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Transcription on lampbrush chromosomes of a centromerically localized highly repeated DNA in pigeon (*Columba*) relates to sequence arrangement

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A highly repetitive, centromerically localized DNA sequence (PR1) has been isolated from the genomic DNA of two species of pigeon (*Columba livia* and *C. palumbus*). PR1 is approximately 900 bp long. It includes a sequence that is similar to the CENP-B box of mammals. It represents about 5% of the genome in *C. livia* and 2% in *C. palumbus*. In both species, tandem arrays of PR1 form part of larger repeating units. The organization of PR1 repeats and the larger repeating units is strikingly different in the two species. The large repeating units in *C. livia* include long (at least 14 units) tandem arrays of PR1 interspersed with relatively short intervening sequences. The large repeats of *C. palumbus* have much shorter (4 units or fewer) PR1 arrays interspersed with longer sections of non-PR1 DNA. PR1 is transcribed on short lampbrush loops in the centromeric regions of all lampbrush bivalents of *C. palumbus*. In *C. livia*, it is not transcribed at any of the major pericentromeric sites at which it is known to be present, although it is transcribed at one minor centromeric site on chromosome 2. It is proposed that transcription of the noncoding PR1 sequence on lampbrush chromosomes of pigeons relates to its genomic organization. The proposal is discussed with regard to the 'read-through' hypothesis for transcription on lampbrush loops.

Key words: *Columba*, lampbrush chromosomes, fluorescence *in situ* hybridization, repeated DNA sequences, RNA transcription

Introduction

Lampbrush chromosomes (LBCs) are found in diplotene of the prophase I of meiosis during oogenesis in many invertebrates and vertebrates, including birds (Callan 1986). These giant chromosomes are characterized by widespread RNA transcription, which takes place on numerous lateral loops.

Most previous studies of LBCs have been carried out on the chromosomes of amphibians in which between 5% and 10% of the entire genome seems to be simultaneously transcribed on several thousand lateral loops. Most of these transcribed sequences are noncoding and highly or moderately repeated (Diaz *et al.* 1981, Epstein *et al.* 1986, Penrad-Mobayed *et al.* 1991). Among these are some sequences that are either species-specific or genus-specific (Wu *et al.* 1986, Diaz & Gall 1985). In particular, this is the case with the centromeric satellite found in some *Triturus* species (Baldwin & Macgregor 1985; Varley *et al.* 1990). The predominantly centromeric satellite 1 from *Notophthalmus* is not transcribed at centromeric loci, although it is transcribed at two non-centromeric sites (Gall *et al.* 1981).

The LBCs of birds have been studied much less extensively and only a few attempts have been made to hybridize repeated DNA sequences to them (Hutchison 1987, Tarantul *et al.* 1989, Solovei *et al.* 1993, 1994, Hori *et al.* 1996), notwithstanding the relatively large number of bird sequences that have been isolated and cloned (Matzke *et al.* 1990, Chen *et al.* 1991, Shapira *et al.* 1991, Saitoh *et al.* 1991, Hori *et al.* 1996),

Information about the centromeres of bird chromosomes is limited. A family of centromeric repeats has been found in a variety of bird species (Longmire *et al.* 1988, Chen *et al.* 1989, Madsen *et al.* 1992 a,b, 1994). Centromere regions have not been reliably identified on bird lampbrush chromosomes. It has been suggested that the centromeres are situated in LBC regions with very short lateral loops (Chelysheva *et al.* 1990, Solovei *et al.* 1993). In pigeons (*Columba*) and some other birds these regions are associated with so-called protein bodies (PBs), which are round bodies of variable size attached to the chromosome axis (Gaginskaya & Gruzova, 1969, 1975, Gaginskaya, 1972, Khutinaeva *et al.* 1989, Solovei *et al.* 1993). The nature of these bodies has not yet been determined. They resemble the sphere organelles of amphibian LBCs (Callan 1986, Gall *et al.* 1995) in appearance, but it is not known whether the two structures share any functional homology. Assignment of the centromeres of bird LBCs to the regions of short lateral loops and protein bodies is based solely on the fact that the positions of these

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A

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dpD3   GAATTCCGCT TCTTAGGGGA AGTGAGAAAT TGCTGAGCTG ACACCAAGTC
              *       *
wp3H6  GAATTCCACT TCTTTGGGGA AGTGAGAAAT TGCTGAGCTG ACACCAAGTC

 50    CTGCAGTGTC CCTGTCACCT GCAGAGTCAC CGCATCCTCG CCACGGTAGC
                               *  *             *      *
       CTGCAGTGTC CCTGTCACCT GCCGACTCAC CGCATCCTTG CCACAGTAGC

100    GGGCATTGTG ACAAAGGTTC AATGTCAGCT GGGTGTGGCA GCCAGGCGCC
                             *                      ****
       GGGCATTGTG ACAAAGGTTC TATGTCAGCT GGGTGTGGCA GATGAGCGCC

150    CCCTAGGGGA TGGGGAGCCT GTCAGCCCCG GGCCTGTCAC TGCCTGCTGG
                  *                                         *
       CCCTAGGGGA GGGGGAGCCT GTCAGCCCCG GGCCTGTCAC TGCCTGCTGT

200    CCCTGCTGTC ACCAGCCAGG CCTCTGAAAA CTTGTCAAAC CATGCCCAGA
           *   *                          *
       CCCTCCTGCC ACCAGCCAGG CCTCTGAAAA CTGGTCAAAC CATGCCCAGA

250    CTTGGGGCCA GTTTGGCCCA AAGTCTCCAA AAGACAAGTC GTGAAGAAAG
           **     ***         *         *           *
       CTTGCAGCCA AAGTGGCCCA AMGTCTCCAA CAGACAAGTC AAGAAGAAAG

300    ATATGGGACT GAGGGAGAAA GCGAAGGGAG CAGGAGAAAG GGAAGAAAGA
                *                  *    * *    * *      *
       ATATGGGACA GAGGGAGAAA GCGAAGAGAG MACGAGATAA GGAAGGAAGA

350    ATAAAAAGTA GTGTTGGGCT GCAGGAGTGA GTTGGAGGAA TTGCAAGAAA
                                         *
       ATAAAAAGTA GTGTTGGGCT GCAGGAGTGA GATGGAGGAA TTGCAAGAAA

400    AGAGGCAGGA AGCAGGGGAG TGAGAGAAAA GGAAA----- ---GAAAGAA
                        *                  *
       AGAGGCAGGA AGCAGGAGAG TGAGAGAAAA GGACAGAAGA GAGGAAAGAA

450    GAGACATGCA GCCAGACGAG GGGTTAGTGA GAGGGGATGG GACAGAGAAC
                             *     *       *
       GAGACATGCA GCCAGACGAG AGGTTATTGA GAGAGGATGG GACAGAGAAC

500    AGGATGAGAA GGAAATGAAA CAAGGCAAGG CTAGGCAAGG ----------
                *       * **            ***
       AGGATGAGAG GGAAATAAGG CAAGGCAAGG ACCGGCAAGG ACAGGCAAGG

550    ---------- -----CTAGG CTGCAGGACA TTTTTTTGCT TTATAATTTC
                                                      * *
       CAAGGCTAGG CTAGGCTAGG CTGCAGGACA TTTTTTTGCT TTACAGTTTC

600    TAGCACATTC GTCTTCAGAC TAGGAGGAAA GCCATGAGAC CATATGGATT
                *            *      *        *      *
       TAGCACATTM GTCTTCAGAC GAGGAGGRAA GCCATAAGAC CGTATGGATT

650    ATGCCCAAGC AATAACTCCA GCTTCTTTGA GACACTTAGA CTAAGCCGAA
        *           *   **    *   *                      *
       AGGCCCAAGC AAAAACAGCA GCTAC-TGGA GACACTTAGA CTAAGCCTAA

700    AATACGAAGA TCTCTCCATA AGGATGGTTT TTATACTGTA CT-CCACAAT
            *      *      *
       AATACCAAGA TGTCTCCACA AGGATGGTTT TTATACTGTA CTTCCACAAT

750    ATGCTGGCAG TACAAAAAAA TAAGCTGCAG ATCTCCTCAG GCTGAGTCAT
       *            *                          *
       CTGCTGGCAG TATAAAAAAA TAAGCTGCAG ATCTCCTTAG GCTGAGTCAT

