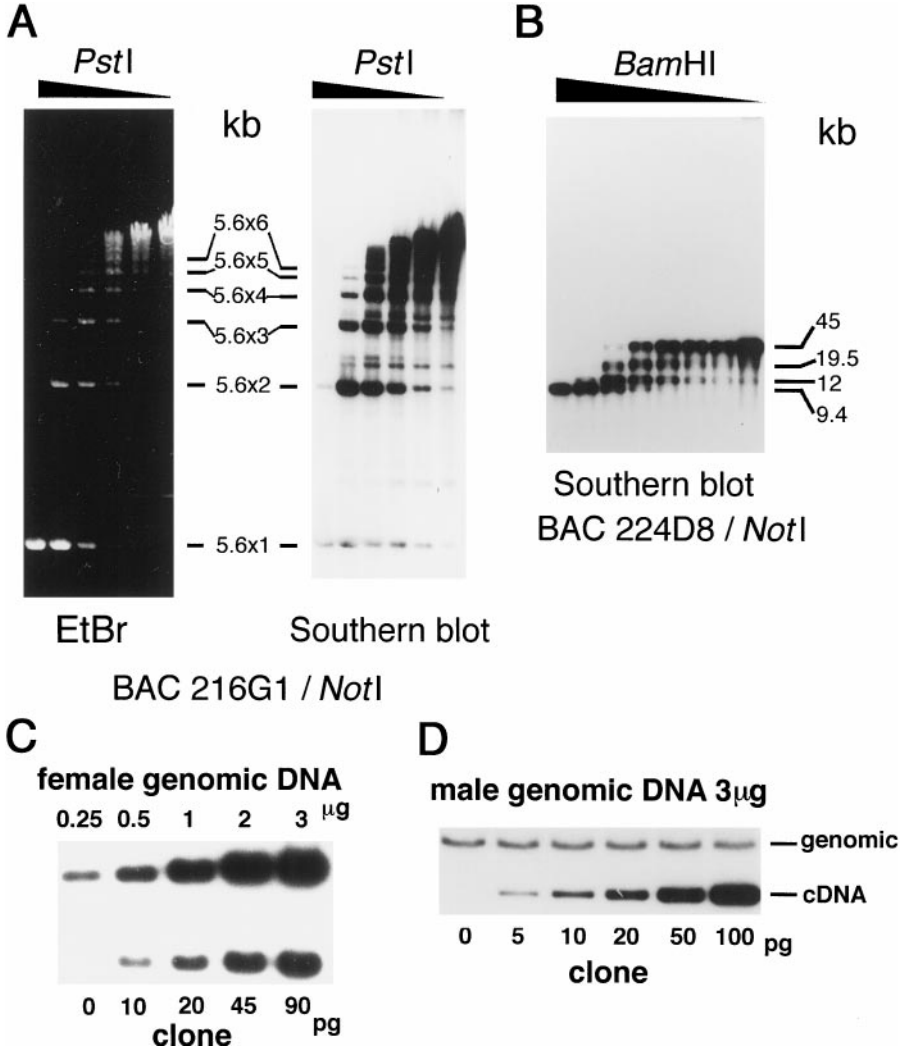

Figure 4. Determination of reiteration frequencies of *Wpkci* and *chPKCI* in the chicken genome. (A) The 140-kb insert containing *Wpkci* genes was obtained from the BAC clone 216G1 by digestion with *Not*I and further digested with *Pst*I at six different concentrations. The digests were separated by agarose gel electrophoresis, stained with ethidium bromide (left panel), and subjected to Southern blot hybridization with the ^{32}P-labeled insert of the cDNA clone fst5.2-5 (right panel; see Figure 2A), which showed signals corresponding to multiples of the 5.6-kb repeating unit. (B) The insert of BAC clone 224D8 containing the *chPKCI* gene sequence was obtained by *Not*I digestion, further digested with *Bam*HI at eight different concentrations, and subjected to Southern blot hybridization with the ^{32}P-labeled cDNA fragment (nucleotide positions 30–410) of *chPKCI*. (C) Different amounts of *Eco*RV-digested genomic DNA of the female chicken (upper panel) and *Eco*RV-digested genomic DNA of the male chicken (3 μg each) mixed with different amounts of the linearized cDNA clone p5fm2 (3.83 kb) (lower panel; see also Figure 2A) were subjected to agarose gel electrophoresis and Southern blot hybridization with the ^{32}P-labeled insert of p5fm2. Comparing the slopes of signal intensities for the former and the latter samples, 1 μg of the female genomic DNA and 72 pg of p5fm2 gave the same signal intensity. (D) The *Bam*HI-digested genomic DNA of the male chicken (3 μg each) and different amounts of the *Hin*dIII-digested, linearized cDNA clone pchPKCI-3 were subjected to electrophoresis and Southern blot hybridization with the ^{32}P-labeled subfragment (nucleotide positions 30–410) of pchPKCI-3. Comparing the mean signal intensity of the former and the slope of signal intensities of the latter samples, 3 μg of the male genomic DNA and 12 pg of pchPKCI gave the same signal intensity.

family sequence on the long arm of the chicken W chromosome (Saitoh *et al.*, 1991; Saitoh and Mizuno, 1992) and pCZTH5-8 for the pFN-1 macrosatellite sequence at the terminal region of the long arm of the chicken Z chromosome (Hori *et al.*, 1996). The results are shown in Figure 3A and summarized in Figure 3B. The reiterated *Wpkci* genes were located close to the terminus of the nonheterochromatic end on the short arm of the W chromosome, and the *chPKCI* gene was located near the centromere on the long arm of the Z chromosome, i.e., at 53.4% of the entire length of the Z chromosome, measured from the end of the short arm.

Reiteration Frequencies of Wpkci and chPKCI Genes

Tandem reiteration of the *Wpkci* gene, as suggested from genomic structural analysis (Figure 2A), was confirmed by Southern blot hybridization. The 140-kb insert of the BAC clone 216G1 was digested partially with *Pst*I and electrophoresed, and the blot was hybridized with the 0.58-kb insert of the cDNA clone pfst5.2-5, which had been derived from the pre-mRNA and consisted of sequences of a part of intron-2 and exon III of *Wpkci* (Figure 2A) as a probe. The probe hybridized to bands corresponding to multiples of the 5.6-kb *Pst*I fragment (Figure 4A). When the BAC clone 224D8, which contains the *chPKCI* sequence, was digested with *Not*I, further digested with *Bam*HI at eight different concentrations, and subjected to pulsed-field gel electrophoresis and Southern blot hybridization with the ^{32}P-labeled *chPKCI* cDNA fragment (nucleotide positions 30–410; Figure 1B), 9.4-kb and three higher-molecular-mass bands were detected, but the sizes of the latter bands were not multiples of 9.4 kb (Figure 4B).

