Chromosome Research 1996, **4**, 411–426

Characterization of DNA sequences constituting the terminal heterochromatin of the chicken Z chromosome

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Received 18 December 1995; received in revised form 29 April 1996; accepted for publication by M. Schmid 29 April 1996

Two clones, pCZTH5-8 and pCZTH12-8, were isolated from a female chicken genomic library by screening with sequences obtained from genomic libraries which had been constructed from a terminal region of a single Z chromosome of chicken utilizing laser microbeam irradiation and PCR amplification. Fluorescence *in situ* hybridization to the mitotic Z chromosome and the lampbrush ZW bivalent of chicken demonstrated that both the cloned sequences are located in the heterochromatic region of the Z chromosome at the end opposite to the pairing region with the W chromosome. The sequences pCZTH5-8 and pCZTH12-8 are distributed widely on both the telomeric bow-like loops (TBL) and the region I (short loops region) of the Z lampbrush chromosome. These clones, pCZTH12-8 particularly notably, hybridized also to the TBLs of lampbrush bivalents 1–4 of chicken. Both sequences are transcribed in the lampbrush stage oocytes on the Z chromosome and on other macrobivalents. The subfragment of pCZTH5-8 which hybridizes to the TBLs and the insert of pCZTH12-8 contain regions that are closely similar in sequence. The pCZTH5-8 sequence has no internal repeats and may be part of the 24-kb macrosatellite repeating unit that is evident after *Nhe*I digestion of the genomic DNA. A cloned 24-kb unit, pFN-1, does not show significant DNA curvature, but cytosines of its CpG dinucleotides may be highly methylated *in vivo*. This contrasts with the repeat sequences of the W heterochromatin which not only have highly methylated CpG but are also strongly curved. The 24-kb unit is repeated about 830 times in the diploid genome of a female chicken, suggesting that nearly the entire terminal heterochromatin on the Z chromosome consists of this macrosatellite family. Sequences of the greater part of the pCZTH5-8 are restricted to the genus *Gallus* but the sequence of one subregion which hybridizes to TBLs is present in the genomes of the order Galliformes.

Key words: fluorescence *in situ* hybridization, *Gallus*, heterochromatin, macrosatellite DNA, Z chromosome

Introduction

In a mitotic chromosome set of a female chicken, the most prominent heterochromatic regions are on the two sex chromosomes, about two-thirds of the W chromosome and a terminal region of the Z chromosome (Saitoh & Mizuno 1992, Saitoh *et al.* 1993). The W heterochromatin shows conspicuous late replication (Suka *et al.* 1993) and its major DNA components are *Xho*I- and *Eco*RI-repetitive families, both of which are confined to the W chromosome of the genus *Gallus*. These two families consist of tandem repeats of a sequence unit averaging 21 bp in length and behave as markedly curved DNA in solution because of alternate occurrences of A and T clusters at nearly every pitch of the DNA helix (Mizuno *et al.* 1993, Suka *et al.* 1993). In the W chromosome, the major fraction of the *Eco*RI family is present in one arm but a minor fraction is distributed around the middle of the other arm. The *Xho*I family occupies a wide region around the centromere of the W chromosome (Saitoh & Mizuno 1992, Solari & Dresser 1995). Although the locations of these families on the arms of the W chromosome are thought to be partially overlapping (Solari & Dresser 1995), they are not intermingled with each other on a megabase scale (Saitoh *et al.* 1991).

On the other hand, the nature of DNA in the terminal heterochromatin of the Z chromosome has not yet been investigated. It has been speculated that properties of its sequence and/or its chromatin structure may be different from those of the W heterochromatin, mainly because the time of replication of the Z heterochromatin does not seem to be as late as that of the W heterochromatin (Suka *et al.* 1993). In the present study, we have obtained genomic clones from the terminal heterochromatic region of the chicken Z chromosome, characterized their DNA sequences and determined their locations on both mitotic and lampbrush chromo-

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somes. To this end, we first constructed and screened a region-specific genomic library from one end of a single chicken Z chromosome.

Materials and methods

Preparation and screening of genomic libraries from the terminal region of the chicken Z chromosome and isolation of genomic clones

Metaphase chromosome spreads were prepared from the chicken female embryonic fibroblasts according to Saitoh and Mizuno (1992) and stained lightly with Giemsa. A well-spread set was irradiated with a computer-controlled microbeam of argon-ion laser using the chromosome dissection microscope system C3144 by Hamamatsu Photonics. All the chromosomes except one end region of a single Z chromosome were destroyed. DNA was extracted from the single Z chromosome end region with 1 μl of a pick-up solution [10 mM Tris-HCl (pH 8.3), 0.1% SDS (sodium dodecyl sulphate), 1 mg/ml proteinase K] and amplified by three steps of PCR: six cycles of 90°C for 5 min/22°C for 120 min/22–50°C in 20 min/50°C for 20 min; 11 cycles of 92°C for 1.5 min/50°C for 1 min/72°C for 2 min; 30 cycles of 92°C for 1.5 min/55°C for 1 min/72°C for 2 min; adding at the beginning of each step the single unique primer (5'-TAGATCTGATATCTGAATTCCC-3') (Hadano *et al.* 1991), dNTPs and *Taq* DNA polymerase.

A portion of each of the PCR products in the 200–500 bp range was examined by Southern blot hybridization with the ^{32}P-labelled W chromosome-specific *Xho*I family probe (pUGD0600, Kodama *et al.* 1987) or a ^{32}P-labelled total genomic DNA probe from the male chicken. Lots of PCR products to which the *Xho*I family probe did not hybridize but to which the total male genomic DNA probe did hybridize, were selected. Two lots (nos. 5 and 12) were selected and their DNA fragments were digested with *Eco*RI (an *Eco*RI site is present in the primer sequence), ligated to λ gt10 arms and subjected to *in vitro* packaging using Gigapack II gold (Stratagene) to yield Z5 and Z12 libraries.

One hundred plaques were randomly picked up from the Z5 library, mixed and their inserts were amplified by PCR using the forward (5'-GCTGGGTAGTCCCCACCTTT-3') and reverse (5'-CTTATGAGTATTTCTAGGGTA-3') λ gt10 primers. The amplified DNA fragments were digoxigenin (DIG) labelled by nick translation using DIG-11-dUTP (Boehringer Mannheim) and their localization to one end of the Z chromosome was confirmed by fluorescence *in situ* hybridization (FISH) according to Saitoh & Mizuno (1992). Those one hundred phage clones were ^{32}P-labelled with [α-^{32}P]dCTP using random primers and *Escherichia coli* DNA polymerase I Klenow fragment and used as probes to screen a λGEM12 genomic library which had been constructed from the female chicken blood DNA digested partially with *Sau*3AI (*Mbo*I). Positive clones were digested with *Not*I and the recovered inserts were cloned using a pBluescript KS (+) vector. One of those clones, pCZTH5-8 containing about 13-kb insert, was used in this study.

Individual inserts of randomly selected phage clones from the Z12 library were amplified by PCR as described above but in the presence of [α-^{32}P]dCTP and each hybridized to the slot-blot of genomic DNAs from male and female chickens. One clone, λZ12-8, which gave about 2:1 signal intensities to the male and female DNA blots, was selected. The λ GEM12 female chicken genomic library was screened with this clone as a probe, and one clone containing an insert of about 12 kb was obtained and recloned into pBluescript KS (+) to yield pCZTH12-8.

Southern blot hybridization and DNA sequencing were carried out as described previously (Saitoh *et al.* 1993).

Isolation and cloning of a macrosatellite repeating unit

Female chicken embryonic fibroblasts were suspended in phosphate-buffered saline (PBS) (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l) at a concentration of 4×10^7 cells/ml, mixed with an equal volume of 1% solution of Incert agarose (FMC BioProducts) at 42°C, poured into a plug mould, and kept at 4°C for 20 min. Agarose plugs thus formed were digested twice with 1 mg/ml proteinase K in 0.5 M EDTA, 1% sodium lauroylsarcosinate at 50°C for 24 h, washed in TE [10 mM Tris-HCl (pH 7.6), 1 mM EDTA], treated twice with 1 mM PMSF (phenylmethylsulphonyl fluoride) in TE at 37°C for 30 min, and washed three times in TE. Five agarose plugs containing about 6 μg DNA were soaked in 1 × reaction buffer [10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol], suspended in 300 μl of 1 × reaction buffer and digested with 0.4 units/μl of *Nhe*I at 37°C overnight, washed in TE, and subjected to pulsed-field gel electrophoresis using GeneLine (Beckman) and ethidium bromide staining. The electrophoresis was carried out on a 1% low melting temperature agarose (SeaPlaque GTG, FMC BioProducts) in 0.5 × TBE [45 mM Tris-borate, 1 mM EDTA] at 10°C under the following electrical conditions: 140 mA, ramped pulse times from 1 to 12 s, 84 min per 1-s step. The gel region containing about 24-kb DNA fragments was cut out and the gel was melted in 1 × β-agarase buffer (FMC BioProducts) at 68°C and digested with β-agarase (1 unit/100 mg 1% agarose, FMC BioProducts) at 40°C for 1 h. The solution was adjusted to 0.5 M NaCl, chilled in ice for 15 min and centrifuged at 15 000 × *g* for 10 min. DNA fragments were precipitated by adding three volumes of ethanol to the supernatant, rinsed with 70% ethanol and dissolved in TE. The 24-kb DNA fragment was ligated to the *Xba*I cut and CIAP (calf intestine alkaline phosphatase, Toyobo)-treated charomid 9-28 vector (Saito & Stark 1986) with T4 DNA ligase, subjected to *in vitro* packaging using Gigapack II Gold (Stratagene) and transfected into *E. coli* DH5α. Four thousand colonies were screened with the ^{32}P-labelled insert of pCZTH5-8 and four positive clones were isolated. One clone, cFN-1, was digested with *Sal*I to remove a spacer sequence, self-ligated and transformed *E. coli* XL1-Blue to yield a clone pFN-1.