800    GGTGCTTTGG TGCAGGGACC ATGCATATCA TGGTAGTATT TCACCATTTC
           *   *          *                *  *
       GGTGGTTTCG TGCAGGGATC ATGCATATCA TGGGAATATT TCACCATTTC

850    TGCTGTGGTT TTCAGTGTAT TACACCAAAA CACCCACTTG ATGACATCCT
           *                      *            *      *   ***
       TGCTCTGGTT TTCAGTGTAT TACACAAAAA CACCCACCTG ATGGCACAAT

900    TTCAAGAGCA AAGGCAGCAA TTTGCAgaat tc
           *
       TTCAGGAGCA AAGGCAGCAA TTTGCAgaat tc
```

Figure 1. Sequence of PR1. **A** Comparative alignment of sequences from domestic pigeon (dpD3) and wood-pigeon (wp3H6). **B** The region of similarity with mammalian CENP-B box with the sequence in the reverse orientation to that in **A**. Two sequences from domestic pigeon (dp) and three from wood-pigeon (wp) are shown, together with known mammalian sequences (from human and two species of mouse, after Kipling et al. 1995). Consensus/p, consensus of pigeon sequences; NNBC, nucleotides necessary for binding CENP-B (after Masumoto *et al.* 1993); bar, match; asterisk, transition; blank, transversion.

B

P

```
dpD3            GTCTTTTGGAGACTTTGGGC
dpB3            GTTTATTGGAGATTTTGGGC
wp3H6           GTCTGTTGGAGACKTTGGGC
wp3H1           GTCTGTTGGAGACTTTGGGC
wp3A12          GTCTGTTGGAGACTTTGGGC
consensus/p     GTcTgTTGGAGActTTGGGC
                 |**||||||*||   |||
human           CTTCGTTGGAAAC---GGGA
M.musculus      ATTCGTTGGAAAC---GGGA
M.caroli        TTTCGTCTAATGC---GGGT