To estimate the reiteration frequency of the *Wpkci* gene, Southern blot hybridization was carried out with the ^{32}P-labeled insert of the p5fm2 cDNA clone, which had been derived from the pre-mRNA (Figure 2A), as a probe to different amounts (0.25–3 μg) of *Eco*RV-digested genomic DNA of the female chicken (Figure 4C, upper panel) and to a fixed amount (3 μg) of *Eco*RV-digested genomic DNA of the male chicken mixed with different amounts (10–90 pg) of

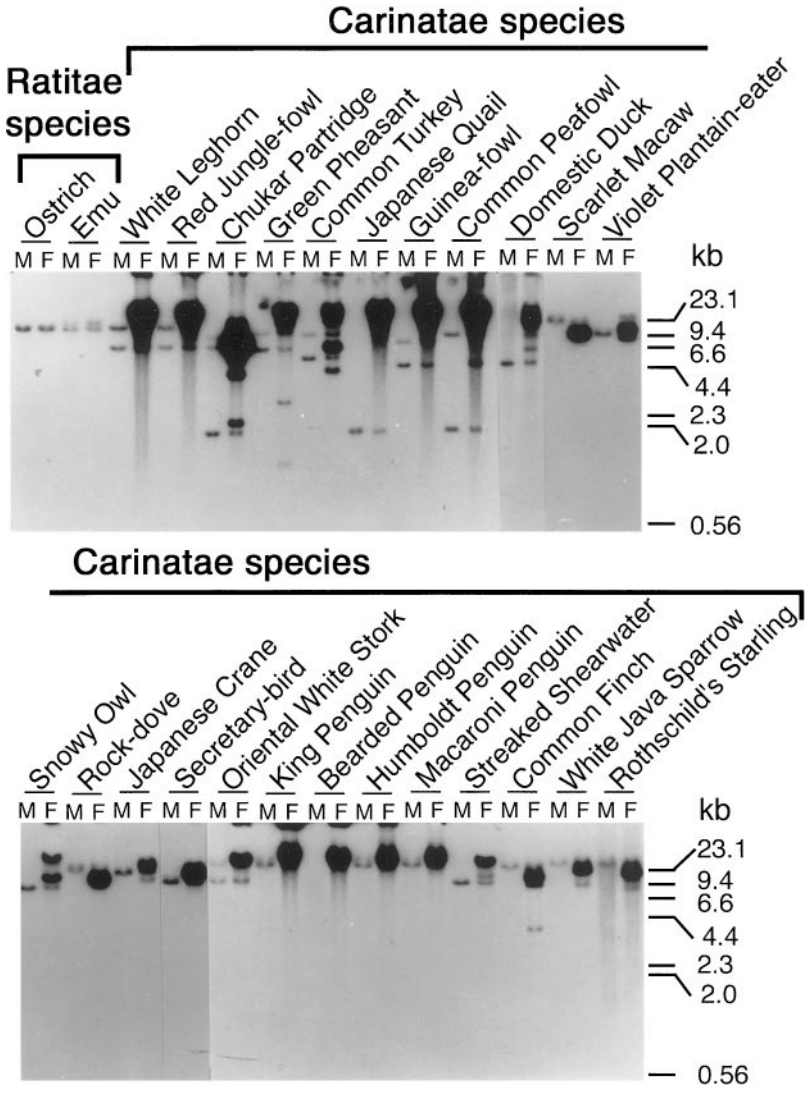

Figure 5. Presence of the reiterated *Wpkci* gene in the female genomes of Carinatae birds. *Eco*RI-digested genomic DNA preparations from male (M) and female (F) species of birds as indicated were subjected to agarose gel electrophoresis and Southern blot hybridization with the ^{32}P-labeled genomic fragment StHi-0.37 (see Figure 2A).

the linearized p5fm2 DNA (Figure 4C, lower panel). Hybridization signals were quantified by the image analyzer, which converted levels of radioactivity to fluorescence intensity values. The reiteration frequency of the *Wpkci* gene was calculated to be 44 times per diploid genome of the female, or 44 copies on the W chromosome, by comparing slopes of fluorescence intensity values obtained from the two sets of reactions described above.

To estimate the number of *chPKCI* genes per genome, a fixed amount (3 μg) of the *Bam*HI-digested genomic DNA of the male chicken and different amounts (5–100 pg) of the *Hin*dIII-digested pchPKCI-3 DNA were subjected to Southern blot hybridization with the ^{32}P-labeled subfragment (nucleotide positions 30–410) of pchPKCI-3 (Figure 4D). By comparing the intensity of hybridization to the genomic DNA with the slope of signal intensities to the cloned DNA, the copy number of *chPKCI* gene per haploid male genome was calculated to be 1.3. These results indicated that a single copy of the *chPKCI* gene existed on the Z chromosome.

Conservation of the Wpkci Gene in the Female Genomes of Carinatae Birds

Southern blot hybridization was carried out with the cDNA probe for the exon III region (StHi-0.37; Figure 2A) of the *Wpkci* gene, under stringent conditions, to the *Eco*RI-digested genomic DNA preparations from 26 pairs of male and female species belonging to 12 different orders of Carinatae birds and 1 order of Ratitae birds (ostrich and emu). The results demonstrated that the *Wpkci* gene was present and reiterated in all of the genomes of the female species of Carinatae birds tested (Figure 5). The extent of reiteration seemed to be similar among the species belonging to the order Galliformes (chicken, red jungle fowl, chukar partridge, green pheasant, common turkey, Japanese quail, Guinea fowl, common peafowl) but somewhat less so in the species belonging to other orders (Figure 5). The band(s) hybridized in each sample from the male was interpreted to represent the sequence of *chPKCI* gene on the Z chromosome, because overall similarity of the exon III se-