Estimation of repetition frequencies by quantitative slot-blot hybridization

A plasmid clone containing an insert, whose repetition frequency in the diploid genome was to be determined, was linearized by digesting with an appropriate restriction enzyme, purified, dissolved in TE and the DNA concentration was determined from A_{260nm}. Samples containing 50, 100 and 200 pg of each of the linearized recombinant plasmid DNA were mixed with 100, 200 and 400 ng, respectively, of DNA from human placenta (carrier). Samples containing 100, 200 and 400 ng of genomic DNA of the female chicken or of human in TE were also prepared. Each set of DNA samples (a recombinant plasmid DNA, female chicken genomic DNA, human DNA) were heat-denatured and fixed on a sheet of Hybond N^+ membrane (Amersham) by slot-blotting in 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The

DNA on the membrane was denatured again in 1.5 M NaCl, 0.5 M NaOH, neutralized in 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl, washed in 2 × SSC and dried. Each set of DNA blot was hybridized with ^{32}P-labelled insert of the same recombinant plasmid as fixed on the membrane in the presence of unlabelled, denatured, sheared female chicken genomic DNA amounting to eight times as much as the total amount of fixed DNA on a sheet of membrane (in order to make a probe excess condition) in 0.5 M sodium phosphate buffer (pH 7.2) containing 7% SDS, 1 mM EDTA and 300 μg/ml sheared, denatured salmon sperm DNA at 65°C overnight. The DNA blot was washed in 2 × SSC, 0.1% SDS, at room temperature for 10 min twice and at 65°C for 30 min twice. Intensities of hybridization signals converted to fluorescence intensities by the imaging plate system were measured in FUJIX Bioimage analyser BAS2000 (Fuji Film Co.). Repetition frequencies were obtained from the linear portion of the graph (fluorescence intensity versus amount of the fixed DNA) by applying the following relation:

$$N_{gen} \times X : N_c = S_{gen} : S_c$$

where X = repetition frequency (times per diploid genome), N_{gen} = diploid genome equivalence of the genomic DNA fixed on the membrane, N_c = molecular number of the recombinant plasmid fixed on the membrane, S_{gen} and S_c = intensities of hybridization signals for the genomic DNA and the recombinant plasmid, respectively, assuming that the diploid genome size of chicken is 2.54 pg (Mizuno *et al.* 1978) and a mean molecular weight of one base pair is 650.

Two-dimensional gel electrophoresis to examine DNA curvature

The 12.8-kb insert was cleaved from pCZTH5-8 with *Not*I and the 24-kb insert was cleaved from pFN-1 with *Sal*I plus *Sma*I. The 0.7-kb *Xho*I family sequence was obtained from pUGD0600 (Kodama *et al.* 1987) by digestion with *Bam*HI plus *Hin*dIII. These inserts were recovered from the agarose gel after electrophoresis. The inserts of pCZTH5-8 and pFN-1 were digested with *Hae*III. These digests and the 0.7-kb *Xho*I family sequence were subjected to two-dimensional electrophoresis. The first dimension was on 2% agarose gel of 2 mm thickness and electrophoresed at 4°C in 0.5 × TBE, in which DNA fragments migrated according to their sizes. After electrophoresis, the lane was cut out and applied on top of the second dimensional 4% polyacrylamide gel (acrylamide–bisacrylamide = 29:1) and the electrophoresis was carried out at 4°C in 0.5 × TBE, in which migration of curved DNA molecules was retarded depending upon the degree of curvature. The DNA fragments in the gel were stained with ethidium bromide.

Chromosome preparation and fluorescence *in situ* hybridization

Mitotic metaphase chromosomes were obtained from female or male chicken embryonic fibroblasts and prepared for FISH as described in Saitoh & Mizuno (1992) except that the hypotonic treatment was carried out by suspending cells for 20 min at room temperature in medium diluted four-fold with distilled water. Lampbrush chromosomes were isolated from the chicken oocytes with diameters of 1–3 mm and spread for FISH as described in Solovei *et al.* (1993).

pCZTH5-8 and its subclones and pCZTH12-8 were labelled as whole plasmids by nick translation using either DIG-11-dUTP (Boehringer Mannheim) or biotin-16-dUTP (Boehringer Mannheim) as described in Saitoh and Mizuno (1992), and used as probes. FISH reactions for mitotic chromosome preparations and immunofluorescence detection were carried out as described by Saitoh and Mizuno (1992). Results were observed with an Olympus BH2-RFL microscope or a Leica DMRB fluorescence microscope followed by image processing with CytoVision (Applied Imaging). FISH to the chicken lampbrush chromosomes and observation with a confocal laser scanning microscope was carried out as described by Solovei *et al.* (1993, 1994).

Results

Localization of the two cloned genomic sequences to the terminal heterochromatin of the chicken Z chromosome

Two genomic clones, pCZTH5-8 containing a 12.8-kb insert and pCZTH12-8 containing a 12.2-kb insert, were obtained from a female chicken genomic library by screening with clones, as probes, isolated from the Z chromosome terminal region-specific libraries as described in Materials and methods. FISH to mitotic chromosomes from male and female chicken embryonic fibroblasts demonstrated that the cloned sequence in pCZTH5-8 is localized in the conspicuous heterochromatic region at one end of the Z chromosome (compare Figure 1A & B and C & D). Similarly, the cloned sequence in pCZTH12-8 showed its major localization at the terminal heterochromatic region of the Z chromosome but, interestingly, localization of this sequence was also detectable at the ends of the long arms of chromosomes 1, 2, 3 and 4 (Figure 1E–H). Although the signal intensity was much less, distributions of this sequence at the ends of the short arms of chromosomes 1–3 could occasionally be detected by faint FISH labelling (Figure 1E–H).

The heterochromatic end of the Z chromosome to which pCZTH5-8 and pCZTH12-8 hybridize is known to be opposite to the end that undergoes meiotic pairing with the W chromosome (Solovei *et al.* 1993) (Figure 2). FISH to the chicken lampbrush ZW bivalent with a mixed probe comprising the insert of pCZTH5-8 and the W chromosome-specific 0.7-kb *Xho*I family repeating unit, confirmed that the cloned sequence in pCZTH5-8 is localized widely at the terminal region of the Z chromosome that is opposite to the pairing region with the W chromosome (Figure 3A). The terminal region where the pCZTH5-8 sequence is located was further proved to be the terminal heterochromatic region of the Z chromosome by comparing its location with that of the gene for IREBP (iron-responsive element-binding protein). The latter gene is located very close to the boundary of the terminal heterochromatin on the mitotic chicken Z chromosome (Saitoh *et al.* 1993). In Figure 3B, it is evident that on the lampbrush Z chromosome, the pCZTH5-8 sequence occupies one end of the region of the chromosome that is adjacent to

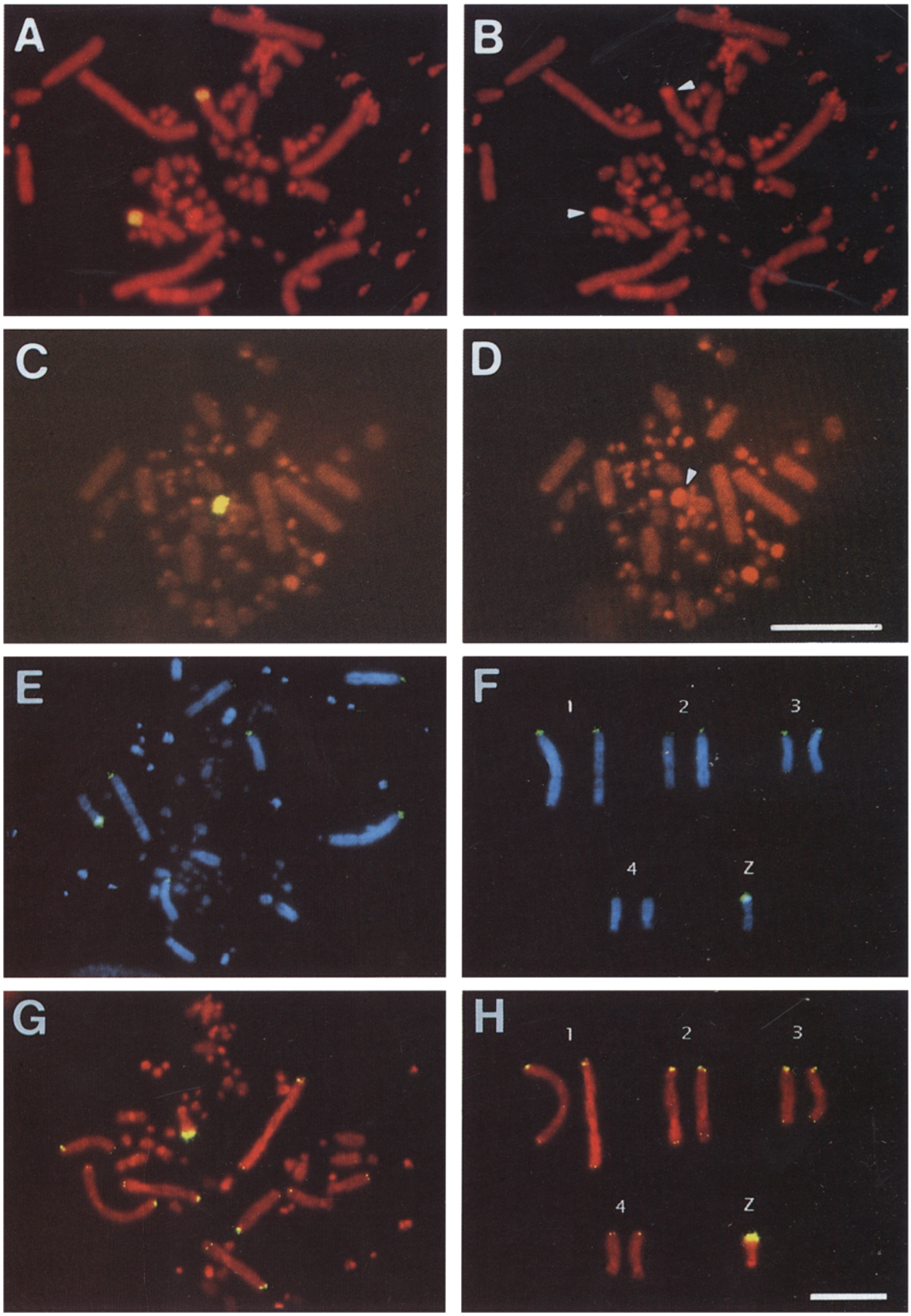
A
B
C
D
E
F
1
2
3
4
Z
G
H
1
2
3
4
Z

Figure 1. Localization of pCZTH5-8 and pCZTH12-8 sequences in the terminal heterochromatic region of the mitotic chicken Z chromosome. FISH to metaphase chromosome sets from embryonic fibroblasts of male (**A**) and female (**C** & **E**) White Leghorn or female Rhode Island Red (**G**) with digoxigenin (DIG)-labelled pCZTH5-8 (**A** & **C**) or DIG-labelled pCZTH12-8 (**E** & **G**). Hybrids were detected by immunoreactions with sheep anti-DIG Fab fragment followed with rabbit FITC-conjugated anti-sheep $F(ab')_2$ (E. Y. Laboratories). Chromosomes were counterstained with propidium iodide (PI) (**A–D**, **G** & **H**) or with 4′, 6-diamidino-2-phenylindole (DAPI) (**E** & **F**). **B** and **D** show the same PI-stained chromosome sets as in **A** and **C**, respectively, but without FITC fluorescence to show densely stained heterochromatic regions. The terminal heterochromatin of the Z chromosome is indicated with a white arrowhead. **F** and **H** show paired chromosomes 1–4 and a Z chromosome taken from **E** and **G** respectively. Bar = 10 μm.