NNBC            .TTCG....A..C---GGG.
                 |**|    |  |   |||
consensus/p     GTcTgTTGGAGActTTGGGC
```

Figure 1B.

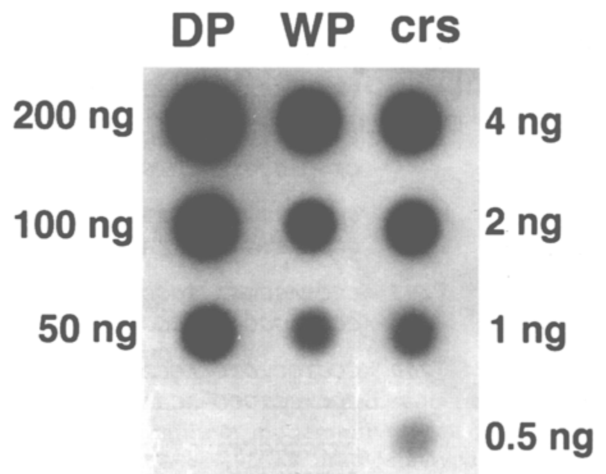


Figure 2. Quantification of the PR1 sequence in genomic DNAs of domestic pigeon (DP) and wood-pigeon (WP) by comparison with plasmid dpD3-containing PR1 sequence (crs). Labelled PR1 sequence excised from the same clone was used as the probe. Figures on the left are amounts of genomic DNA per well; figures on the right are the amounts of PR1 DNA (calculated from lengths of PR1 and pUC18) per well.

regions on LBCs, as a rule, correspond to those of centromeres in mitotic chromosomes (Chelysheva *et al.* 1990, Solovei *et al.* 1993). Transcription of bird centromere repeats at the LBC stage has never been investigated.

This article is about a highly repetitive centromeric DNA sequence (PR1) from two species of pigeon (*Columba*). We describe its distribution on the chromosomes and show that it is abundantly transcribed in one of the species studied but not in the other. This difference in transcription correlates with a different genomic organization of the repeat in the two species.

Materials and methods

The birds used in this study were domestic pigeon (rock dove), *Columba livia*, and wood-pigeon, *Columba palumbus*. Females of *C. livia* were bought from commercial stock. *C. palumbus* were shot locally in the Leicestershire countryside.

DNA was extracted from blood fixed with 100% ethanol from one specimen of domestic pigeon and one specimen of wood-pigeon as described by Bruford *et al.* (1992). DNA digestion with *Eco*RI and *Bam*HI (Promega) was performed following the manufacturer's instructions. Southern blotting was performed following standard protocols (Bruford *et al.* 1992) with successive post-hybridization washes in × 2, × 1 and × 0.1 saline sodium citrate (SSC) at 64°C.

Genomic DNA of each species (about 5 μg) was digested with *Eco*RI and *Bam*HI (Promega) overnight at 37°C, fractionated in 0.8% agarose gel and transferred to a nylon filter (Hybond, Amersham). Probes labelled with α-^{32}P were prepared, either of a 900-bp-long fraction of domestic pigeon genomic DNA digested in the same way and isolated from gel by 'death-wish' electro-elution (see Bruford et al. 1992), or of pure PR1 DNA (domestic pigeon, clone D3) isolated also by 'death-wish' electro-elution from plasmid DNA with PR1 insert digested with *Eco*RI.

For dot-blots, the genomic DNA was transferred to Hybond filters using a vacuum manifold and then probed with pure PR1 DNA probe. The amount of labelled DNA was measured using a Phosphor Imager (Molecular Dynamics).

Cloning and sequencing

The 900-bp-long fractions of genomic DNA digested with *Eco*RI were isolated by 'death-wish' electro-elution from agarose gel and cloned into the pUC18. ABI sequencing was performed using ABI Prism Dye Terminator kit (Perkin Elmer) following the manufacturer's instructions. For sequencing, a pair of M13 primers was used and four intermediate primers, two direct (5-CACCCAGGCCTCTGAAAACT and 5-GCTTTATAGTTTCTAGCAGATTCGTCTT) and two reverse (5-GATCTGCGTATTTTAGGCTTAGTC and 5-GCAGCCCAACACTACTTTTTATTC).

Preparation of chromosomes

Pigeon mitotic chromosomes were obtained by a standard method from primary fibroblast cultures. Cultures were made from 4- to 5-day-old embryos. Cells were grown in Dulbecco's minimum essential medium (DMEM), 15 mmol Hepes, and with 10% fetal calf serum in CO_2 incubator at 37°C.

Lampbrush chromosomes from both species were isolated manually according to the standard technique (Solovei *et al.* 1993, 1994). After brief fixation in 2% paraformaldehyde and post-fixation in 70% ethanol, some preparations were stained with Coomassie blue R250; others were dried from 100% ethanol and kept at −70°C before using for fluorescence *in situ* hybridization (FISH) or primed *in situ* labelling (PRINS).

Fluorescence *in situ* hybridization

The plasmid carrying the PR1 repeat was labelled with biotin-16-dUTP (Boehringer Mannheim) by nick translation or by random priming. After labelling and ethanol precipitation, probes were dissolved in hybridization mixture (50% forma-

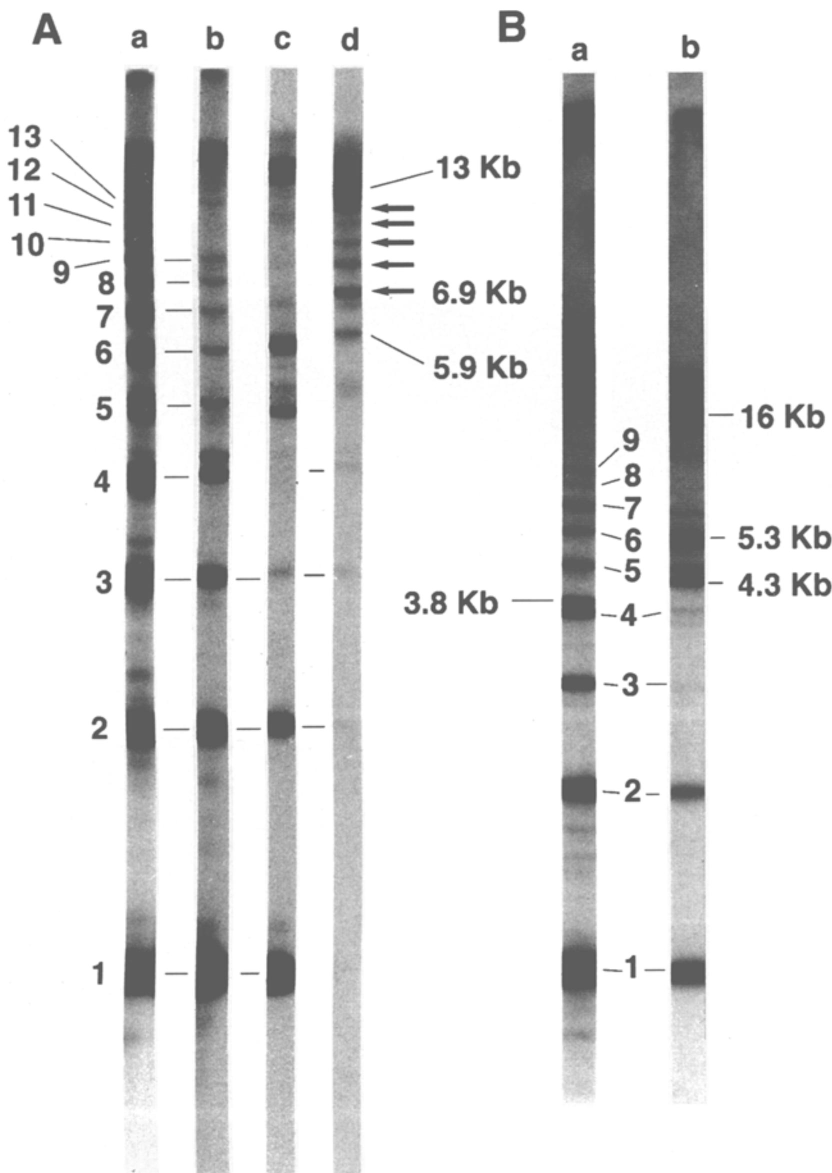


Figure 3. Genomic organization of the PR1 sequence. **A** Digested genomic DNA of domestic pigeon (a & b) and wood-pigeon (c & d) probed with approximately 900-bp fraction extracted from agarose gel after digestion with *Eco*RI. a, *Bam*HI digestion; b & c, *Eco*RI digestion. Figures on the left (1–13) are lengths in repeat units of PR1; arrows on the right point to the ladder with 1 repeat unit distance between the bands based upon a 6.9-kb band. **B** Genomic DNA of domestic pigeon (a) and wood-pigeon (b) probed with dpD3 plasmid DNA, *Eco*RI digestion. Figures between the lanes are lengths in repeat units; figures on left and right are lengths of additional bands.

mide, 2 × SSC, 10% dextran sulphate) at a concentration of 10 ng/μl.

The DNA of mitotic chromosomes was denatured by dipping preparations into 70% formamide in 2 × SSC at 70°C for 1 min. For lampbrush chromosomes, the time of denaturation was increased to 3 min. Hybridization was carried out at 37°C in dry chambers under coverslips sealed with rubber cement for 12–18 h.

After hybridization, preparations were washed in 50% formamide in 2 × SSC at 42°C, in 2 × SSC at 42°C and in 2 × SSC at room temperature. Detection of probe hybridization was performed with avidin DN conjugated with fluorescein isothiocyanate (FITC; Vector Laboratories), followed by one round of amplification. Chromosomes were counterstained with propidium iodide (Ockleford *et al.* 1981) and DAPI.