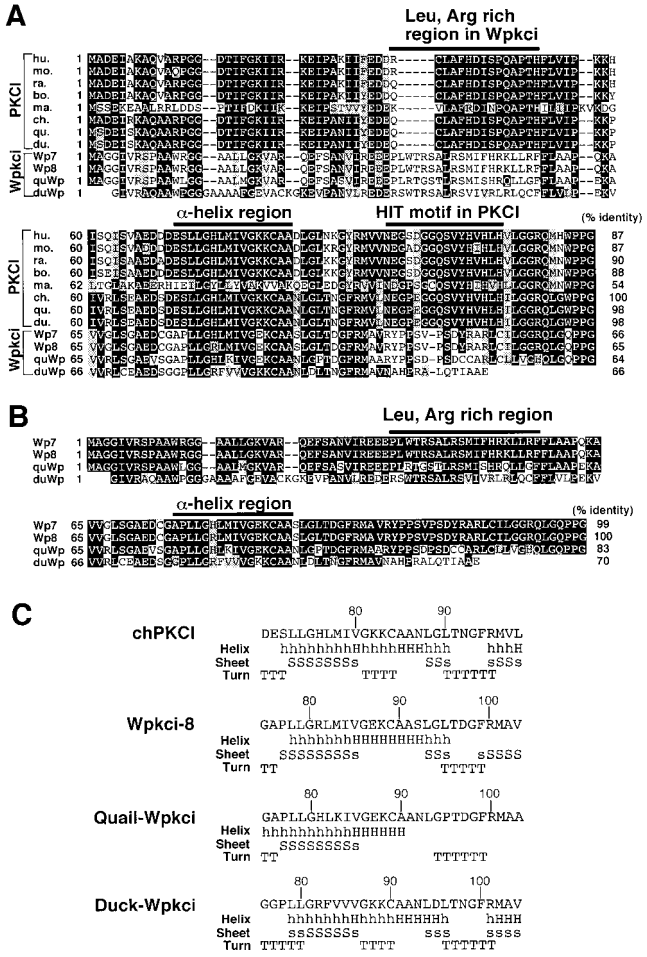

Figure 6. Comparison of deduced amino acid sequences of mammalian, plant, and avian PKCI and avian Wpkci, and prediction of the α-helix for the fourth region of avian PKCI and Wpkci. (A) Deduced sequences of PKCI from human (hu.), mouse (mo.), rabbit (ra.), bovine (bo.), maize (ma.), chicken (ch.), Japanese quail (qu.), and domestic duck (du.) and of Wpkci from chicken (Wp7 and Wp8), Japanese quail, and domestic duck, abbreviated as above, were aligned, and three characteristic regions or a motif are indicated. Identical residues are shown with white letters on a black background and similar residues are shown on a stippled background. The overall level (%) of identity relative to the sequence of chicken PKCI is indicated at the end of each deduced sequence. Only a single residue is different between Wpkci-7 (Wp7) (H-81, minor type) and Wpkci-8 (Wp8) (R-81, major type) of chicken. (B) Alignment and comparison of the deduced sequences of Wpkci from chicken (Wp7 and Wp8), Japanese quail (qu), and domestic duck (du). Wpkci of domestic duck lacks the last 14 residues. (C) Prediction of α-helix (H or h) formation for the fourth region (see Figure 1C) of chPKCI and Wpkci of chicken (Wpkci-8), Japanese quail, and domestic duck. The cDNA sequences for Japanese quail PKCI (AB033882), domestic duck PKCI (AB033884), Wpkci-7 (AB033880), Japanese quail Wpkci (AB033881), and domestic duck Wpkci (AB033883) are deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession numbers shown in parentheses.

quence between *Wpkci* and *chPKCI* was ~73%. The reiteration of *Wpkci*-related sequence in the female genome was not observed for the two Ratitae species; thus, we were unable to conclude from these results if the *Wpkci* gene was present in the genomes of these species.

Comparison of Deduced Amino Acid Sequences of PKCI from Mammals, Birds, and a Plant with Those of Wpkci from Birds

The deduced sequences of PKCI from human (GenBank accession number [GB] U51004), mouse (GB U60001), rabbit (GB Y11175), bovine (GB U09405), maize (GB Z29643), chicken, quail, and duck (this study) and of Wpkci from chicken, quail, and duck (this study) were aligned and compared (Figure 6, A and B). For the chicken Wpkci, sequences deduced from two different cDNA clones were compared in which only a single amino acid change was noticed: His-81 (Wpkci-7; minor type) or Arg-81 (Wpkci-8; major type). The deduced sequence of chicken PKCI was almost identical (98% identity) to the sequences of quail and duck and was highly similar (87–90% identity) to those of mammals. On the other hand, the deduced sequences of Wpkci of birds and those of PKCI were much less similar, except for the α-helix region and the C-terminal region (Figures 1C and 6A), although the duck PKCI lacked the C-terminal 14 residues (Figure 6, A and B).

The highly conserved α-helix region has been suggested to be the site of intermolecular contact in the formation of a homodimer of PKCI (Lima *et al.*, 1996). When the deduced sequences of this region of Wpkci for chicken, quail, and duck, and the corresponding region of chPKCI, were analyzed for the secondary structure by the method of Chou and Fasman (1974), all of these sequences were predicted to form α-helices (Figure 6C).

Transcriptional Expression of Both Wpkci and chPKCI Genes Is Higher during Early Stages of Embryonic Development

The levels of expression of *Wpkci* and *chPKCI* genes were examined by Northern blot hybridization to poly(A)$^+$ RNA preparations from whole embryos at 3 to 14.5 d of incubation (stages 20–40) or from different tissues of 80-d chickens with the use of the PCR-generated probe for a part of the exon III sequence of each gene, which detected *Wpkci* mRNA or *chPKCI* mRNA specifically. The results shown in Figure 7, A (*Wpkci* mRNA) and B (*chPKCI* mRNA), demonstrate that both genes were expressed as ~0.65-kb mRNAs and that the levels of both mRNAs were higher during the 3- to 6-d stages (stages 20–29) than in later stages of embryonic development or in different tissues of 80-d chickens. The higher-level expression of both *Wpkci* and *chPKCI* genes in stage 20–29 embryos was shown more clearly when the ratios of *Wpkci* mRNA, or *chPKCI* mRNA, to *GAPDH* mRNA were plotted as in Figure 7D (a and b).