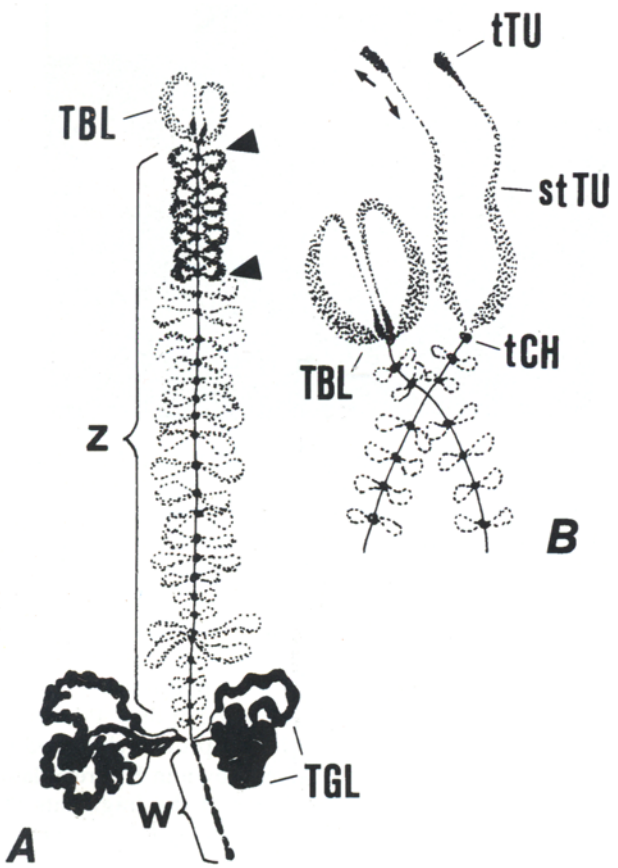

Figure 2. A Schematic drawing of the whole ZW bivalent of chicken. The limits of the Z and W chromosomes are shown by the large (z) bracket and the smaller (w) bracket respectively. The chromosomes are joined by a single terminal chiasma, the position of which is marked by the telomeric giant loops (TGL) of the Z chromosome. Region 1, characterized by short loops, is situated towards the non-chiasmate end of the Z and is marked by two arrowheads. The Z chromosome ends at the telomeric bow-like loops (TBL). **B** Diagram to show the arrangement of the TBLs in the close loop and the open-ended forms. These loops consist of two transcription units (TU): a long subterminal TU (stTU) and a short terminal TU (tTU). They originate from the last chromomere on the Z chromosome (tCH).

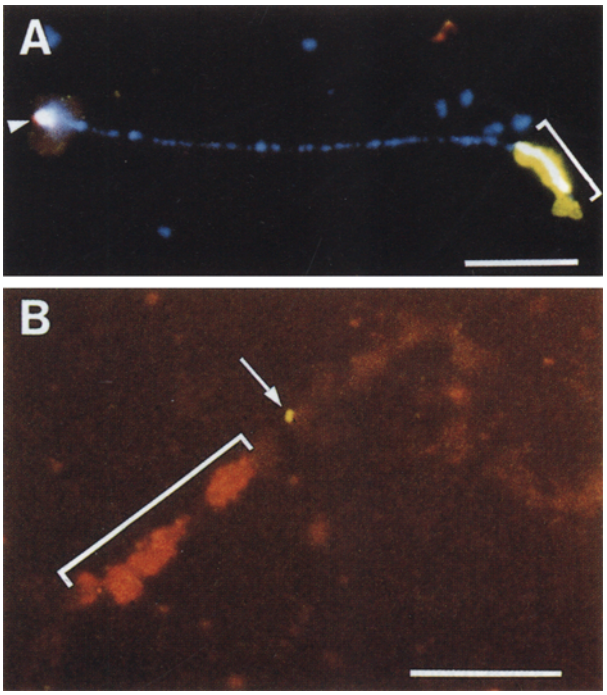

Figure 3. A wide distribution of the pCZTH5-8 sequence at the terminal region of the Z chromosome which is opposite to the chromosomal end pairing with the W chromosome in the chicken lampbrush ZW bivalent. **A** FISH with two mixed probes: biotinylated pCZTH5-8 and DIG-labelled *Xho*I family 0.7-kb repeating unit. Hybridization of the former probe (bracket) was detected by a series of reactions with FITC-labelled avidin DCS, biotinylated goat anti-avidin D and FITC-avidin DCS, and that of the latter probe (arrowhead) was detected as in Figure 1 but using rhodamine-conjugated anti-sheep $F(ab')_2$. Chromosomes were counterstained with DAPI. **B** FISH with two mixed probes: DIG-labelled pCZTH5-8 and biotinylated pGIREBP, a genomic clone containing a part of the chicken *IREBP* gene (Saitoh *et al.* 1993). Hybridization of the former probe (bracket) was detected by immunoreactions with sheep anti-DIG Fab fragment followed with rabbit rhodamine-conjugated anti-sheep $F(ab')_2$ and that of the latter probe (arrow) was detected as in **A**. Bar = 10 μm.

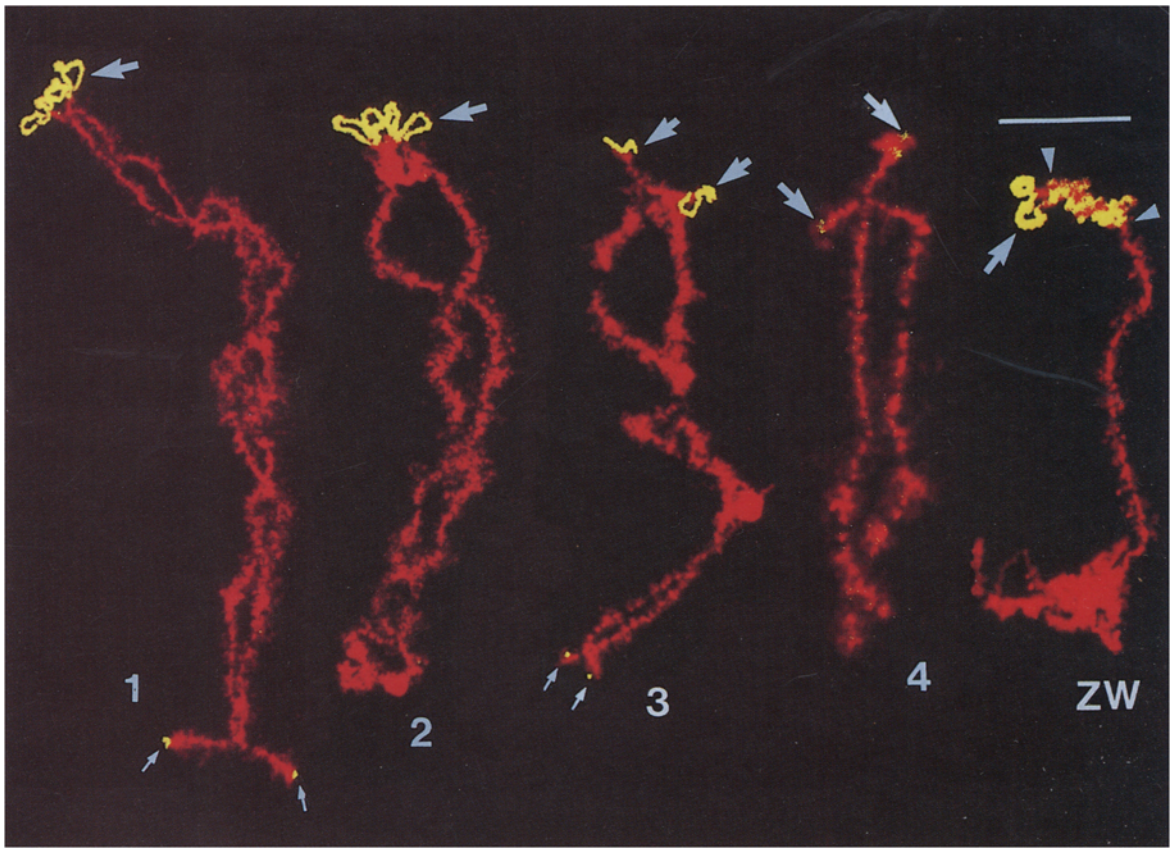

Figure 4. Localization of the pCZTH12-8 sequence on the chicken lampbrush chromosomes. FISH with DIG-labelled pCZTH12-8 and detection of hybrids with FITC fluorescence were carried out as in Figure 1. The FITC signals are detected at the small loops region (region marked by two arrowheads) and telomere bow-like loops (TBLs, large arrow) (see Figure 2) on the lampbrush Z chromosome in the ZW bivalent (**A**) and at TBLs of bivalents no. 1, 2, 3 and 4, and at the ends of the short arms of bivalents no. 1, 2 and 3 (small arrow). Lampbrush chromosomes were counterstained with PI. Bar = 25 μm.

the IREBP gene. The patterns of hybridization shown in Figure 3A & B suggest that the pCZTH5-8 sequence is highly repetitive and present both in the region I (a small loops region) and in the TBLs (telomere bow-like loops) at the heterochromatic end of the Z chromosome (Solovei *et al.* 1993; see Figure 2); the latter localization is particularly notable in Figure 3A.

FISH to the lampbrush ZW bivalent with the insert of pCZTH12-8 as a probe under conditions favouring the formation of DNA/DNA and DNA/RNA-transcript hybrids showed that this sequence is enriched in the TBLs of bivalents 1, 2, 3 and 4 and in the short loop region and TBLs of the Z chromosome (Figure 4). Our observations on FISH to lampbrush chromosomes support a conclusion that signals observed at the end of mitotic chromosomes 1 to 4 with the same probe (Figure 1E–H) are at the TBL loci which contain a common sequence represented in the insert of pCZTH12-8.