Some slides with LBCs were treated with ribonuclease A (100 μg/ml in 2 × SSC, 1 h at 37°C) before hybridization to prevent DNA/RNA transcript hybridization.

Primed *in situ* labelling

For primed *in situ* labelling, the following mixture was applied to each slide: 50 μl of *Taq* buffer with 1 unit of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.01% bovine serum albumin (BSA), 5% glycerol, 0.1 mM each of dATP, dCTP, dGTP and 0.05 mM biotin-16-dUTP, and 250 ng of each primer (one direct and one reverse at a distance of about 300 bp from each other). The programme was as follows: 94°C for 1 min, 55°C for 3 min and 70°C for 10 min. Sites of DNA synthesis were detected by incubation with avidin DN conjugated with FITC.

Microscopy

After FISH or PRINS, chromosomes were studied using a conventional fluorescence microscope (Opton) and a Biorad MRC600 confocal scanning laser attachment to a Zeiss Axiovert microscope. The grey-scale images were false coloured using the 'autumn look-up table'. Images were processed and assembled into plates using Adobe Photoshop software.

Results

The PR1 sequence

A highly repetitive sequence of about 900 bp (PR1) was found in both species of pigeon. One clone from each species was sequenced (Figure 1A) (EMBL accession numbers X97402 and X97403). To extend these data, one further clone from domestic pigeon and three clones from wood-pigeon were sequenced partially or fully in one direction only. These all proved to be very similar to one another, leading to the conclusion that the sequence of PR1 is the same in both species.

At positions 260–280, a sequence was identified in PR1 that is similar to the CENP-B box of mammals (Figure 1B). This is followed (or preceded, if the 5 to 3 orientation of CENP-B box is adopted) by a long polypurine/polypyrimidine tract (nucleotides 290–515). Within this region, there is an insertion/deletion of about 10 bp. The two sequenced clones from domestic pigeon and one of the clones from wood-pigeon did not have these 10 nucleotides. The internal repeat with the GGCAA to GGCTA motif starts at position 519 (Figure 1A). The above-mentioned deletion and variation in the number of copies of the internal repeat (about 5–12) more or less account for differences in the total length of the cloned copies: 892 bp and 903 bp in domestic pigeon, 893–925 bp in wood-pigeon. Outside these two regions, there are only single nucleotide variations scattered over the entire length of PR1.

Genomic organization of PR1

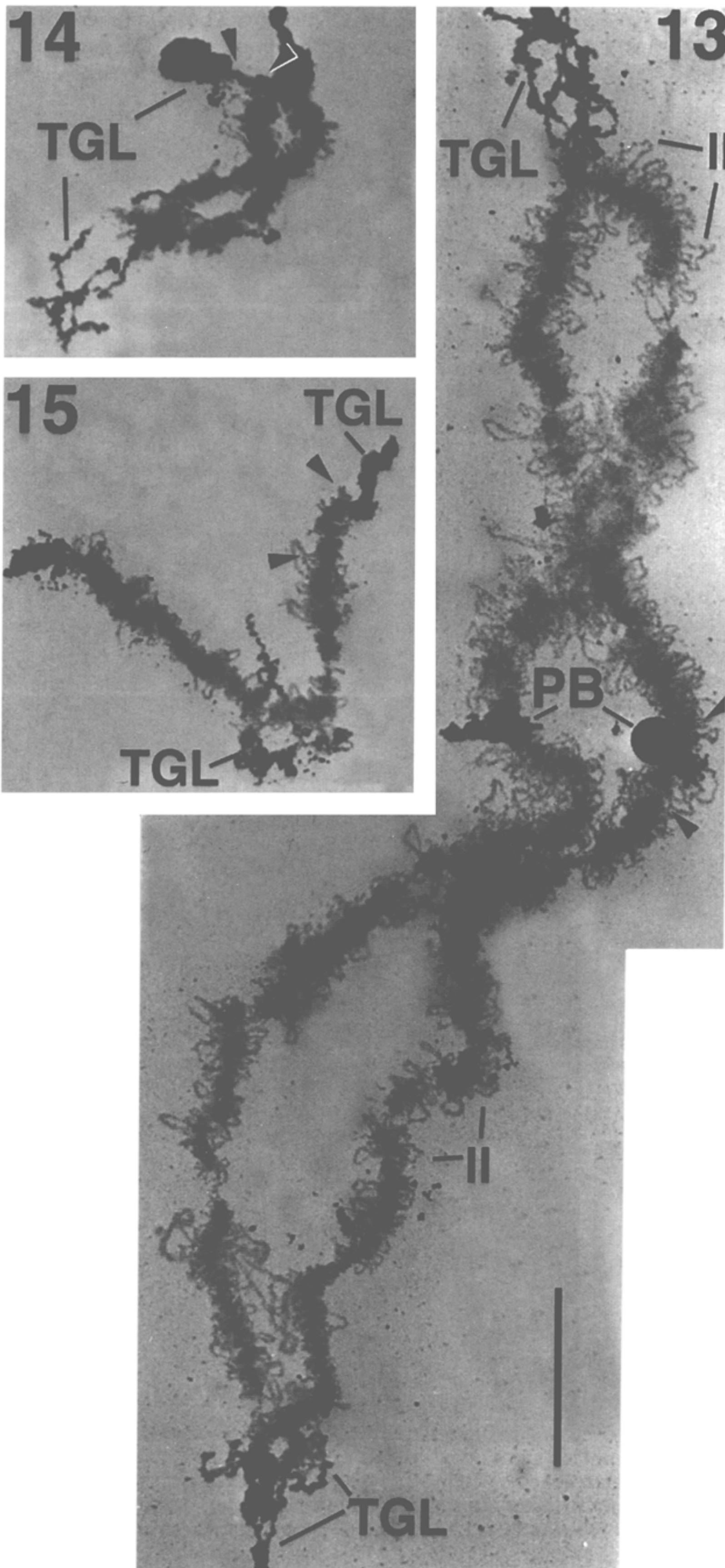
The relative amounts of PR1 DNA in the genomes of the two species were estimated from dot-blot hybridizations of known amounts of genomic DNA from each species and DNA from a plasmid containing PR1 (dpD3) as a standard, with labelled PR1 cut out from the same plasmid. Direct measurement of radioactivity on the blot (Figure 2), using a Phosphoimager, indicated that PR1 represents about 5% of the genome in domestic pigeon and about 2% in wood-pigeon.

The two species differ with respect to the genomic organization of the PR1 repeat. In domestic pigeon, conspicuous ladders are present in blots after digestion with restriction enzymes (Figure 3). Labelled fragments of up to nine PR1 repeats were observed on blots after digestion with *Eco*RI (Figure 3A, lane b and Figure 3B, lane a). Even longer fragments, up to 13 PR1 units, were observed after digestion with *Bam*HI (Figure 3A, lane a): this higher number probably indicates that the *Bam*HI site is present in a smaller proportion of the PR1 copies than the *Eco*RI site.

Long ladders are not evident on autoradiographs of gel blots after digestion of DNA from wood-pigeon. After digestion with *Eco*RI, even the band corresponding to only four tandem copies of PR1 was weak (Figure 3A, lane c and Figure 3B, lane b). After digestion with *Bam*HI (Figure 3A, lane d), the band corresponding to the basic monomer was scarcely visible and bands corresponding to 2–4 repeats were weak. However, an additional ladder of up to five bands, based on a relatively strong band at 6.9 kb, was present. Each band of this ladder was one PR1 unit longer and weaker than the previous one. Tandem arrays of more than four or five units of PR1 were never seen on blots of *Eco*RI- or *Bam*HI-digested wood-pigeon DNA.

In both species, restriction enzymes also generate fragments of lengths not proportional to that of the basic PR1 unit but recognized by PR1 probes. Such 'out-of-ladder' bands were first observed on a blot probed with a 900-bp fraction of DNA extracted from a gel after digestion of genomic DNA by *Eco*RI (Figure 3A). The presence of PR1 in these bands was confirmed by probing with the pure cloned PR1 sequence (Figure 3B). Hence, the out-of-ladder fragments contain both PR1 DNA and some other DNA and that is why their lengths are not proportional to that of the basic PR1 unit. It is important to note that the intensities of all out-of-ladder bands were of the same order as those of the middle and upper bands of the true PR1 ladder. The non-PR1 components of these fragments are, therefore, also repetitive.

Figures 4–12. Results of FISH (4, 6 & 8–12) and PRINS (5 & 7) with PR1 probe on nuclei, mitotic and meiotic chromosomes from domestic pigeon. **Figures 4–6.** Interphase nuclei from cultured pigeon fibroblasts. Note that localization of PR1 (label on Figure 4A) does not fully correspond to heterochromatic lumps (white spots on Figure 4B) revealed in the same nucleus by propidium iodide staining. In Figures 4 & 5, scale bars = 10 μm. In Figure 6, scale bar = 5 μm. **Figures 6 & 7.** PR1 repeat revealed on six pairs of medium-sized chromosomes (their numbers are marked by green figures on Figure 6). Scale bars = 5 μm. **Figure 8.** Late diplotene bivalents 6–11 isolated from the same oocyte nucleus; note condensed chromosome axes. Scale bar = 10 μm. **Figures 9 & 10.** Medium-sized chromosomes at well-developed lampbrush stage with labelled chromomeres at the centromeric regions. Arrowheads point to the borders of regions with short loops; large arrow indicates the protein body; TL, telomere loops. Scale bar = 10 μm. **Figures 11 & 12.** Lampbrush chromosomes 2. A pair of tiny loops (small green arrows) at the locus of the PBs (white arrows) is labelled (Figure 11), while no labelling is detectable if the chromosomes are pretreated with RNAase before the hybridization (Figure 12). Scale bar = 10 μm.