The higher-level expression of both *Wpkci* and *chPKCI* genes in the 5- to 6-d (stages 27–29) embryos was also demonstrated for the poly(A)$^+$ RNA preparations from mixed tissues of undifferentiated gonads plus mesonephroi (Figure 7, C and D, c and d). It was of interest to note that both *Wpkci* and *chPKCI* genes were expressed at higher levels during stages 20–29 of embryonic develop-

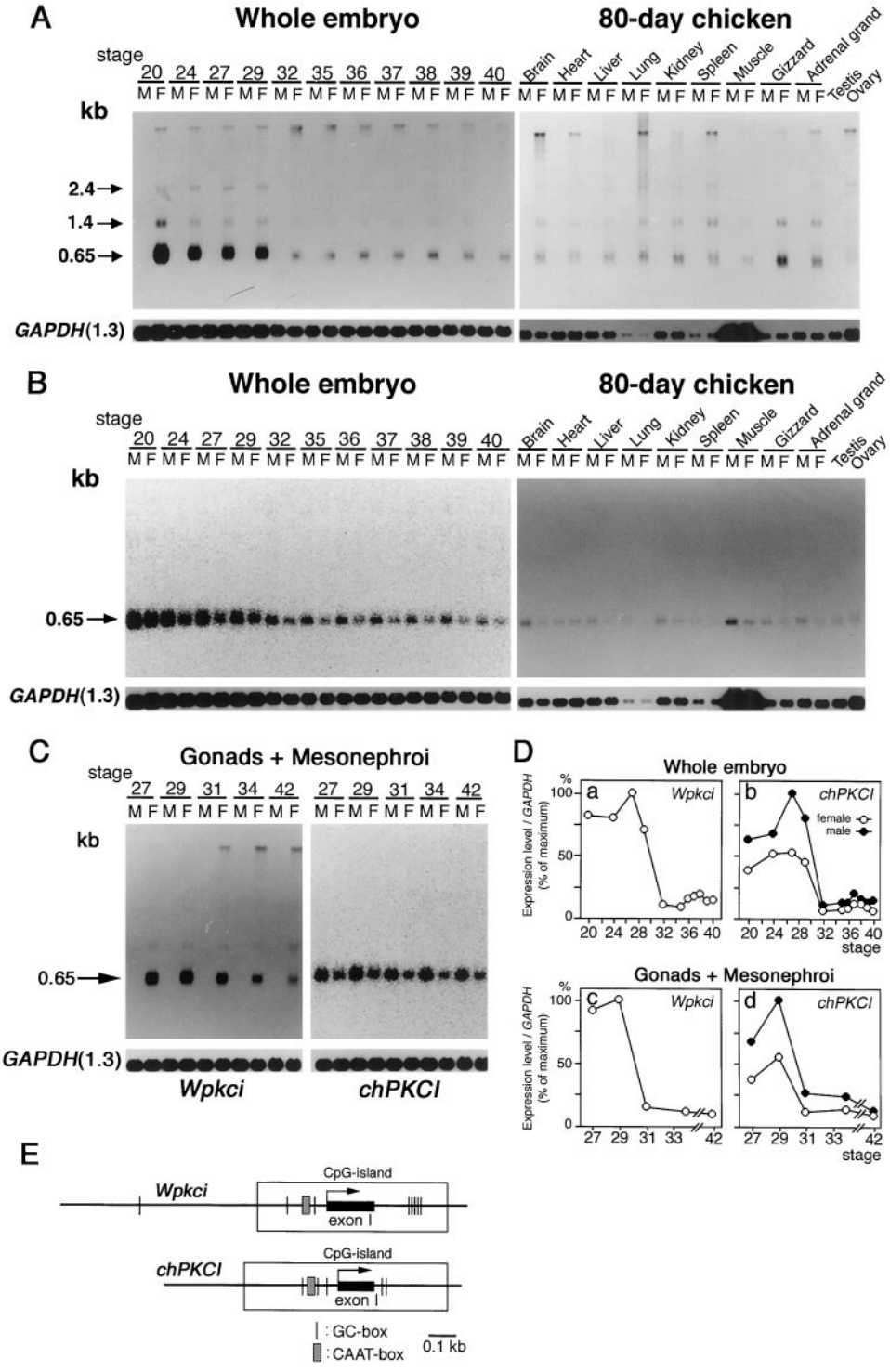

Figure 7. Transcripts of both *Wpkci* and *chPKCI* genes are present at higher levels in early embryonic stages. (A and B) Poly(A)$^+$ RNA preparations from 3- to 14.5-d (stages 20–40) male (M) and female (F) chicken whole embryos or from different tissues of 80-d chickens, as indicated, were subjected to Northern blot hybridization with the ^{32}P-labeled cDNA probe specific for *Wpkci* (63-bp probe) (A) or *chPKCI* (72-bp probe) (B) and with ^{32}P-labeled *GAPDH* cDNA probe (A and B), followed by autoradiography (A and the right panel of B for the 80-d chicken) or fluorescence image analysis (the left panel of B for the whole embryo). (C) Poly(A)$^+$ RNA preparations from undifferentiated gonads plus mesonephroi of 5- to 16-d (stages 27–42) male (M) or female (F) chicken embryos, as indicated, were subjected to Northern blot hybridization with the ^{32}P-labeled *Wpkci*-specific, *chPKCI*-specific, or *GAPDH* cDNA probe, followed by autoradiography (the left panel for *Wpkci*) or fluorescence image analysis (the right panel for *chPKCI*). (D) Relative values of the signal intensity of *Wpkci* mRNA or *chPKCI* mRNA to *GAPDH* mRNA, determined from the same Northern blot analyses, were plotted for female (○) and male (●) embryos at different stages. (E) Some common transcriptional elements around exon I of *Wpkci* and *chPKCI* predicted from the genomic sequences determined for pGP-3 and pGH3.3-3 (see Figure 2).

ment, which were before the onset of gonadal differentiation in the female embryo; a thickening of the germinal epithelium in the left gonad was seen after 7 d of incubation (stage 31 or later) (Romanoff, 1960). As expected from the linkage of *Wpkci* to the female-specific W chromosome but that of *chPKCI* to the male/female common Z chromosome, the expression of the *Wpkci* gene was limited to the female (Figure 7, A and C). The expression of the *chPKCI* gene took place in both sexes, but the intensity of hybridization was ~2:1 (male [ZZ]:female [ZW]) (Figure