Characterization of the two cloned DNA sequences

Partial restriction maps of the inserts of pCZTH5-8 and pCZTH12-8 are illustrated in Figure 5. The insert of pCZTH5-8 was cleaved into eight fragments (fragments [1]–[8]) and that of pCZTH12-8 into four fragments ([9]–[12]) with *Eco*RI. When *Eco*RI-digested pCZTH5-8 was subjected to agarose gel electrophoresis and Southern blot hybridization with each one of the ^{32}P-labelled probes of fragments [1] to [8], each probe hybridized to a single band of its own size as shown in Figure 6 (lane 1 of each panel). Although sizes of the fragments [4] and [7] (1.7 kb) and those of fragments [1] and [6] (2.0 and 1.9 kb) are close and they were not separated well on the electrophoresis shown in Figure 6 (lane 1 in the ethidium bromide-stained panel), only the fragment [7] probe but not the fragment [4] probe detected the fragment [7], and the fragment [6] probe but not the fragment [1] probe detected the fragment [6] in the mixture of subcloned fragments [6] and [7] (Figure 6, lane 2 in each panel; cf. [4] and [7], and [1] and [6]). These results indicate that each one of the subfragments of the cloned 12.8-kb region in pCZTH5-8 is unique with respect to its sequence. In Figure 6 (lane 3 of each panel) the *Eco*RI-digested insert of pCZTH12-8 was electrophoresed and hybridized with ^{32}P-labelled probes [1] to [8]. Only the probe [7] detected a 2.6-kb band (corresponding to fragment [9] in Figure 5) and the probe [8] detected a 2.2-kb band (corresponding to

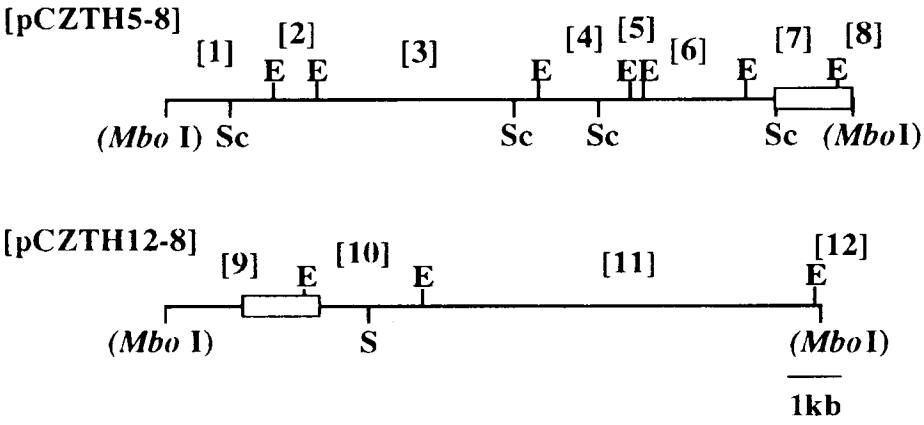

Figure 5. Partial restriction maps for the inserts of pCZTH5-8 and pCZTH12-8. Inserts were cleaved from pBluescript KS(+) vector by *Not*I digestion and restriction sites for *Eco*RI (E), *Sac*I (Sc) and *Sal*I (S) were determined. Both ends of these inserts are *Mbo*I sites because these inserts were derived from the genomic library that had been constructed with DNA fragments partially digested with *Mbo*I. Lengths (kb) of *Eco*RI fragments are: [1], 2.0; [2], 0.8; [3], 4.1; [4], 1.7; [5], 0.25; [6], 1.9; [7], 1.7; [8], 0.31; [9], 2.6; [10], 2.2; [11], 7.4; [12], 0.1. Common sequence regions in the two clones are boxed.

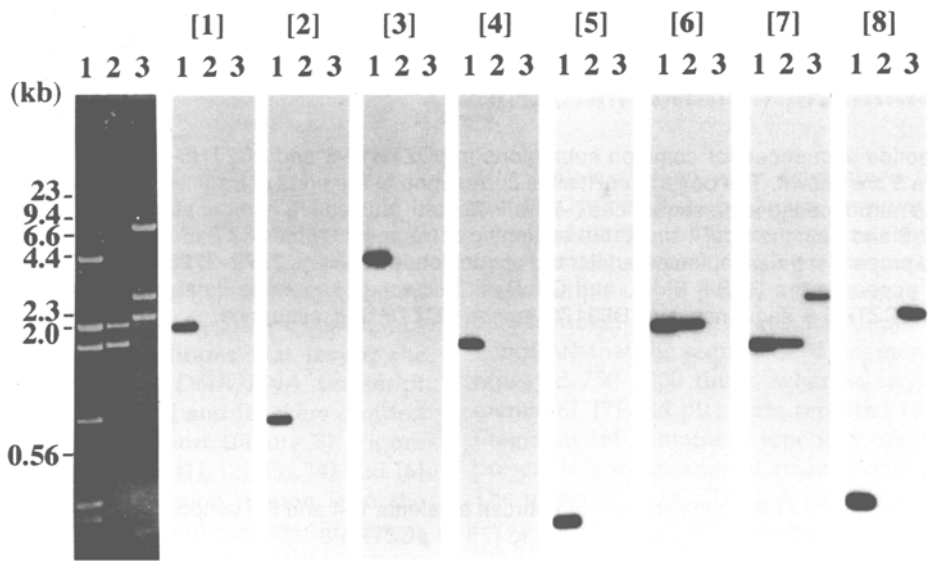

Figure 6. Absence of an internally repetitive structure in the 12.8-kb pCZTH5-8 sequence and presence of similar sequence regions in the inserts of pCZTH5-8 and pCZTH12-8. The insert of pCZTH5-8 recovered by *Not*I digestion and further digested with *Eco*RI (lane 1 of each panel), a mixture of fragments [6] and [7] (Figure 5) recovered from subclones in pBluescript KS (+) by *Sma*I plus *Eco*RV and *Eco*RV plus *Pst*I digestion respectively (lane 2 of each panel), and the insert of pCZTH12-8 recovered by *Not*I digestion and further digested with *Eco*RI (lane 3 of each panel) were electrophoresed and stained with ethidium bromide (leftmost panel) or subjected to Southern blot hybridization with the ^{32}P-labelled fragments [1] to [8]. The stringency of hybridization was estimated to allow 30% base pair mismatches as calculated according to Meinkoth & Wahl (1984) and Saitoh *et al.* (1991). Size markers are λDNA digested with *Hin*dIII.

fragment [10] in Figure 5) among the digest of the insert of pCZTH12-8, suggesting that the sequence of the fragments [7] to [8] region is similar to the sequence of the fragments [9] to [10] region.

Nucleotide sequences of fragment [9] and a part of fragment [10] (down to the *Sal*I site indicated in Figure 5) of pCZTH12-8 and of fragments [7] and [8] of pCZTH5-8 were determined. Highly similar sequences of about 1.4 kb (boxed in Figure 5) are present in these two cloned regions as shown in Figure 7. The common sequence has no obvious internal repeats but contains many short stretches of one of the four bases. One relatively long open reading frame encoding 150 amino acid residues is present in the complementary strand within this common region (nucleotide numbers 2278–2728) but its significance is unknown.

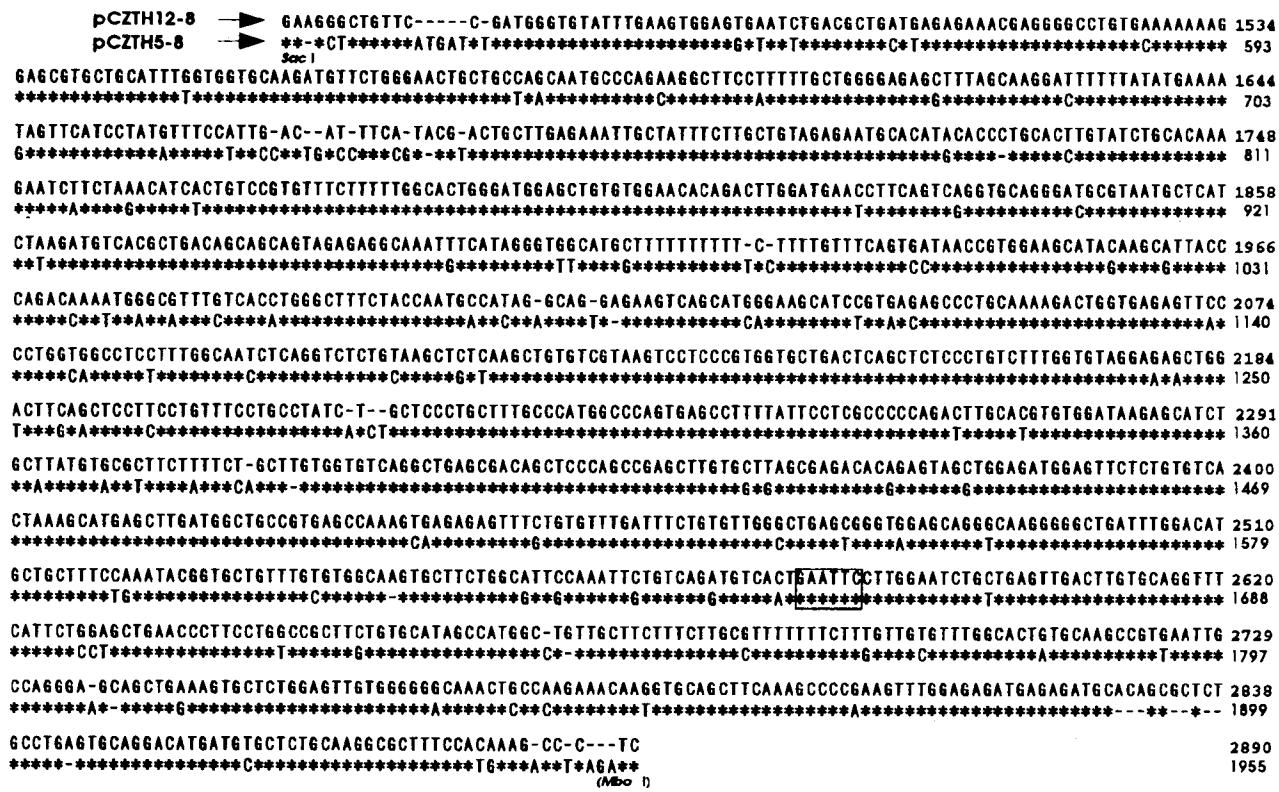

Figure 7. Nucleotide sequences for common subregions in pCZTH12-8 and pCZTH5-8. Sequences for the open-boxed regions in Figure 5 are shown. The boxed *Eco*RI sites correspond to the ends of fragments [7] and [9] (Figure 5). Common base (*) and gap introduced to align sequences (–) are indicated. Nucleotide number starts from the *Mbo*I end of fragment [9] for pCZTH12-8 and from the *Eco*RI site at the beginning of fragment [7] for pCZTH5-8 (Figure 5). A relatively long open reading frame is present in the complementary strand of nucleotide positions 2278–2728. The entire nucleotide sequences determined will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession numbers D63169 for the pCZTH5-8 sequence and D63170 for the pCZTH12-8 sequence.