Figures 13–15. Lampbrush chromosomes from domestic pigeon oocytes in permanent preparations stained with Coomassie R250: macrobivalent (Figure 13) and medium-sized bivalents (Figures 14 & 15). Centromeric regions of chromosomes are marked by arrowheads; PB, protein body; ll, lateral loops, TGL, telomere giant loops. Scale bar = 25 μm.

In domestic pigeon, there was only one out-of-ladder fragment with a length of 3.8 kb after digestion with *Eco*RI. It cannot be excluded, however, that another weak band of the same kind overlaps with the regular 7-unit PR1 band, which would make the latter somewhat stronger than the regular 6-unit PR1 band (Figure 3B, lane a). After digestion with *Bam*HI, no out-of-ladder bands were observed in the domestic pigeon.

In wood-pigeon, after digestion with *Eco*RI, there were prominent out-of-ladder bands at 5.3 kb and 16 kb and a less prominent one at 4.3 kb. The 16-kb band was wide and probably included some 15-kb material (Figure 3A, lane c, and Figure 3B, lane b). After digestion with *Bam*HI, there were a weak out-of-ladder band at 5.9 kb and a major out-of-ladder band at about 13 kb in addition to the 6.9-kb band and the ladder based on it, as mentioned above (Figure 3A, lane d). All the steps in this ladder are out of phase with those of the regular (pure PR1) ladder, because of the 6.9-kb tail in each band.

Chromosome localization of PR1

Interphase nuclei and mitotic chromosomes

Interphase nuclei in splash preparations of cultured pigeon fibroblasts and nuclei of pigeon erythrocytes have been subjected to FISH and PRINS with PR1. The labelled chromatin occupies a large area in the nuclei of both types (Figures 4 & 5) and is only partially colocalized with clumps of condensed heterochromatin (Figure 4A & B).

Both conventional FISH and PRINS on mitotic chromosomes from cultured fibroblasts show that in domestic pigeon PR1 occupies pericentromeric regions on six pairs of acrocentric medium-sized chromosomes, numbers 6–11 (Figures 6 & 7). FISH on lampbrush chromosomes revealed one more minor site in the centromere region of chromosome 2 (see next section). Unfortunately, we were unable to obtain mitotic chromosome preparations from wood-pigeon. FISH to lampbrush chromosomes provided evidence for PR1 in the putative centromeric regions of all chromosomes in this species (see next section).

Lampbrush chromosomes

Lampbrush chromosomes (LBCs) from pigeon oocytes show the usual lampbrush organization with an axis consisting of chromomeres, each of which carries one or more pairs of lateral loops. The loops are of different lengths and are sites of intensive transcription of RNA from the DNA axis of the loop (Figures 13–18). A peculiar feature of LBCs in pigeons is the organization of the loops that spring from the very last terminal chromomere. In the majority of bird species from which LBCs have been examined, telomere loops on some or nearly all the chromosomes are large, have a distinctive morphology and have been called telomere giant loops (TGLs) (Khutinaeva *et al* 1989, Solovei *et al.* 1993, 1994). Telomere loops in birds are also often open-ended, the loop being attached to its parent chromomere only at one end, so that the tip of the other end represents the true tip or telomere of the meiotic chromatid (Solovei *et al.* 1994, 1995). In pigeons, TGLs are present at both ends of nearly all chromosomes and are extraordinarily large, in some cases reaching hundreds of microns in length and having an unusually dense RNP matrix that stains intensely with Coomassie R250 (Figures 13–15). The RNP matrices of TGLs often fuse, so that the LB bivalents of domestic pigeon are often joined to one another at their ends.

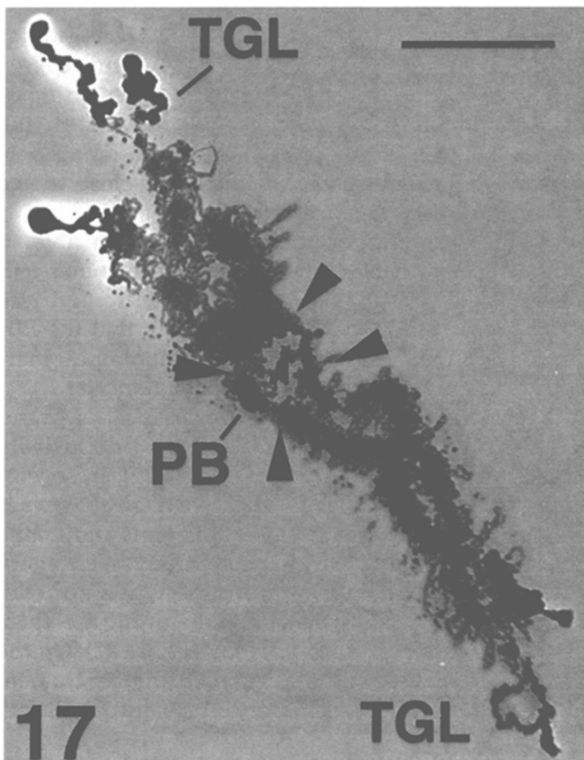
The TGLs of wood-pigeon are spectacularly long and nearly all of them are fused together forming gigantic star-like structures (Figure 16). One bivalent (four chromatid ends) can participate in three or even four of these star-like structures, so that all the chromosomes form a complicated network in which the TGLs represent by far the most massive objects in the entire germinal vesicle (Figures 16 & 21).

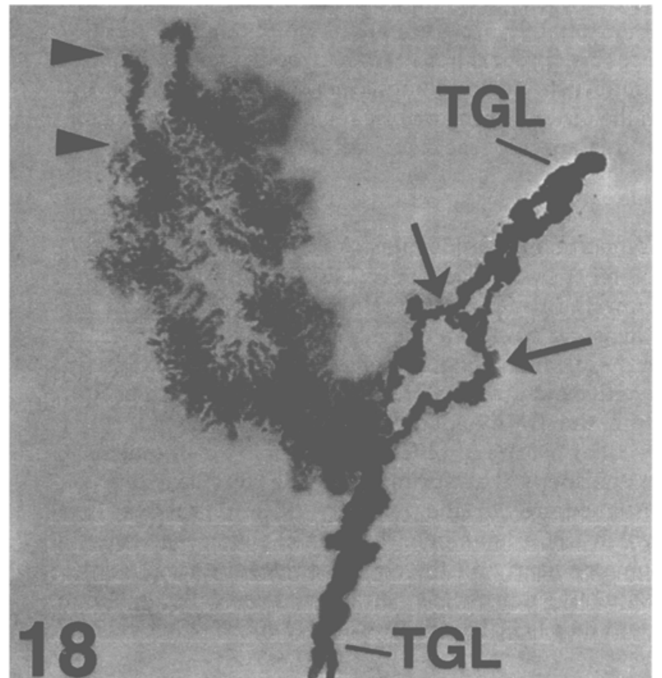
Another important feature of LBCs of domestic pigeon and wood-pigeon is the presence of so-called protein bodies (PBs) (Figures 13 & 18). These are perfectly round objects of variable size and absolutely characteristic appearance that are regularly associated with specific regions on all the LBCs of domestic and wood-pigeon. The PBs are always located in regions of the LBCs that have uniformly short loops: this is especially so in wood-pigeon (Figures 16 & 18). To judge from the positions of centromeres on metaphase chromosomes, the lampbrush centromeres might be expected to be in the same regions of the chromosomes as the PBs and the short loop regions (Khutinaeva *et al.* 1989).

FISH performed on LBCs of domestic pigeon under conditions favouring both DNA/DNA and DNA/RNA hybridization produced labelling on chromosomes 6–11 in the short loop regions to which the PBs were attached (Figures 8–10). Chromosome 2 was also labelled on one pair of small loops close to the locus of the PBs (Figure 11).