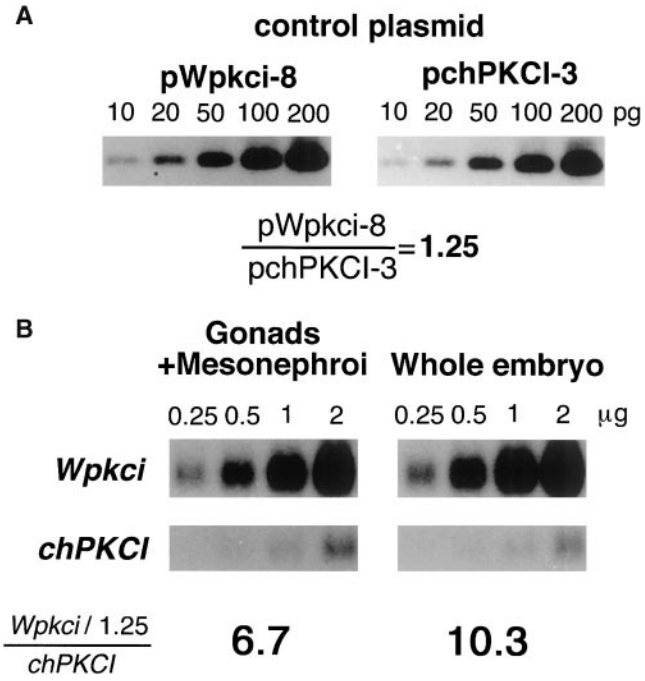

Figure 8. Determination of the molar ratio of mRNAs for *Wpkci* and *chPKCI*. Different amounts of the linearized cDNA clone, pWpkci-8 or pchPKCI-3 (A), or different amounts of poly(A)$^+$ RNA from gonads plus mesonephroi of 6-d (stage 29) female embryos or 6-d female whole embryos (B) were electrophoresed, blotted onto nylon membranes, and hybridized together with ^{32}P-labeled cDNA probe specific for *Wpkci*, *chPKCI*, or *GAPDH*, respectively, and subjected to fluorescence image analysis. The ratio of signal intensity in A was obtained by comparing slopes of signal intensity for each cDNA clone. In B, the signal intensity for *Wpkci* mRNA or *chPKCI* mRNA was first corrected with that for *GAPDH* mRNA (the intensity of *Wpkci* mRNA was multiplied by 1.003 for the sample from whole embryo and by 1.38 for the sample from gonads plus mesonephroi), the slope of signal intensity for *Wpkci* mRNA was corrected by the ratio obtained in A, and the molar ratio of mRNAs for *Wpkci* and *chPKCI* was calculated.

7, B and C). The coordinate expression of *Wpkci* and *chPKCI* genes may be related to the common features of the 5′ upstream sequences of both genes, i.e., the absence of a TATA box but the presence of a CAAT box and GC boxes (Figure 7E), but the mechanisms of the higher-level expression during the early embryonic stages remain to be elucidated.

In the Northern blot patterns for the transcripts of the *Wpkci* gene (Figure 7, A and C), transcripts of higher molecular mass (1.4 kb, ~2.4 kb, and a band at the top of the gel) were detected as minor components. The 1.4- and 2.4-kb components were likely mRNA species having longer 3′ untranslated regions, because the downstream cDNA probe, 5fm3 (Figure 2A), hybridized to the 1.4- and 2.4-kb bands but not to the 0.65-kb band, and sequencing of the 3′ untranslated region of the 1.4-kb component suggested that an alternative polyadenylation signal in the downstream region was used.

The Abundance of Wpkci Transcripts Exceeds That of chPKCI Transcripts in Early Embryos

It was suggested that the *Wpkci* mRNA was more abundant than the *chPKCI* mRNA in the early-stage embryos, because the intensity of the 0.65-kb bands shown in Figure 7A was obtained after 24 h of exposure of the x-ray film but the intensity of the bands shown in Figure 7B was obtained after 8 h of exposure of the imaging plate for the bioimage analyzer, which was ~30 times as sensitive as autoradiography. To confirm this notion, molar ratios of both mRNA species in the whole 6-d (stage 29) female embryos and in the undifferentiated gonads plus mesonephroi from the 6-d female embryos were determined (Figure 8). In this determination, the intensity values of *Wpkci* and *chPKCI* mRNA bands in the Northern blot hybridization were corrected by the intensity values of *GAPDH* mRNA and further by the ratio of signal intensity values of pWpkci-8 and pchPKCI-3 cDNA clones in Southern blot hybridization (Figure 8, A and B). The corrected molar ratio (*Wpkci* mRNA/*chPKCI* mRNA) was 10.3 for the whole embryo and 6.7 for the undifferentiated gonads plus mesonephroi.

In Situ Detection of Wpkci and chPKCI Transcripts in the Undifferentiated Gonads of Chicken Embryos

To examine the presence of *Wpkci* and *chPKCI* transcripts in undifferentiated gonads, the part of an embryo containing undifferentiated gonads and mesonephroi was dissected out from the 4.5-d (stage 25) female or male embryo and subjected to whole mount in situ hybridization with the use of the DIG-labeled antisense or sense riboprobe for *Wpkci* or *chPKCI* (Figure 9). The antisense riboprobe for *Wpkci* detected *Wpkci* transcripts in the undifferentiated left and right gonads and mesonephroi in the female embryo, but not in the male embryo (Figure 9A). The antisense riboprobe for *chPKCI* detected *chPKCI* transcripts as for the *Wpkci* transcripts but in both female and male embryos (Figure 9B). The level of expression of *chPKCI* in undifferentiated gonads seemed to be higher in the male than in the female, which was consistent with the results of Northern blot hybridization (Figure 7C). The sense riboprobe did not give signals of hybridization in each case (Figure 9, A and B).

To confirm that *Wpkci* and *chPKCI* genes were expressed in undifferentiated gonads in the female embryo, paraffin sections of embryonic tissues containing the undifferentiated gonads were prepared from the 5-d (stages 26 to 27) female embryo and subjected to in situ hybridization with the DIG-labeled antisense riboprobe for *Wpkci* or *chPKCI* (Figure 10). It was evident that transcripts of both *Wpkci* and *chPKCI* genes were detectable in the left and right undifferentiated gonads, mesonephric tubules, spinal cord, spinal ganglion, and myotome. In the undifferentiated gonads of the female embryo, transcripts of both *Wpkci* and *chPKCI* genes were detected widely in the tissue, except for some large cells (Figure 10, b, c, e, and f). However, we could not conclude in this study that the latter cells were primordial germ cells.