Table 1. Summary of FISH to the chicken lampbrush bivalents 1–4 and the lampbrush Z chromosome probed with pCZTH5-8, pCZTH12-8 or subclones ([1] to [7]) of pCZTH5-8

Lampbrush bivalent	Sub region	pCZTH 12-8	pCZTH 5-8	[1]	[2], [3], [4] or [6]	[7]	[1+2+3+4 +6]
1	TBL	++	+	–	–	++	–
	TSL	(+)	–	–	–	–	–
2	TBL	++	+	–	–	++	–
	TSL	(+)	–	–	–	–	–
3	TBL	++	+	–	–	++	–
	TSL	(+)	(+)	(+)	–	–	(+)
4	TBL	(+)	–	–	–	–	–
ZW	TBL	++	(+)	–	–	++	–
	LR1	++	++	++	++	++	++

Key: ++, strong signals; +, weaker but reproducible signals; (+) weak signals observed occasionally; –, no signals observed.
TBL, telomere bow-like loops (long arm); TSL, telomere small loops (short arm); LR1, loops on region I of lampbrush Z chromosome.

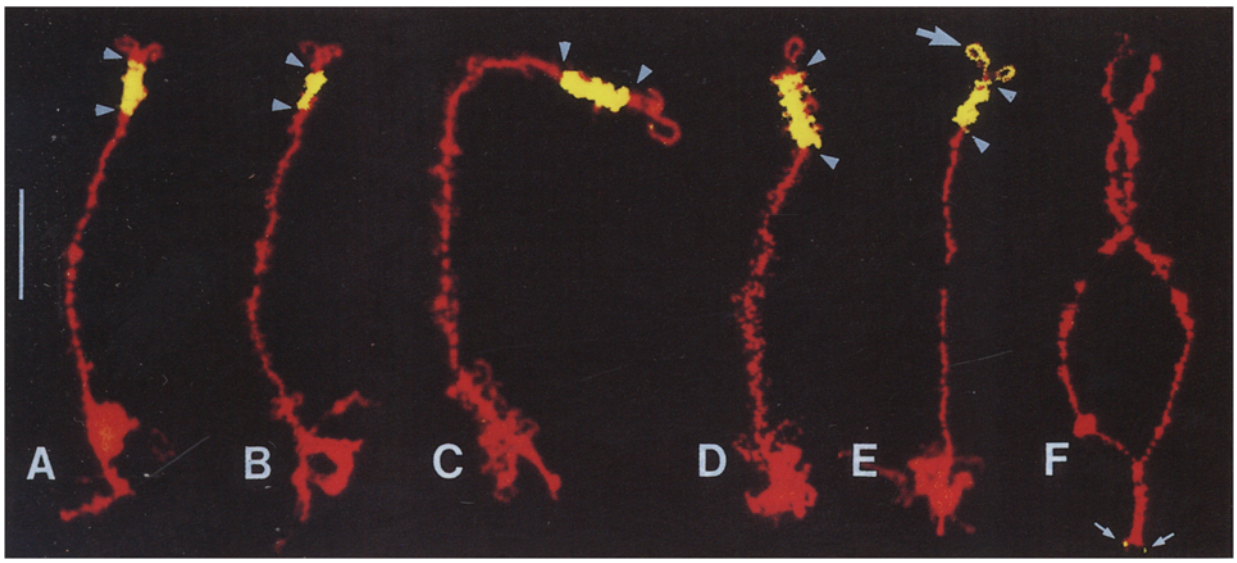

Figure 8. Distributions of the subfragments of pCZTH5-8 to different morphological subregions in the terminal region of the lampbrush Z chromosome or bivalent 3. FISH to the chicken lampbrush ZW bivalent was performed with DIG-labelled subclones in pBluescript KS (+) vector containing each one of the subfragments of the insert of pCZTH5-8: fragment [1] (**A**), fragment [2] (**B**), fragment [3] (**C**), a mixture of fragments [1], [2], [3], [4] and [6] (**D** & **F**), or fragment [7]. Probe was hybridized to a ZW bivalent (**A–E**) or a bivalent 3 (**F**). Hybrids were detected with FITC fluorescence as in Figure 1 and chromosomes were counterstained with PI. TBLs on the Z chromosome (large arrow) and telomere small loops on the bivalent 3 (small arrows) are indicated. Arrowheads mark the limits of the region 1 on the Z lampbrush chromosome Bar = 20 μm.

Different patterns of localization within the Z terminal heterochromatic region and different repetition frequencies among subfragments of the insert in pCZTH5-8

Fragments [1], [2], [3], [4], [6] and [7] of the insert of pCZTH5-8 were used as probes in FISH to the lampbrush ZW bivalent under conditions that favour the formation of DNA/DNA and DNA/RNA transcript hybrid molecules. Fragments [5] and [8] were omitted because their sizes were too short (Figure 5). Figure 8A–D shows that the fragments [1], [2], [3], [4] and [6] hybridize to the small loops region (region I) in the heterochromatic end of the Z chromosome. Fragment [7], which contains a sequence common to the sequence of fragment [9] in pCZTH12-8, hybridizes to most of the region I and to the TBLs (Figure 8E). These results, together with the FISH patterns with pCZTH12-8 probe (shown in Figure 4), suggest that the sequence common to fragments [7] to [8] and [9] to [10] is particularly abundant in the TBLs. The mixed probe consisting of fragments [1], [2], [3], [4] and [6] hybridizes to the telomere small loops on one end of bivalent 3 (Figure 8F), and only to them, in addition to the region I of the Z chromosome (Figure 8D). We have been able to show that it is fragment [1] that is responsible for this particular pattern of FISH on bivalent 3 (data not shown).

The entire range of results with FISH to lampbrush chromosomes employing probes derived from pCZTH12-8 and pCZTH5-8 are summarized in Table 1.

Repetition frequencies of the individual subfragments of the insert of pCZTH5-8 in the diploid genome of a female chicken were determined by quantitative slot-blot hybridization. Again, fragments [5] and [8] were omitted for the same reason as above. Instead, the insert of pR1.4 which consists of sequences of a part of fragment [7] and of fragment [8] (Figure 9) was used as an additional probe. The results summarized in Figure 9 indicate that the sequences of fragments [1] to [4] are repeated 750–1000 times, whereas sequences of fragments [6], [7] and pR1.4 are repeated 1500–1800 times. Fragment [6] contains a repetitive sequence which is present in a wide range of avian species as shown later. The presence of pCZTH12-8 sequence, which contains common sequence regions to fragments [7] and [8], in the TBLs of several macrochromosomes (Figure 4) may relate to their higher repetition frequencies.

Relatively recent evolutionary origin of the Z terminal heterochromatin

The ^{32}P-labelled fragments [1] to [8] of pCZTH5-8 were hybridized individually to female genomic DNAs of chicken, red jungle fowl, pheasant, duck and to human DNA as a control. As shown in Figure 10A, fragments [1], [2], [3], [4], [5] and [8] hybridized only to the DNAs of chicken and red jungle fowl (genus *Gallus*). The fragment [6] gave smeared signals to all the bird DNAs tested, suggesting that it contains a repetitive sequence which is widely distributed among a wide range of avian species. The fragment [7] hybridized not only to the genomic DNAs of the genus *Gallus* but also to the DNA of pheasant (lane 3). Sequences related to that of

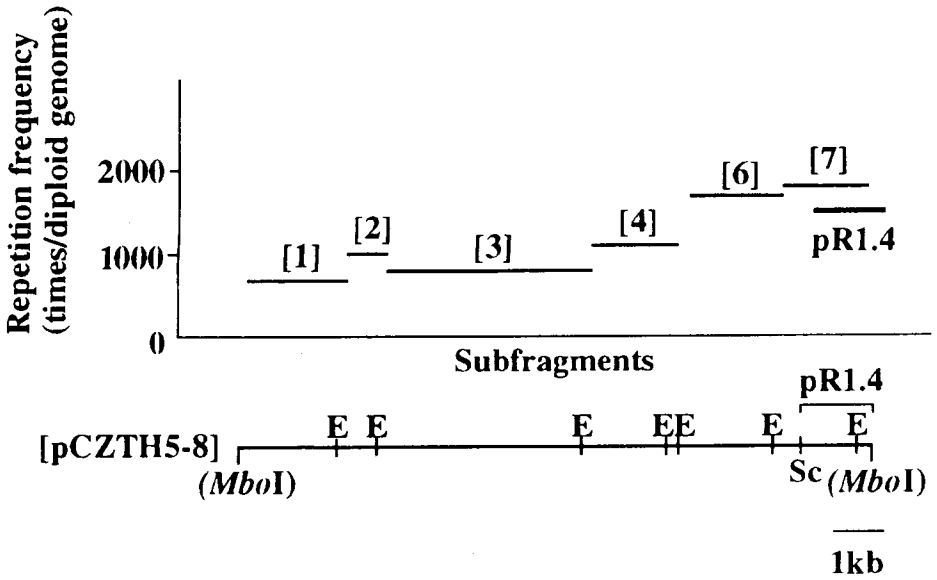

Figure 9. Repetition frequencies of the sequences represented by the *Eco*RI subfragments of pCZTH5-8 in the diploid genome of a female chicken as determined by quantitative slot-blot hybridization. Restriction sites are abbreviated as in Figure 5. The location of the pR1.4 subclone is indicated.

the fragment [7] are present and restricted to the order Galliformes as shown in Figure 10B; the fragment [7] probe hybridized to genomic DNAs of three species of the genus *Gallus* (lanes 1–3) and of six other species of the order Galliformes (lanes 4–9) but not of other avian species (lanes 10–15). These results suggest strongly that amplification of the most part of the pCZTH5-8 sequence has occurred rather recently on the evolutionary time-scale after the divergence leading to the genus *Gallus*, although a part of the sequence (fragment [7]) had been evolved much earlier in the ancestral species leading to the order Galliformes.

Macrosatellite DNA constituting the Z terminal heterochromatin

The FISH patterns with the pCZTH5-8 probe (Figure 3) suggest that sequences highly similar to this cloned sequence are present over the entire small loops region (region I) and in the TBLs (Solovei *et al.* 1993), implying that the cloned sequence of 12.8 kb is highly repetitive, although it does not contain an internally repetitive structure. From this notion, it was speculated that the cloned sequence in pCZTH5-8 was a part of a larger repeating unit. In order to examine this possibility, extremely high molecular weight DNA was prepared from female chicken embryonic fibroblasts, digested with a number of relatively rare cutting restriction enzymes whose recognition sites do not contain a CpG sequence, and the digests were separated by pulsed field gel electrophoresis and subjected to Southern blot hybridization with the ^{32}P-labelled insert of pCZTH5-8 as a probe. As shown in Figure 11A, genomic DNA fragments of approximately 5 to 50 kb produced with different restriction enzymes were hybridized with this probe. Among them, fragments produced by digestion with *Nhe*I (lane 5) showed a relatively homogeneous size of about 24 kb. These fragments were then cloned using a charomid 9-28 vector and clones hybridized with the ^{32}P-labelled insert of pCZTH5-8 were selected. One of those positive clones was converted to a plasmid form, pFN-1.