We therefore consider that we have been able to identify the positions of the lampbrush centromeres in domestic pigeon using FISH with a centromerically located probe (PR1), and we have shown that the PBs and short loop regions are reliable morphological markers for lampbrush centromeres in this species.

In wood-pigeon, the label after DNA/DNA and DNA/RNA hybridization was also found exclusively in the region of the PB loci (Figures 20–22). In several preparations of wood-pigeon LBCs we were able to see almost the entire set of lampbrush bivalents and, in the course of our studies, we have certainly seen every bivalent many times over. Accordingly, we are confident that the PB/short loop regions of all the chromosomes label with PR1 after FISH (Figure 21). On the basis of our reliable identification of the centromere regions in domestic pigeon and the general similarity


TGL
PB
17
TGL


TGL
18
TGL

of the karyotypes and lampbrush markers in the two species, we conclude that the lampbrush regions to which PR1 binds in wood-pigeon are also the centromeric regions in this species.

Transcription of PR1 at the LBC stage

Transcription of PR1 on LBCs was investigated by FISH under conditions favouring DNA/RNA hybridization, such that the probe binds to the nascent RNA transcripts that are associated with and attached to the DNA axes of the lampbrush loops. Essentially, the chromosomal DNA is not denatured and the chromosomes are not pretreated with ribonuclease, so that single-stranded RNA is available to hybridize with the single-stranded DNA probe.

In domestic pigeon, no PR1 transcripts are detectable at the lampbrush stage at any of the six PR1 sites found on mitotic chromosomes. Only one site of RNA transcription was located in domestic pigeon on a single pair of small loops (4–5 μm long) right next to the PBs on chromosome 2 (Figure 11).

In wood-pigeon LBCs, PR1 transcripts were easily detectable at sites at which the sequence was located. All the loops in the short loop/PB regions of all chromosomes were labelled (Figures 20–22). As well as the small loops that are characteristic of these regions, at least one microbivalent also showed labelling of a pair of big lateral loops arising from the PB region (Figure 22).

After control DNA/RNA transcript FISH preceded by ribonuclease treatment, no labelling was detectable either at the PB locus of the second bivalent from domestic pigeon (Figure 12), or on any loops in the short loop/PB regions of LBCs from wood-pigeon.

It is also worth mentioning that, in wood-pigeon, the label in PR1-positive chromosome regions of LBCs after DNA/DNA hybridization is weak or absent because much of the PR1-containing DNA is decondensed to form the axes of lateral loops. The DNA/DNA hybrids registered in these regions can be detected only in more mature oocytes in which the lampbrush transcription has declined, the loops have retracted and the chromosome axis has undergone considerable shortening and condensation (Figure 19). In domestic pigeon, there is no transcription of PR1 on chromosomes 6–11, and chromomeres in the short loop/PB regions of these chromosomes are always well labelled (compare Figure 8 with Figures 9 & 10).

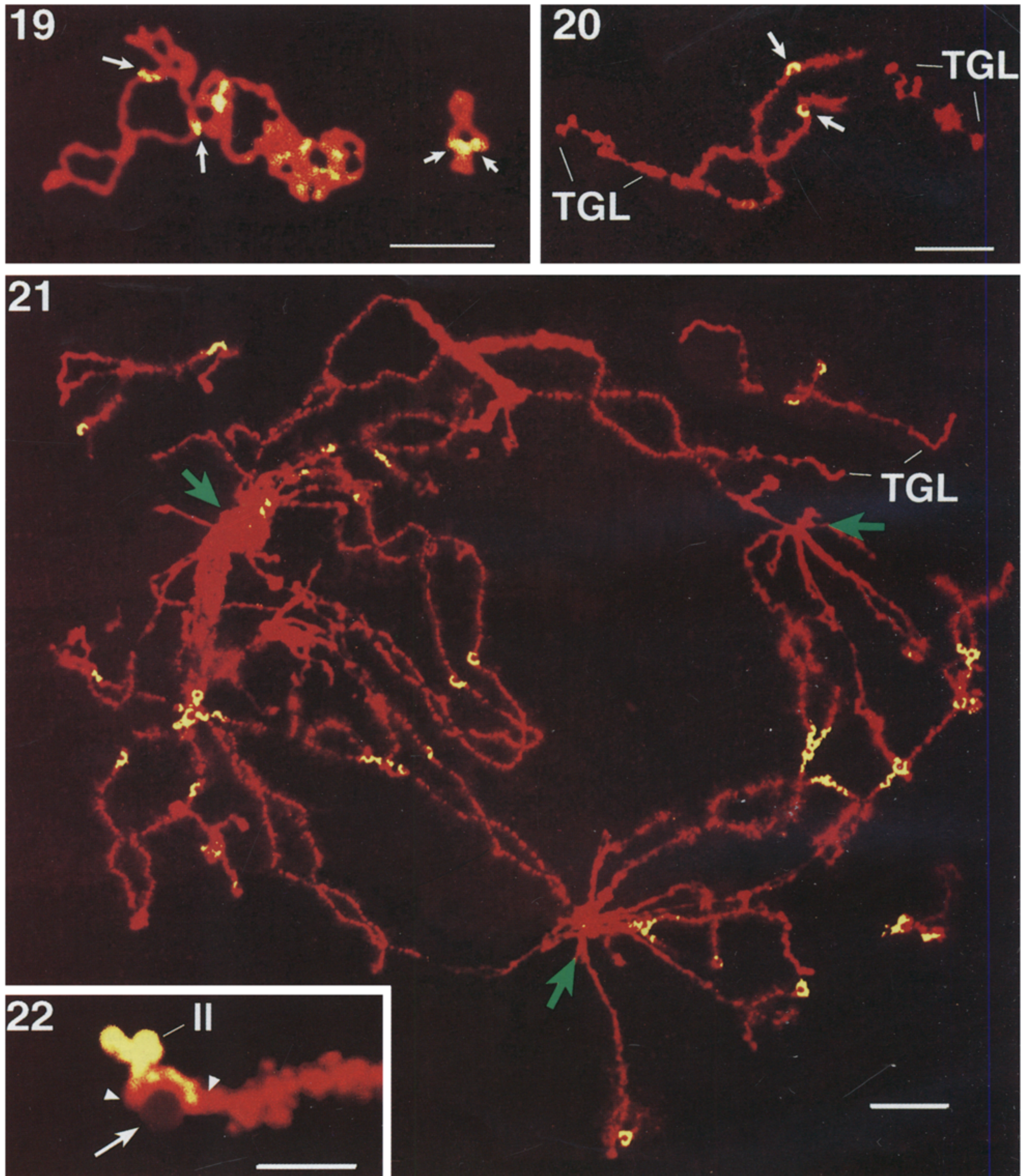
Discussion

PR1 is a new satellite repeat so far only recorded in two species of pigeon. The sequence of PR1 is almost identical in both species. PR1 does not resemble any other DNA sequence currently listed in EMBL or GENEBANK. Nor does it resemble the other centromerically localized repeated DNA sequences, the 190-bp polymorphic repeat family, that have been identified and characterized in other species of birds (Longmire *et al.* 1988, Chen *et al.* 1989, Madsen *et al* 1992a,b, 1994).

Without any exceptions, PR1 is concentrated around the centromeric regions of pigeon chromosomes. It is detectable by FISH on all the chromosomes of the wood-pigeon, but on only six chromosomes at the centromeric site and one other minor site, also centromeric, in domestic pigeon. It is noteworthy that PR1 contains a sequence that has some similarity to the CENP-B box (Figure 1B) that is recognized by the centromeric protein, CENP-B, in mice and humans (Masumoto *et al* 1989, Kipling *et al* 1995). This sequence is a likely candidate for the avian analogue of the CENP-B box. On the other hand, our inability to detect the PR1 sequence by FISH or PRINS on the majority of chromosomes from domestic pigeon does not fully correspond to this suggestion.

On LBCs from both species, the regions at which PR1 was detectable by FISH always have small uniform loops and PBs. Centromere regions on the LBCs of other species of bird have so far invariably been characterized by the same short loops and, in the majority of species, also by PBs (Chelysheva *et al.* 1990, Khutinaeva *et al.* 1989), and it seems that PBs, which are morphologically highly distinctive and easily identifiable, may be a reliable marker for centromere positions on the lampbrush chromosomes of many birds. Chicken and other Galliformes do not have PBs.