Nuclear Localization of GFP-fused Forms of Wpkci and chPKCI Expressed in Chicken Embryonic Fibroblasts

The intracellular localization of Wpkci and chPKCI was estimated by expressing their full-length cDNA sequences,

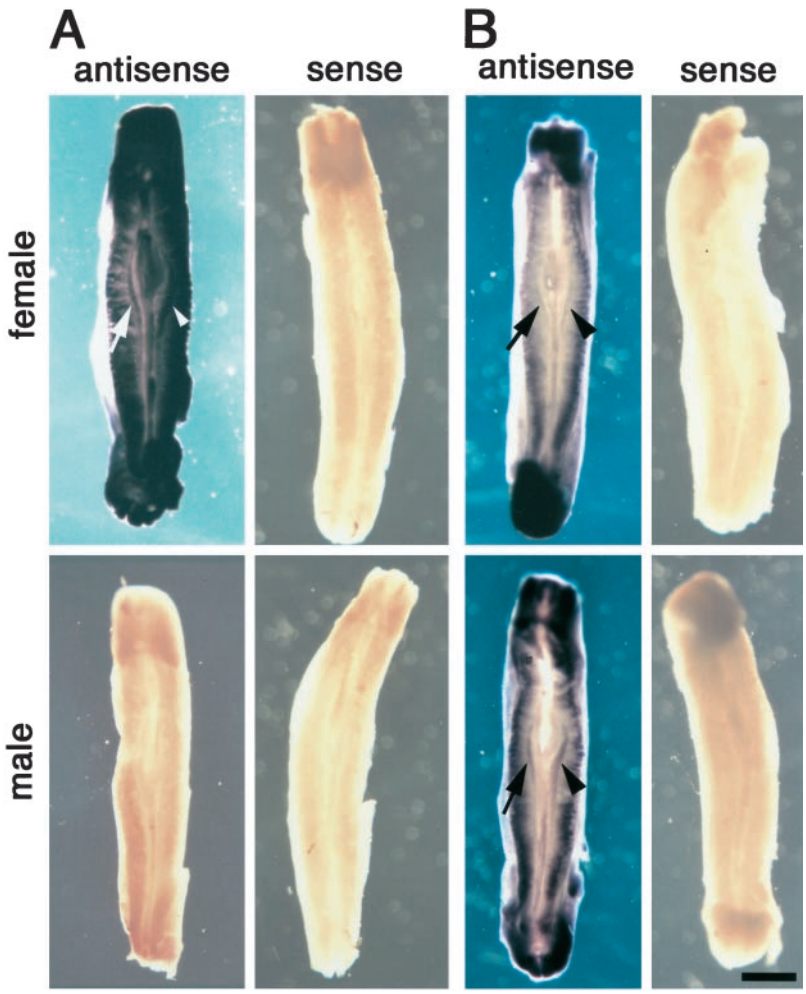

Figure 9. Whole mount in situ hybridization to the part of a 4.5-d (stage 25) female or male chicken embryo containing mesonephroi and undifferentiated left (arrowhead) and right (arrow) gonads, with DIG-labeled antisense or sense riboprobe for *Wpkci* (A) or *chPKCI* (B). *Wpkci* transcripts were detected only in the female embryo, and the *chPKCI* transcripts were detected in both female and male embryos with the antisense probe, followed by the reaction with anti-DIG antibody–coupled alkaline phosphatase. Bar, 1 mm.

ligated in frame to the 3′ end of the GFP-encoding sequence, in the male chicken embryonic fibroblasts. The GFP-fused form of Wpkci was found exclusively in the nucleus (Figure 11, A–C). The GFP-fused form of chPKCI was distributed in both nucleus and cytoplasm (Figure 11, D–F), but its nuclear distribution seemed to be more conspicuous compared with the distribution of GFP alone (Figure 11, G–I).

DISCUSSION

Sex Chromosome Linkage of Wpkci and chPKCI

In the present study, two cDNA clones, p5fm2 and p5fm3, were isolated from the subtracted (i.e., female minus male) cDNA library prepared from the mixed tissues of undifferentiated gonads and mesonephroi of 5-d (stages 26–28) chicken embryos. The full-length cDNA clone was then obtained with the use of p5fm2 as a probe and identified as that for a gene encoding an altered form of PKCI because of its substantial homology with the cDNA sequences of mammalian *PKCI*. This gene was designated *Wpkci* because of its location near the terminus of the nonheterochromatic end of the W chromosome, the female-specific sex chromosome, of chickens and its encoding a related but not identical protein with PKCI.

The female-specific *Wpkci* gene was conserved among all the Carinatae species of birds examined. Subsequently, a cDNA clone for the chicken homologue of *PKCI* (*chPKCI*) was identified because its deduced sequence was nearly 90% identical with those of mammalian PKCI and it contained the conserved HIT motif HVHLH (residues 110–114) near the C terminus. The chPKCI also contained the conserved His-51, which was suggested to participate in the binding of zinc with His-112 and His-114 based on analysis of the three-dimensional structure of hPKCI-1 by x-ray crystallography (Lima *et al.*, 1996).

The *chPKCI* gene was located near the centromere of the long arm of the Z chromosome, the sex chromosome common to the female and the male. The human *hPKCI-1* gene was mapped to 5q31.2 (Brzoska *et al.*, 1996). In view of the recently advanced notion that the chicken Z chromosome and human chromosome 9 share a number of genes in common (Nanda *et al.*, 1999), the location of *hPKCI-1* on chromosome 5 does not conform to this evolutionarily conserved synteny.