When the extremely high molecular weight genomic DNA of a female chicken was digested with restriction enzymes, separated by pulsed field gel electrophoresis and subjected to Southern blot hybridization with the ^{32}P-labelled, linearized pFN-1, similar patterns of hybridization as in Figure 11A were obtained (Figure 11B), indicating that pFN-1 is a clone representing the major repeating unit containing the pCZTH5-8 sequence.

Figure 12A shows that all of the fragments [1] to [8] of pCZTH5-8 hybridized to one or two bands produced by digesting pFN-1 with *Sal*I plus *Bam*HI (lane 1), *Sal*I plus *Eco*RI (lane 2) or *Sal*I plus *Saç*I (lane 3). When compared with ethidium bromide-stained bands, some bands were noted not to be hybridized with the subfragments of pCZTH5-8, indicating that the approximately 24-kb pFN-1 insert consists of pCZTH5-8-related and unrelated sequences. Assignment of bands hybridized in Figure 12A to restriction fragments of pFN-1 is shown in Figure 12B.

Repetition frequency of the insert of pFN-1 was estimated by quantitative slot-blot hybridization as for Figure 9. The sequence in pFN-1 was calculated to be repeated about 830 times per diploid genome of a female White Leghorn. Accordingly, we suggest that the terminal heterochromatin of the chicken Z chromosome consists mostly of the type of macrosatellite repeats as represented by the insert of pFN-1 (see Discussion).

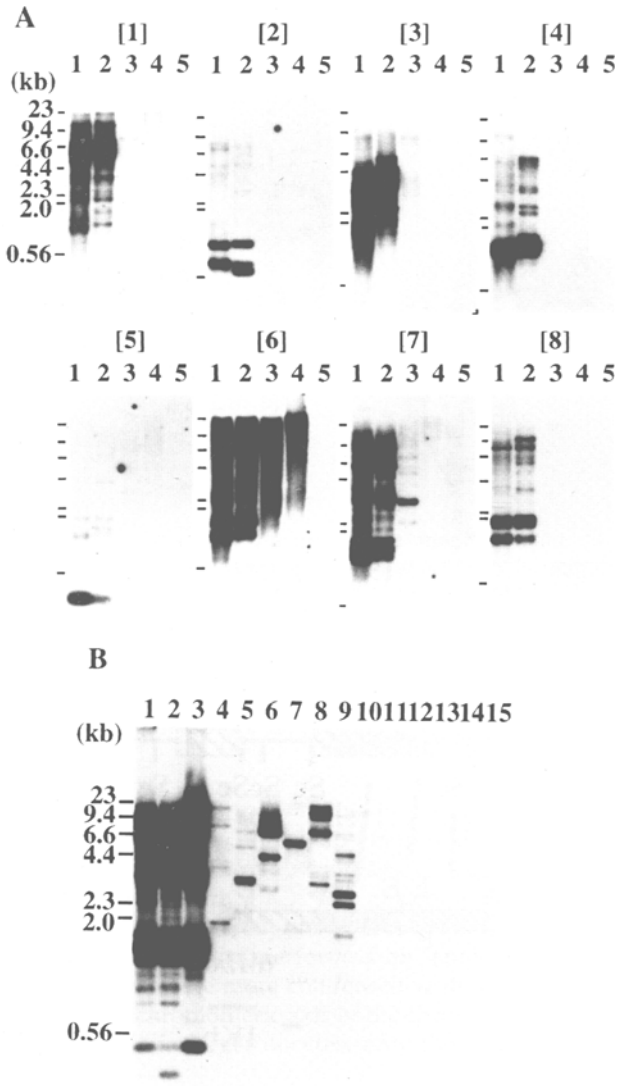

Figure 10. The presence of related sequences to pCZTH5-8 is restricted to taxonomically close species. **A** *Eco*RI-digested female genomic DNAs of chicken (*Gallus g. domesticus*) (lane 1), red jungle fowl (*Gallus gallus*) (lane 2), Japanese common pheasant (*Phasianus versicolor*) (lane 3), duck (*Anas platyrhynchos*) (lane 4) and human (lane 5) were electrophoresed on 1% agarose gel and subjected to Southern blot hybridization with the ^{32}P-labelled fragment [1] to [8] of pCZTH5-8. **B** Southern blot hybridization of the ^{32}P-labelled fragment [7] to *Eco*RI-digested female genomic DNAs of chicken (1), red jungle fowl (2), grey jungle fowl (*Gallus sonnerati*) (3), chukar partridge (*Alectoris graecea*) (4), Japanese common pheasant (5), turkey (*Meleagris gallopavo*) (6), Japanese quail (*Coturnix c. japonica*) (7), guinea fowl (*Numida meleagris*) (8), peafowl (*Pavo cristatus*) (9),Chinese button quail (*Turnix susciator*) (10), duck (11), rock dove (*Columba livia*) (12), budgerigar (*Melopsittacus undulatus*) (13), Java sparrow (*Padda oryzivora*) (14) and bearded penguin (*Pygoscelis antarctica*) (15). Size markers are as in Figure 6.

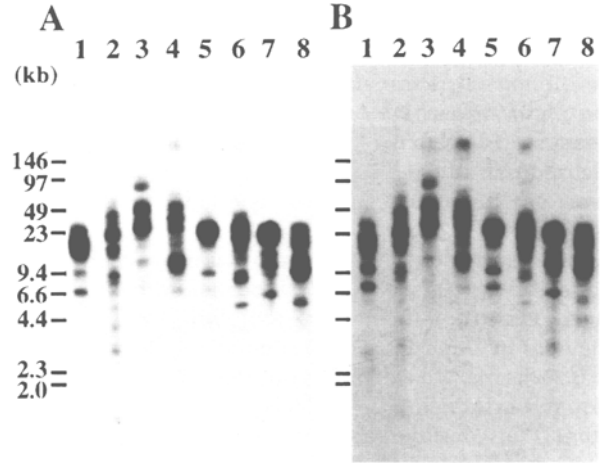

Figure 11. Macrosatellite repeating units as revealed by pulsed field gel electrophoresis and Southern blotting. **A** Extremely high molecular weight DNA from the female chicken embryonic fibroblasts was digested with *Apa*I (lane 1), *Ase*I (lane 2), *Eco*RV (lane 3), *Kpn*I (lane 4), *Nhe*I (lane 5), *Spe*I (lane 6), *Stu*I (lane 7) or *Xba*I (lane 8), separated by pulsed field gel electrophoresis as described in Materials and methods and subjected to Southern blot hybridization with the ^{32}P-labelled insert of pCZTH5-8. **B** As in **A** but probed with the ^{32}P-labelled insert (a 24-kb *Nhe*I fragment) of pFN-1. Size markers are a mixture of *Hin*dIII-digested λDNA and λconcatemers (low-range PFG markers, New England BioLabs).

Extensive CpG methylation but no significant DNA curvature for the macrosatellite repeating unit

The *Xho*I and *Eco*RI family sequences constituting the chicken W heterochromatin are characterized by extensive CpG methylation and strong DNA curvature owing to their intrinsic sequence organizations (Tone *et al.* 1984, Saitoh *et al.* 1991, Suka *et al.* 1993). In order to examine the level of CpG methylation for the repetitive sequences constituting the Z terminal heterochromatin, digestibilities of the macrosatellite repeats in the genomic DNA with methylation-sensitive *Hpa*II and methylation-resistant *Msp*I were compared. Figure 13 shows that the macrosatellite repeats in both male (lanes 1 and 2) and female (lanes 3 and 4) chicken genomic DNAs, probed with the insert of either pCZTH5-8 (Figure 13A) or pFN-1 (Figure 13B), were extensively digested with *Msp*I (lanes 2 and 4) but were cleaved to a much lower extent with *Hpa*II (lanes 1 and 3), whose activity has been shown to be inhibited when the cytosine of CpG in its recognition site (CCGG) is methylated (Waalwijk & Flavell 1978).

In order to examine the extent of DNA curvature for the macrosatellite sequence, the inserts of pCZTH5-8 and pFN-1 were digested with *Hae*III, and separated by two-dimensional gel electrophoresis at a low temperature. The first-dimension electrophoresis was carried out on an agarose gel to separate according to fragment sizes and the second dimension was carried out on a polyacrylamide gel so that the mobility of a fragment is