Figures 16–18. Lampbrush chromosomes from wood-pigeon oocytes under a phase-contrast microscope (Figure 16) and in permanent preparations stained with Coomassie R250 (Figures 17 & 18). **Figure 16.** One of the star-like centres (SLCs) formed by joining of the ends of at least seven half-bivalents. Two telomeric loops of one end of one chromosome (arrows) fuse together and make one of the arms of the star-like centre. Note the microbivalent on the right (Mi), both half-bivalents of which send their telomere loops to the same SLC. The other ends of the microchromosomes are centromeric regions. They do not have telomere giant loops, but bear PBs that often fuse and make one common PB. The macrobivalent on the upper left (Ma) is subacrocentric (probably number 3); it has four pairs of telomeric loops, two of which go to the same SLC shown on the photograph; one pair join another SLC (not shown but out of the picture to the top), and the fourth pair of loops lie free, probably owing to disturbance of the chromosome net during removal of the nuclear envelope. ll, lateral loops. **Figures 17 & 18.** Medium-sized bivalent (Figure 17) and microbivalent (Figure 18) with clearly visible centromeric regions with short loops (arrowheads). Note that chromosomes, even of the same bivalent, can differ with respect to the presence or absence of PBs (PB, Figure 17), or may lack them altogether (Figure 18). Scale bars = 25 μm.



Perhaps the most striking aspect of our observations is that the arrangement of PR1 repeats is entirely different in the two species of pigeon. The presence of out-of-ladder bands after restriction digestion shows that, in both *C. livia* and *C. palumbus*, tandem arrays of PR1 form part of large repeating units of at least 16 kb in *C. livia* (domestic pigeon) and about 23 kb in *C. palumbus* (wood-pigeon). Such large repeating units have also been reported in other parts of bird genomes. The DNA of the heterochromatin of the long arm of the chicken Z chromosome, for example, is organized in long (24 kb) repeats (Hori *et al.* 1996).

These large centromeric repeats in pigeons are different in the two species with regard to the number of PR1 repeats that they contain and the lengths of the non-PR1 out-of-ladder restriction fragments that are visible in blots probed with PR1. Our schemes for the arrangement of PR1 in the large repeats are shown in Figure 23, where we have attempted to relate the relative positions and arrangements of PR1 and other sequences to the observed restriction fragments produced by *Eco*RI and *Bam*HI. Specifically, with regard to our observations using PR1 as a FISH probe on lampbrush chromosomes of pigeons, we consider it especially noteworthy that in domestic pigeon (*C. livia*) the lengths of uninterrupted tandem arrays of PR1 are at least 14 units of the sequence, while in wood-pigeon (*C. palumbus*) they rarely exceed 4 units, as visualized on our gels, which show the products of digestion under the same controlled conditions.

With regard to the transcription of PR1, as inferred from the FISH-detectable presence of PR1-complementary RNA on lampbrush loops, PR1 is transcribed on many short loops in the pericentromeric regions of all chromosomes of the wood-pigeon, whereas in the domestic pigeon it is not transcribed at any of the major pericentromeric sites at which we know it to be present, although it is transcribed at one minor site on chromosome 2 in this species.

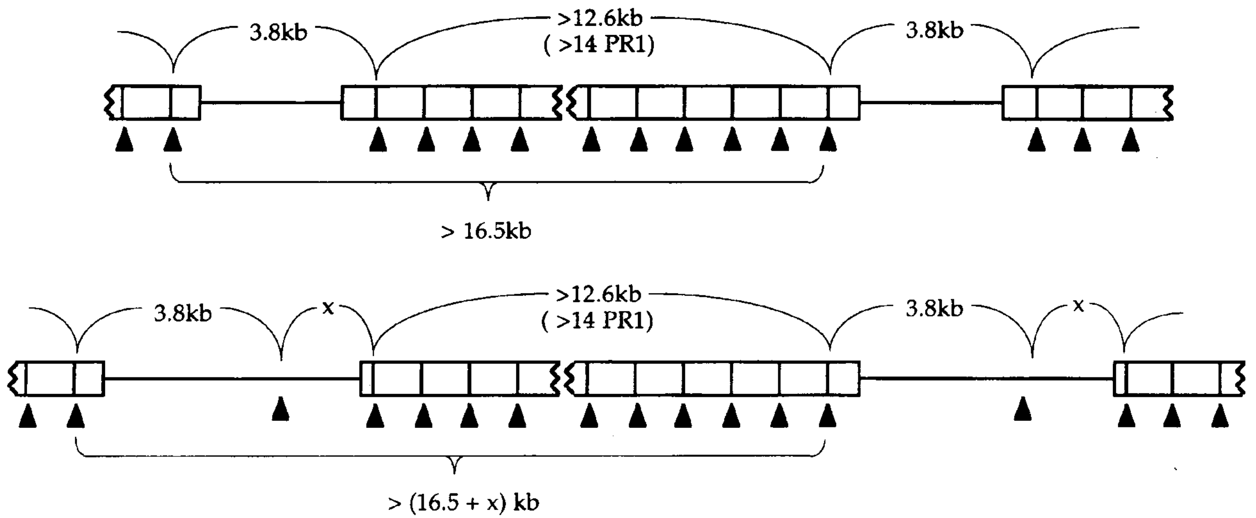
Transcription of non-coding repeated DNA sequences on lampbrush chromosomes has been explained on the basis of the 'read-through' hypothesis (Varley *et al.* 1980, Gall *et al.* 1983). The strongest basis for this hypothesis is found in the extremely elegant work of Gall and his colleagues on the S1 satellite and the histone genes of the North American newt *Notophthalmus* (Gall *et al.* 1983). We consider that our observations on PR1 in avian lampbrush chromosomes have some bearing on this hypothesis.

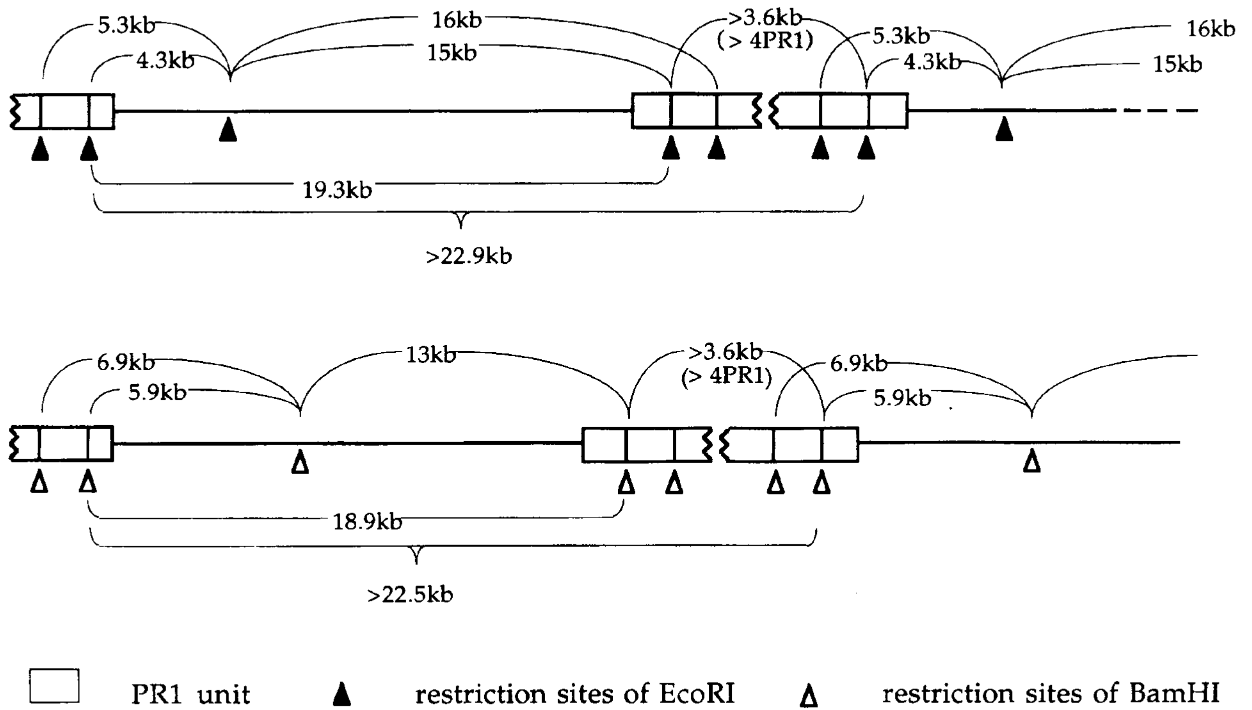
Discussion of this question is perhaps best introduced by consideration of another avian macrorepeat (CZMR) that is known to be localized on the long arm of the Z chromosome in chickens (Hori *et al.* 1996). CZMR is about 24 kb long. There are about 400 copies of it per haploid genome. It represents approximately 1% of the chicken genome. The terminal heterochromatin region of the Z chromosome has also been estimated to contain about 1% of the entire chromosomal DNA. Most of the Z-terminal heterochromatin in chicken must, therefore, consist of CZMR. FISH, under conditions favouring hybridization to nascent RNA transcripts on lampbrush loops, has shown that CZMR is transcribed on about 20 short loops in the Z-terminal heterochromatin region. These loops are about 5 μm long. The DNA axis of each loop is, therefore, unlikely to contain much more than 20 kb, which is equivalent to less than one copy of CZMR. It seems certain, therefore, that only a few copies of the macrorepeat are transcribed. These observations are consistent with the notion that transcription is not initiated in the CZMR repeat itself, but on some low copy number sequences, perhaps genes, that are interspersed between the CZMR repeats.

The same general principles can be applied to the PR1 situation in pigeons. We found no difference between the PR1 of wood-pigeon and that of domestic pigeon. Hence, initiation of transcription within the PR1 repeat in one species but not the other seems unlikely. Yet the sequence is liberally transcribed on lampbrush chromosomes from wood-pigeon, but scarcely at all in domestic pigeon. We must, therefore, suppose that transcription of PR1 is initiated in some non-PR1 sequences, perhaps some genes that occur either within the PR1-containing macrorepeats or in regions flanking the macrorepeats themselves (see Figure 24). Poor FISH to chromomeres in the PR1 lampbrush regions suggests that most, if not all, the PR1 sequences are pulled out to form the lateral loops. It is not possible to determine the exact number of PR1-containing lateral loops, nor is it possible to know exactly how much DNA is present in the axis of each loop. Nevertheless, the crucial point here is that PR1 transcripts are detectable on many small loops in the centromeric regions of all chromosomes in wood-pigeon, suggesting a regular repeated association of transcription initiation points (promoters) and PR1 macrorepeats (Figure 24). Accordingly, unless transcription is initiated in non-coding sequences, we would have to suppose that a considerable number of genes are located within the centromeric heterochromatin of wood-pigeon.