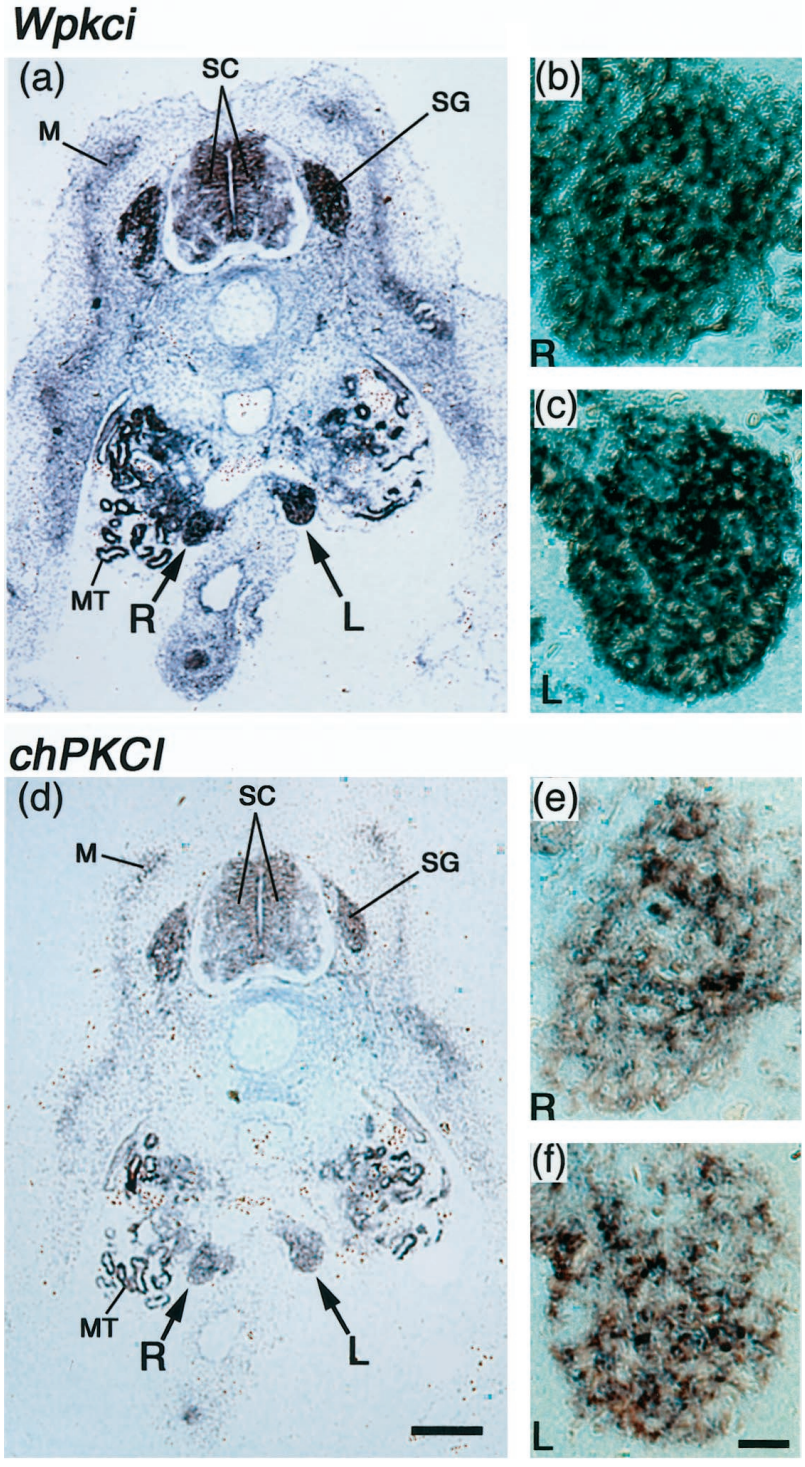

Figure 10. In situ detection of *Wpkci* and *chPKCI* transcripts in the tissue section of 5-d (stages 26 to 27) female chicken embryo. In situ hybridization was carried out as in Figure 9 to the 4-μm-thick paraffin sections containing left (L) and right (R) undifferentiated gonads with the antisense riboprobe for *Wpkci* (a–c) or *chPKCI* (d–f). Significant signals of hybridization were detected on both sides of gonads (a and d, and enlarged in b, c, e, and f), mesonephric tubule (MT), spinal cord (SC), spinal ganglion (SG), and myotome (M). Bar in d, 500 μm; bar in f, 50 μm.

When the deduced sequences of Wpkci and chPKCI are compared, there are two substantially different regions (the second and fifth regions from the N terminus; Figure 1C). The second region of Wpkci is characterized by the relatively high content of Leu and Arg (4 residues each out of 20 residues), which may give a unique property to Wpkci and/or cause the distinct nuclear localization of Wpkci, as suggested from the localization of the GFP-fused form of Wpkci (Figure 11). The fifth region contains the HIT motif in chPKCI, but this motif is absent in Wpkci.

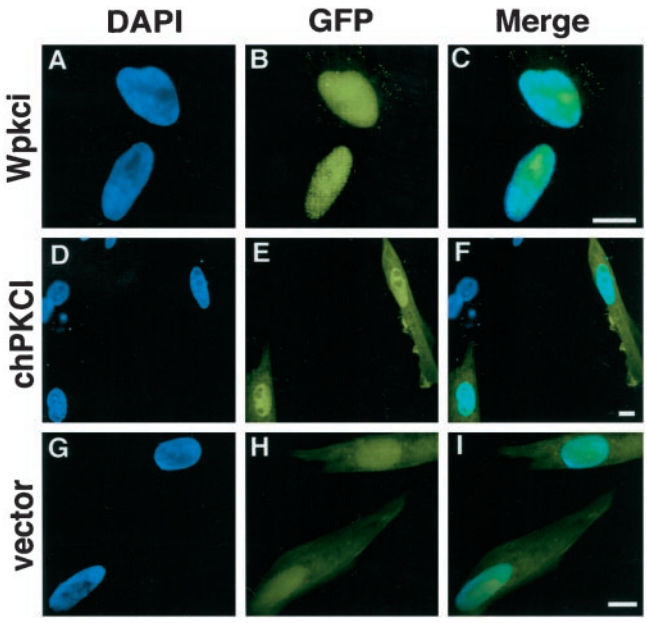

Figure 11. Intracellular distribution of the GFP-fused form of Wpkci or chPKCI in male chicken embryonic fibroblasts. DAPI-stained nuclei (left panels), GFP fluorescence of the protein expressed (middle panels), and merged images of DAPI staining and GFP fluorescence (right panels) are shown for cells transfected with the expression vector for GFP-Wpkci (A–C), GFP-chPKCI (D–F), or GFP alone (G–I). Bars, 10 μm.

Wpkci also does not contain His-51. Thus, Wpkci should not have zinc-binding ability. Overall, Wpkci does not display the same biological function as chPKCI. However, the presence of highly conserved regions (the fourth region from the N terminus and the C-terminal region; Figure 1C) is notable, because it has been suggested from three-dimensional analysis of hPKCI that it forms a homodimer by means of close contact between the α-helix formed by the fourth region and the C-terminal region of each protomer (Lima *et al.*, 1996).

These structural features of Wpkci suggest that, although it does not have a function comparable to that of chPKCI, it may form a homodimer and/or a heterodimer with chPKCI. Our finding that the region corresponding to the C-terminal region of chicken Wpkci was absent but the fourth region, which was predicted to form an α-helix (Figure 6C), was conserved in duck Wpkci suggests that the α-helix region might be sufficient for intermolecular association. The facts that hPKCI, lacking most of the N-terminal region, was isolated originally by its association with the regulatory domain (N-terminal 317-residue polypeptide) of PKC-β (Lima *et al.*, 1996) in the yeast two-hybrid system and that the length and the sequence of the C-terminal region of PKCI were variable among species (Robinson and Aitken, 1994; Lima *et al.*, 1996) also suggested that formation of a homodimer of Wpkci and/or a heterodimer between Wpkci and chPKCI might occur through contact between the α-helix region of each molecule.