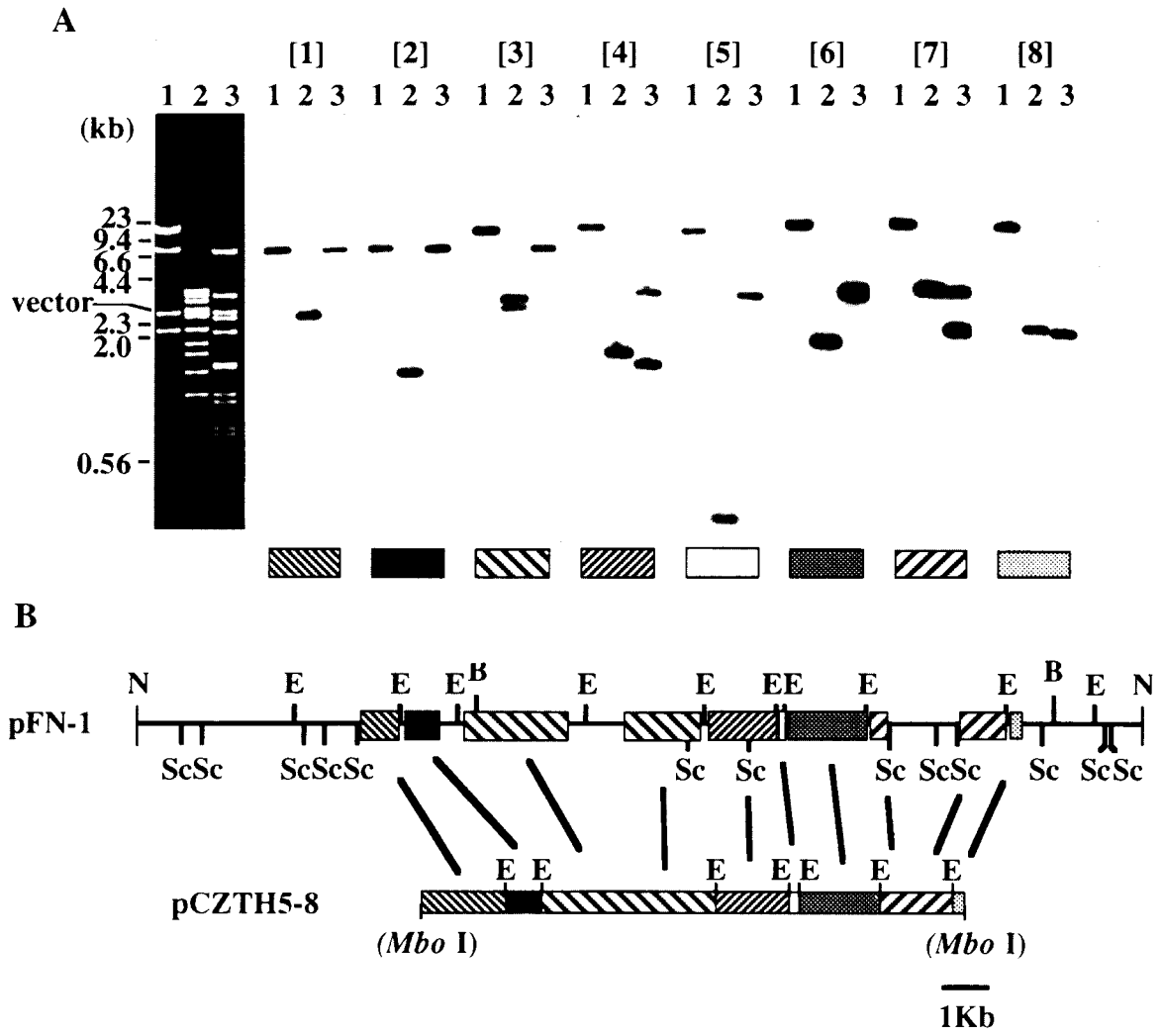

Figure 12. A cloned macrosatellite repeating unit contains all of the sequences of subfragments of pCZTH5-8. **A** pFN-1 digested with *Sal*I plus *Bam*HI (lane 1 of each panel), *Sal*I plus *Eco*RI (lane 2 of each panel) or *Sal*I plus *Sac*I (lane 3 of each panel) was separated by 1% agarose gel electrophoresis (leftmost panel; stained with ethidium bromide, position of the linearized vector indicated on the left) and subjected to Southern blot hybridization with the ^{32}P-labelled fragments [1] to [8] of pCZTH5-8. Size markers are as in Figure 6. **B** A partial restriction map for the insert of pFN-1 and assignment of fragments hybridized with the subfragment [1] to [8] of pCZTH5-8. Restriction sites are abbreviated as follows: *Nhe*I (N), *Eco*RI (E), *Bam*HI (B) and *Sac*I (Sc). *Sal*I sites are present in the vector sequence.

retarded depending upon the extent of its curvature. Figure 14A (pCZTH5-8) and B (pFN-1) demonstrate that most of the fragments produced did not show significant retardation in the second dimension, as compared with the remarkable retardation of the 0.7-kb *Xho*I family sequence under the same electrophoretic conditions (C). These results indicate that the macrosatellite sequences constituting the Z terminal heterochromatin are highly CpG-methylated, like the sequences constituting the W heterochromatin, but they differ from the W heterochromatic sequences in that they do not show significant DNA curvature in solution.

Transcription of DNA sequences in the Z-terminal heterochromatin in lampbrush stage oocytes

RNA transcription is active along all loops of lampbrush chromosomes in amphibian oocytes (Callan 1986). Satellite DNA sequences are transcribed on the lampbrush loops of newts (Varley *et al.* 1980, Diaz & Gall 1985). As the macrosatellite sequences are distributed all along the small loops region and the TBLs on the Z-terminal heterochromatin, we examined their transcription by comparing hybridization patterns with the pCZTH5-8 or pCZTH12-8 probes under different conditions (Figure 15A–E). Fuzzy profiles of hybridization were notable on the small loops region and TBLs

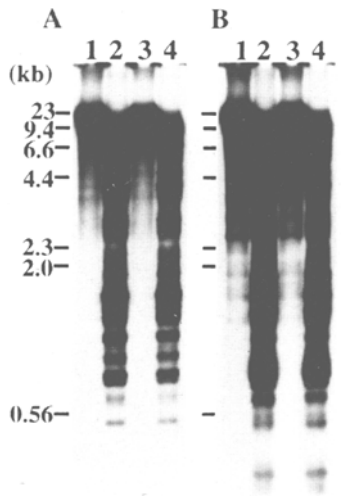

Figure 13. CpG methylation of the macrosatellite sequences *in vivo*. Genomic DNA prepared from the blood of a male chicken (lanes 1 and 2) or of a female chicken (lanes 3 and 4) was digested with *Hpa*II (lanes 1 and 3) or *Msp*I (lanes 2 and 4), separated by 1% agarose gel electrophoresis and subjected to Southern blot hybridization with the ^{32}P-labelled insert of pCZTH5-8 (**A**) or of pFN-1 (**B**). Size markers are as in Figure 6.

under conditions in which the probe hybridized to chromosomal DNA and loop-associated RNA transcripts (Figure 15D); or to loop-associated RNA transcripts only (Figure 15C); the chromomeres of this region were only weakly labelled (Figure 15A). When DNA/DNA FISH was performed on lampbrush chromosomes that were more condensed with loops retracted onto the chromomeric axis of the chromosome, such as is the case in larger oocytes, only the chromomeric axis in region I of the Z chromosomes was labelled (Figure 15B). Similarly, hybridization was observed to the TBLs of other macrobivalents under the conditions favouring hybridization to RNA transcripts (data not shown). These results suggest that parts of the macrosatellite sequences are transcribed in the lampbrush-stage oocytes wherever they are present in the chromosome set.

FISH to lampbrush chromosomes with fragment [7] of pCZTH5-8 under conditions favouring hybridization of probe DNA and loop-associated RNA transcripts showed that this sequence is not detectable in the very small transcription unit that occupies the extreme tip of each TBL when the loops are in the open form (Figures 2B & 15E). The small terminal transcription unit (Figure 2) is known to consist specifically of telomeric DNA (TTAGGG) repeats (Solovei *et al.* 1994).

Discussion

Comparison of W heterochromatin and Z-terminal heterochromatin in chicken

A region representing about two-thirds of the entire W chromosome of chicken consists of *Xho*I and *Eco*RI family repetitive sequences and forms the major heterochromatin in the somatic cell nucleus. The rest of the W chromosome does not contain those repetitive families and does not form heterochromatin (Saitoh & Mizuno 1992, Suka *et al.* 1993). Solari and Dresser (1995) demonstrated that the terminal region of the W chromosome, which is involved in the recombination with the Z chromosome in a meiotic prophase, does not contain detectable amounts of *Xho*I and *Eco*RI family sequences. Solovei *et al.* (1993) showed that the heterochromatic end of the Z chromosome in chicken and in three other species of bird is opposite to the one that

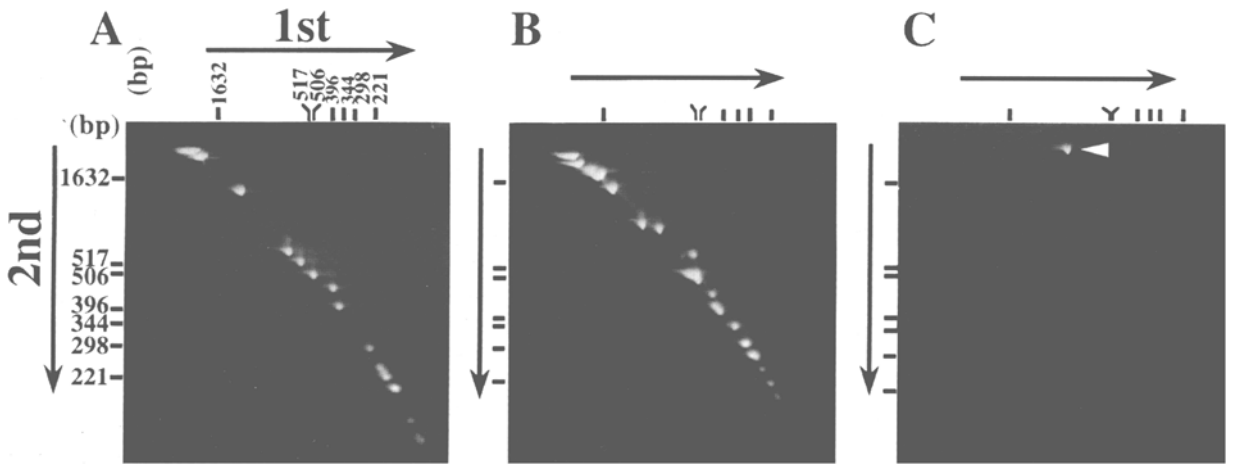

Figure 14. No significant DNA curvature for the macrosatellite sequences as revealed by two-dimensional electrophoresis. DNA samples applied were the insert of pCZTH5-8 digested with *Hae*III (**A**), the insert of pFN-1 digested with *Hae*III (**B**), and the 0.7-kb insert of pUGD0600 (**C**), a remarkably curved *Xho*I family repeating unit (Suka *et al.* 1993). Size markers are pBR322 digested with *Hin*fI. Samples were electrophoresed at 4°C on 2% agarose gel (first dimension) and 4% polyacrylamide gel (second dimension), and stained with ethidium bromide. An arrowhead in **C** indicates the position of the 0.7-kb *Xho*I fragment.