In domestic pigeon, there is no transcription of the PR1 repeats that are present in the centromeric regions of any of the chromosomes, with the sole exception of

Figures 19–22. Results of FISH with PR1 probe on lampbrush chromosomes from wood-pigeon. **Figure 19.** Labelled centromeric regions (arrows) of several late diplotene macro- and medium-sized bivalents with condensed axes that no longer show distinct chromomeric organization. Scale bar = 10 μm. **Figure 20.** A macrobivalent (number 4 or 5) with labelled centromere regions. Note a characteristic semicircular form of the labelled regions (arrows), owing to the fact that they are curved around the PBs (compare Figure 22); the labelled loops at these regions are so short (compare Figures 17 & 18) that they cannot be resolved, and the whole centromere regions have the appearance of brightly labelled curved bars. Scale bar = 20 μm. **Figure 21.** Three joined star-like centres (green arrows) uniting about 22 bivalents (out of a total of 39); another three microbivalents lie free at the top and bottom right corners of the picture. Scale bar = 25 μm. **Figure 22.** Labelled centromere region (its borders are marked with arrowheads) of an acrocentric microchromosome bent around the unstained PB (arrow); small labelled lateral loops at the centromere region (compare Figures 17 & 18) cannot be resolved after hybridization. A pair of big lateral loops (ll), an unusual feature for the centromere region, is also labelled. Scale bar = 5 μm.


A
C. livia (domestic pigeon) DNA digested with EcoRI
3.8kb
>12.6kb
(>14 PR1)
3.8kb
> 16.5kb
3.8kb
x
>12.6kb
(>14 PR1)
3.8kb
x
> (16.5 + x) kb


B
C. palumbus (woodpigeon) DNA digested with EcoRI and BamHI
5.3kb
16kb
>3.6kb
(> 4PR1)
5.3kb
16kb
4.3kb
15kb
4.3kb
15kb
19.3kb
>22.9kb
6.9kb
13kb
>3.6kb
(> 4PR1)
6.9kb
5.9kb
5.9kb
18.9kb
>22.5kb
PR1 unit
restriction sites of EcoRI
restriction sites of BamHI

the minor site on chromosome 2. If transcription in wood-pigeon were initiated in structural genes, these same genes might also be expected to initiate transcription on the LBCs of domestic pigeon. Indeed, there are lampbrush loops in these regions, but they do not carry PR1 transcripts. We therefore propose that in domestic pigeon the arrangement of PR1 and interspersed transcribed sequences is different from that in wood-pigeon, such that the PR1 sequence in domestic pigeon is not involved in transcription (see Figure 24). This model takes account of the relatively large unbroken tandem arrays of PR1 that we have identified in domestic pigeon, the shorter and more interspersed arrays in wood-pigeon and the presence or absence of PR1 transcripts on lampbrush loops in PR1-containing regions. It is the basis of our proposal that transcription of a non-coding repeated DNA sequence on lampbrush chromosomes relates to its genomic organization.

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Figure 23. Proposed schemes for the arrangement of PR1 arrays in larger repeating units. **A** Domestic pigeon. Two possible interpretations of the *Eco*RI digestion data are shown. In the top example, there are no *Eco*RI sites in non-PR1 DNA between the PR1 arrays, so that only a 3.8-kb out-of-ladder band is seen. In the lower example, *Eco*RI sites are present in non-PR1 DNA but one of the two bordering fragments (X) contains too short a portion of the PR1 sequence to be visible on blots. **B** Wood-pigeon. The results of digestion with two different enzymes correspond with respect to the length of non-PR1 DNA situated between PR1 arrays (about 19 kb). This correspondence strengthens the suggestion that the 19 kb value is not an underestimate, such as might result from the presence of more restriction sites and consequent unlabelled fragments.

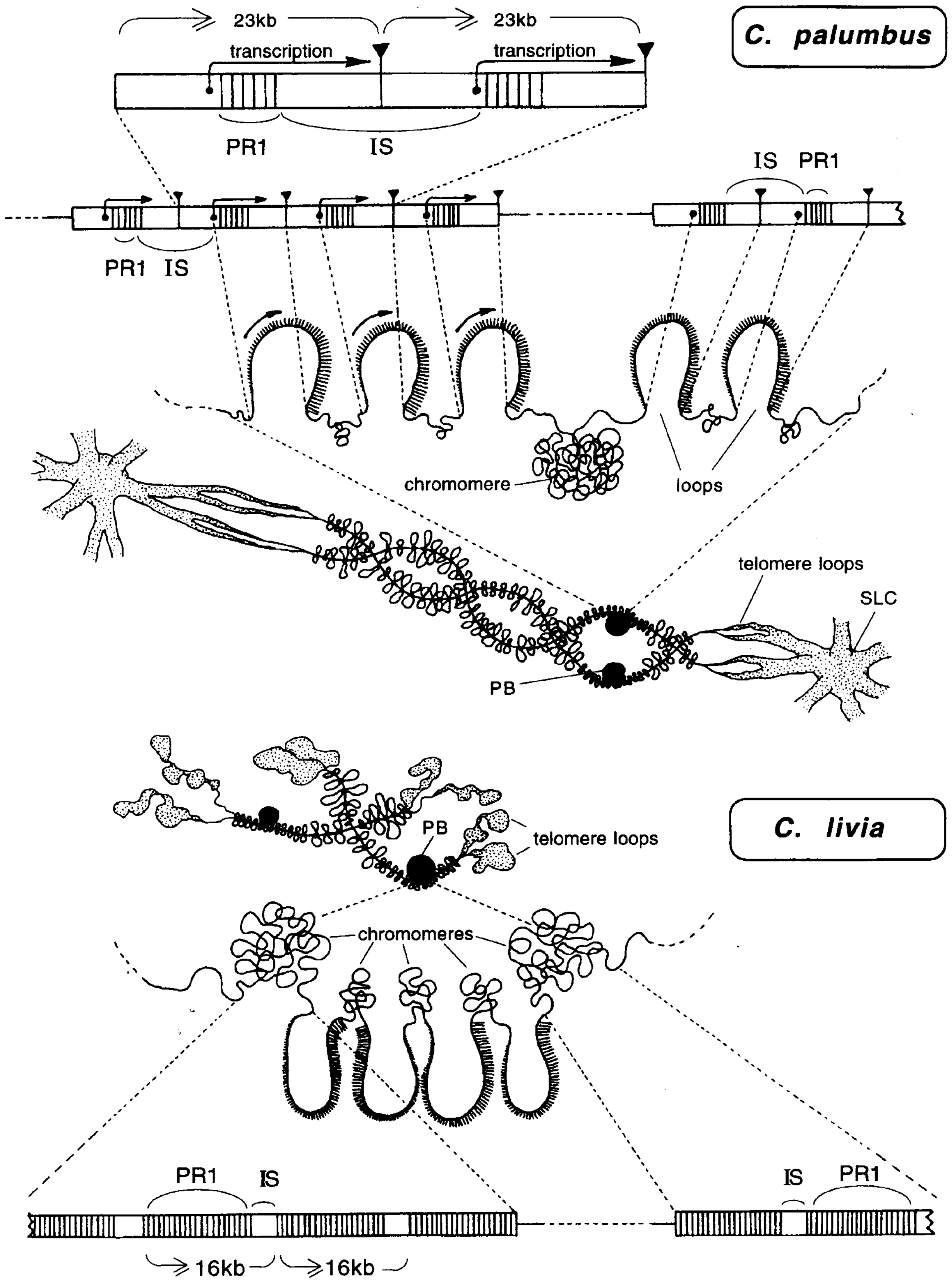
C. palumbus
23kb
transcription
23kb
transcription
PR1
IS
IS PR1
PR1 IS
chromomere
loops
telomere loops
SLC
PB
C. livia
PB
telomere loops
chromomeres
PR1
IS
IS
PR1
16kb
16kb

Figure 24. Hypothetical scheme depicting a possible relationship between the arrangements of PR1 repeats within larger repeating units in domestic pigeon and wood-pigeon genomic DNAs. In wood-pigeon, short tandem arrays of PR1 are interspersed with other sequences that include numerous transcriptional promoters, such that the PR1 sequences are transcribed on the short lampbrush loops in the centromeric regions of the lampbrush bivalents. According to the 'read-through' hypothesis, a promoter will lie at the start of each loop transcription unit and much of the remainder of the loop downstream from the promoter will consist of PR1 sequences and associated transcripts. In domestic pigeon, PR1 occurs in larger tandem arrays with smaller arrays of interspersed sequences and no transcriptional promoters in the immediate vicinity of the PR1 sequences. There is, therefore, no transcription in PR1-containing regions of the chromosomes, and the short loops of the centromeric regions are transcribing non-PR1 DNA. PR1, arrays of PR1 repeats; IS, interspersed sequences; PB, protein bodies; ↱ initiation and direction of transcription; ▼ transcription stop; arrows along lampbrush loops show the directions of transcription. SLC, star-like complexes formed by fusion of the matrices of telomere giant loops from different lampbrush half-bivalents.

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