A Model of the Role of Wpkci in Female Sex Determination

Molecular mechanisms of sex determination in birds have not been elucidated. Considering the female heterogametic sex chromosomes, the following two possibilities may be postulated: 1) the W chromosome contains a gene whose expression in the early embryonic stage triggers the cascade of gene expression toward female sex differentiation; and 2) the double dosage of a gene on the Z chromosome (ZZ in the male) causes male determination, whereas the single dosage (ZW in the female) of the gene causes female sex determination. In the latter case, the W chromosome may be required only for the proper meiotic segregation of the sex chromosomes. At present, there is no strong evidence favoring either of these possibilities.

The W chromosome of chicken is largely heterochromatic, and genes involved in female sex determination, if present, are expected to be located in the terminal nonheterochromatic region on the short arm (Mizuno and Macgregor, 1998). The *CHD1-W* (Ellegren, 1996) and *ATP5A1W* (Fridolfsson *et al.*, 1998) genes, reported to be present on the chicken W chromosome, are not likely to be responsible for female sex determination because of the reason mentioned above (see INTRODUCTION).

The Z chromosome of chicken contains genes that seem to be required for the function of the female gonad: *VLDLR* (Barber *et al.*, 1991) encoding the very-low-density lipoprotein receptor on the plasma membrane of ova, which is responsible for the uptake of VLDL and vitellogenin during oocyte growth, and *ZOV3* (Kunita *et al.*, 1997) encoding an immunoglobulin superfamily glycoprotein located on the plasma membrane of granulosa cells and islets of cells in the theca externa layer of ovarian follicles, both of which are involved in estrogen synthesis. These two genes, however, do not seem to exhibit the triggering role in female sex differentiation. The *DMRT1* gene on the Z chromosome may be a candidate, because its mammalian homologue on chromosome 9 is implicated in the differentiation of the male gonad, and in birds its expression in the genital ridge is more pronounced in the male, as mentioned above.

However, a positive function of the W chromosome may be inferred from studies on triploid chickens. In ZZZ chickens, gonadal and excurrent duct development was normal as in ZZ male chickens, although meiosis and spermiogenesis were somewhat abnormal. On the other hand, in ZZW chickens, the right gonad developed into a testis but the left gonad developed into an ovotestis at hatching, although no excurrent ducts were associated with it and it started to degenerate by 1 wk of age (Lin *et al.*, 1995). These results may suggest that a gene on the W chromosome affected the development of the ovarian component of the left ovotestis during the early stage of development.

In the present study, we found the *Wpkci* gene on the W chromosome, and the following facts, revealed in this study, suggest that Wpkci may function in the process of female sex determination in Carinatae birds. 1) *Wpkci* is located on the female-specific W chromosome, enabling its female-specific expression, and its locus is in the nonheterochromatic end region of this largely heterochromatic sex chromosome, facilitating its gene expression. 2) *Wpkci* is moderately reiterated on the W chromosome, whereas *chPKCI* is a single-copy gene on the Z chromosome. The higher dosage of *Wpkci* may

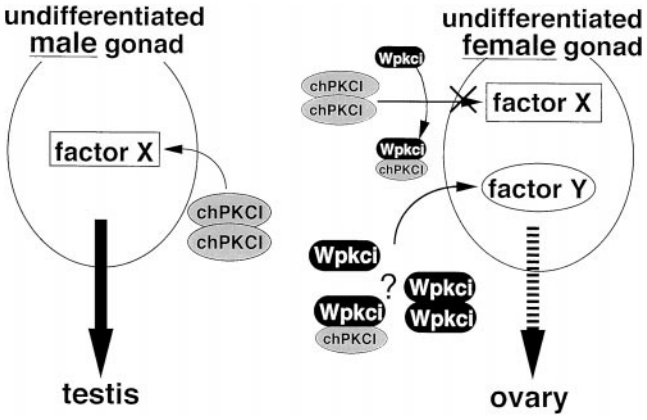

Figure 12. A model suggesting the involvement of chPKCI and Wpkci in triggering gonadal differentiation in chicken embryos.

contribute to the presence of larger amounts of *Wpkci* mRNA over *chPKCI* mRNA in early female chicken embryos. 3) *Wpkci* is present and moderately reiterated in the female genomes of all of the Carinatae species examined (12 orders, 24 species), indicating that its W linkage and reiteration have been maintained during the evolution of modern birds. 4) *Wpkci* is transcribed and its translation products are likely localized in the nucleus, suggesting its possible function within the nucleus. 5) The level of expression of *Wpkci* is significantly greater during the early stages of development of female embryos, which correspond to the period before the onset of gonadal differentiation, and its timing of active expression is about the same as that of the *chPKCI* gene. 6) The deduced sequence of Wpkci lacks the HIT motif and His-51, indicating that Wpkci should have no zinc-binding function, whereas Wpkci has a unique Leu- and Arg-rich region, and the central α-helix region is well conserved, suggesting its ability to form a homodimer or a heterodimer with chPKCI through contact between the α-helix region of each molecule.

However, when a possible role of Wpkci in female sex determination is considered, the fact that its expression is not limited to the undifferentiated gonads needs to be reconciled. In the model presented in Figure 12, it is proposed that the excess Wpkci forms the heterodimer with chPKCI efficiently and thereby interferes with the association of the chPKCI homodimer with a specific target protein (factor X) in undifferentiated gonads. If the association of the chPKCI homodimer with factor X is a key event in triggering the cascade of gene expression toward the differentiation of testis, this interference may, by itself, switch the pathway toward the differentiation of ovary. Another possibility is that the homodimer of Wpkci, the Wpkci monomer, or the Wpkci–chPKCI heterodimer, may play a positive role by interacting with another target protein (factor Y) in undifferentiated gonads, triggering the cascade of gene expression toward the differentiation of ovary.

To assess this model, it is essential to identify the Wpkci homodimer, the Wpkci–chPKCI heterodimer, and/or the target factors X and Y at the level of protein in undifferentiated gonads. It also would be desirable to transfer the *Wpkci* gene into the male embryonic stem cells and examine its effect on the fate of gonadal differentiation during embryogenesis.

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