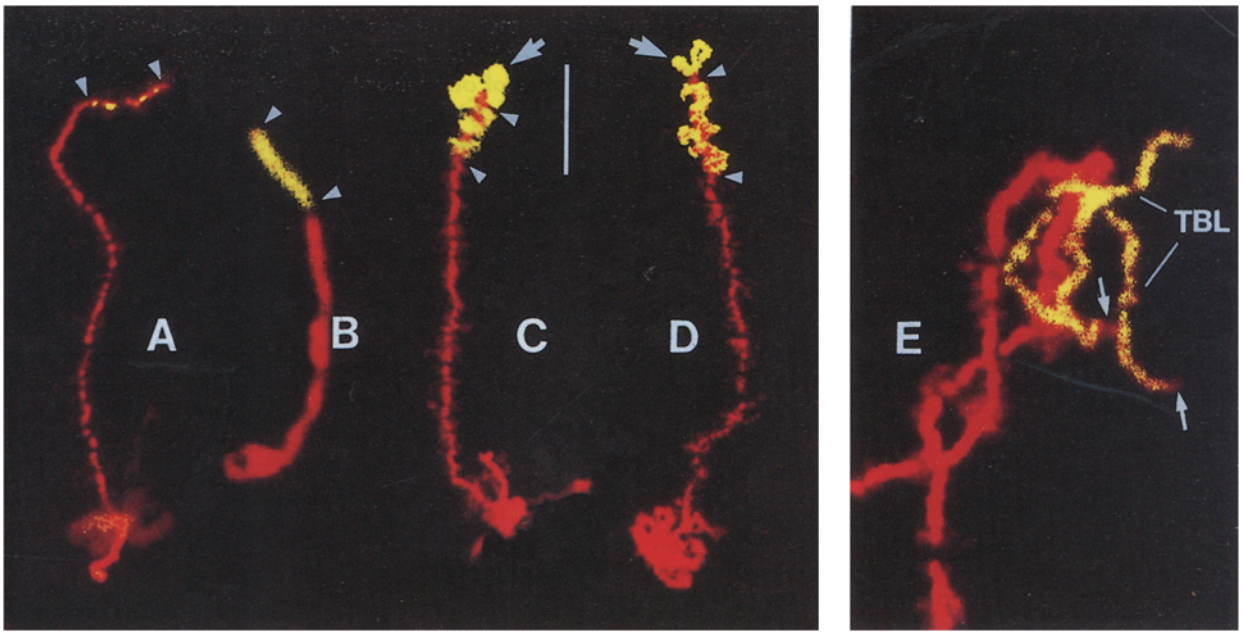

Figure 15. The results of FISH on the chicken lampbrush Z chromosome (**A–D**) with biotinylated pCZTH12-8 (**A**, **C** &**D**) and pCZTH5-8 (**B**) indicating transcription of these sequences on the telomere bow-like loops (TBLs) and on the small loops that characterize region 1 of the chromosome (arrowheads mark the limits of this region). In **A** and **B**, FISH was performed under conditions favouring binding of DNA to DNA and only the chromomeres of the chromosome axis are labelled. In **C**, the conditions favoured binding of DNA probe to loop-associated RNA transcripts and the TBLs and short loops on region 1 are labelled. In **D**, both DNA/DNA and DNA/RNA hybridization occurred and both loops and axis are labelled. Bar = 20 μm. In **E**, FISH with fragment [7] of pCZTH5-8 on the open-ended TBLs of bivalent 1 is shown. Only the large subtelomeric transcription units are labelled. Arrows point to the unlabelled telomeric transcription units (see also Figure 2B).

synapses and forms chiasmate association with the W chromosome (Figure 2). In the present study, FISH to the lampbrush ZW bivalent with a newly isolated probe for the Z-terminal heterochromatin has confirmed that the terminal region of the Z chromosome which is involved in the pairing with the W chromosome is the non-heterochromatic end (Figure 3).

DNA sequences of the W and Z heterochromatins are different not only in their nucleotide sequences but also their sequence organization and conformation. The *Xho*I and *Eco*RI families of sequences constituting the W heterochromatin both consist of tandem repeats of the average 21-bp basic units. Both sequences are strongly curved molecules due to the regular appearances of A and T clusters at almost every pitch of the DNA helix (Suka *et al.* 1993). The DNA sequences constituting the Z-terminal heterochromatin consist of macrosatellite repeating units of about 24 kb which do not contain internal repeats and do not show significant DNA curvature. The only common feature for the DNA sequences of the W and Z heterochromatins is that cytosines in the CpG dinucleotides in both sequences are highly methylated *in vivo*.

At molecular level, we propose that the different sequence characteristics of the Z and W heterochromatins may be at least partly responsible for the different replication timing of the two heterochromatins in interphase nuclei. In addition to the solenoidal conformation of the DNA path, the more or less regular tandem repeats of the 21-bp units might be expected to cause a more orderly array of phased nucleosomes and repeated binding or association of non-histone chromatin proteins along the chromatin fibre in the W heterochromatin, factors that could cause formation of more condensed W heterochromatin and less condensed Z-terminal heterochromatin and significantly later replication of W heterochromatin.

There may, on the other hand, be another, quite different explanation for the difference in the Z and W heterochromatin in interphase nuclei. If the W heterochromatin consisted largely of uniform, uninterrupted tandem arrays of a non-transcribable 21-bp unit, then it would be reasonable to expect it to remain tightly condensed during interphase. We know, however, that the Z heterochromatin is liberally interspersed with transcribable sequences, since it carries numerous loops (= transcription units) when it is in the lampbrush form. Although it remains to be investigated how actively those sequences in the Z heterochromatin are transcribed in the interphase nucleus, the interspersion with potentially transcribable sequences may prevent or discourage tight compaction of the entire region of the Z heterochromatin.

Sequence and morphological characteristics of the Z-terminal heterochromatin

The terminal heterochromatic region of the mitotic Z chromosome of chicken, as revealed by differential staining with PI, was estimated to occupy about 22% of the total length of the chromosome (Saitoh *et al.* 1993). The length of a Z chromosome was measured to be 3.15% of the total chromosome length in a diploid set of a female chicken (Solari 1977) or 3.84% of the total chromosome length in a diploid set of a male chicken (Kaelbling & Fechheimer 1983). Assuming that a diploid genome of a chicken consists of 2300 megabases (Mb) (2.3×10^9 bp; calculated from Mizuno *et al.* 1978), a Z chromosome contains 72.5 Mb, calculated from the former value, or 88.3 Mb, calculated from the latter value, of DNA. Thus, the terminal heterochromatin of the Z chromosome contains 16 Mb (72.5×0.22) or 19.4 Mb (88.3×0.22) of DNA. The 24-kb macrosatellite-repeating unit, cloned in pFN-1, was estimated to be repeated 830 times in a female diploid set (this study). The total DNA region thus occupied by this macrosatellite family should be 19.9 Mb (0.024×830). This amount of DNA corresponds to 124% or 103% of the above estimated amount of DNA in the Z-terminal heterochromatin. Thus, if we assume that DNA packing ratios of heterochromatin and euchromatin in a mitotic metaphase chromosome are not significantly different, then the above calculation indicates that the Z-terminal heterochromatin is mostly made up of the 'pFN-1 type' macrosatellite DNA repeats.

However, all the repeat units of these macrosatellites are obviously not the same as revealed by a number of genomic restriction fragments hybridized with each of the subfragments of pCZTH5-8 (Figure 10), by the unequal repetition frequencies among the subfragments (Figure 9), and by the similar but not identical sequence organization between the inserts of pCZTH5-8 and pFN-1 (Figure 12). A macrosatellite repeating unit has a mosaic structure as illustrated in Figure 12. Some members of the 'pFN-1' type macrosatellite family seem to have different sets of subregional sequences and the locations of these members within the terminal heterochromatin does not seem to be random. The unit containing the common sequence element between the fragments [7] to [8] of pCZTH5-8 and the fragments [9] to [10] of the pCZTH12-8 may be more abundant in the TBL regions.

Although pCZTH12-8 should not contain the sequence derived from the telomere because it was cloned from the *Sau*3AI/*Mbo*I fragment of the genomic DNA, its presence in the TBLs suggested that it represents a subtelomeric sequence (Solovei *et al.* 1994). Present results showing that the pCZTH12-8 sequence hybridizes to the TBLs of several macrochromosomes suggest that those chromosomes contain common subtelomeric sequences.

Solovei *et al.* (1994) have observed that the C-rich strand of the telomere repeat (TTAGGG) is transcribed in lampbrush chromosomes of chicken at the extreme ends of the meiotic chromatids and there are non-transcribed telomere sequences in the terminal chromomere, which lies a little distance inwards from the ends of the chromatids. They also observed that a morphologically identifiable transcription unit which is unlabelled with the telomere probe is present in between the extreme terminal telomeric transcription unit and the terminal chromomere. We think that the present pCZTH12-8 sequence that is enriched and transcribed in the TBLs of the chicken lampbrush Z and other 1–4 macrochromosomes is the one corresponding to the unlabelled subterminal transcription unit described by Solovei *et al.* (1994).

Evolutionary aspects of sex heterochromatins in chicken

Our previous studies on the *Xho*I and *Eco*RI family repetitive sequences in the W chromosome of chicken have shown that highly related sequences to those families are present only in the female genomes of species belonging to the genus *Gallus* (Tone *et al.* 1984), although the *Pst*I and *Taq*I families, having overall sequence similarities of 63% and 57% to the *Xho*I family sequence, are found in the female genomes of turkeys and pheasants, respectively, belonging to the order Galliformes (Saitoh *et al.* 1989). The present study of the macrosatellite sequences constituting the terminal heterochromatin of the Z chromosome tells a similar story. Except for a subregion containing the sequence that is enriched in the TBLs and another subregion containing a repetitive sequence which is widely distributed among avian species, the sequences making up the rest of the pCZTH5-8 repeating unit are confined to the genus *Gallus*. Only the subregional sequence enriched in the TBLs has related sequences in species belonging to the order Galliformes but not to other orders. Thus, the two major heterochromatins on both sex chromosomes in the chicken genome seem to have emerged only in the direct ancestor of the genus *Gallus*.

At early pachytene of the female meiosis, Z and W chromosomes of chicken pair between the sites which are located very close to one end of each chromosome and they form a single recombination nodule within the pairing region, suggesting that recombination is restricted to this end region (Rahn & Solari 1986). The pairing region in the W chromosome has been shown to be distal from the large region containing the two major repetitive families both in pachytene and diplotene ZW pair (Solari & Dresser 1995, Solovei *et al.* 1993). Our present results demonstrate that the pairing region in the Z chromosome is also free from the macrosatellite repeats constituting heterochromatin at the other end of the chromosome. Presence of large heterochromatic blocks which contain DNA sequences specific to each chromosome on the Z and W chromosomes may contribute to prevention of pairing and crossing-over in a large fraction of the ZW pair. In this context, it is of

interest to recall observations in newts where the frequency of chiasma formation between chromosomal arms is remarkably reduced when a terminal heterochromatin is acquired on the arm of one of the homologous chromosomes (Schmid *et al.* 1979, Sims *et al.* 1984). Prevention of meiotic crossing-over may then cause further chromosome-specific changes in the repetitive sequences of Z and W chromosomes.

Acknowledgements

We thank R. J. Etches, A. Shinjo, I. Uchida, M. Naito, M. Minezawa and T. Yajima for providing us with blood samples of various birds for DNA extraction. Shigeki Mizuno is grateful to the support of the Ministry of Education, Science and Culture of Japan with a Grant-in-Aid for Scientific Research on Priority Areas, 07282101, and an International Scientific Research Program, Joint Research, 07044175, and to the support of the Mitsubishi Foundation. Herbert Macgregor and Irina Solovei acknowledge with thanks the support of The Wellcome Trust UK through grant No. 039293/Z/93/A.

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