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Presence of female-specific bent-repetitive DNA sequences in the genomes of turkey and pheasant and their interactions with W-protein of chicken

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Abstract. Two female-specific repetitive DNA units, the 0.4 kb PstI and 0.5 kb TaqI sequences, were detected in the genomic DNA of turkey and pheasant, respectively, by Southern blot hybridization under non-stringent conditions with the W chromosome-specific 0.7 kb XhoI repetitive unit of chicken as a probe. Cloning and sequencing of these two repetitive units revealed that they shared features with the XhoI family repetitive unit of chicken although the overall similarities of the nucleotide sequences were less than 60%. In common with the chicken XhoI family they consisted of tandem repeats of about 21 bp, the majority of which contained $(A)_{3-5}$ and $(T)_{3-5}$ clusters separated by six or seven relatively G+C-rich sequences, and they behaved as bent DNA molecules on polyacrylamide gel electrophoresis at room temperature. W-protein, purified from chicken liver nuclei and shown to bind with high affinity to the XhoI family repetitive unit, also bound with the cloned repetitive units from turkey and pheasant. DNase I footprint analysis suggested that the mode of interaction of W-protein with these units was similar to that with the 0.7 kb XhoI sequence. On the other hand, W-protein did not bind to the female-specific 0.4 kb BamHI repetitive unit from the Bobwhite quail. The 0.4 kb BamHI sequence contained some A and T clusters but these clusters did not appear in phase with the pitch of DNA helix and the repetitive unit did not show DNA bending.

Introduction

Most eukaryotic genomes contain significant amounts of highly repetitive DNA sequences and those sequences are usually located in the constitutive heterochromatin. However, it has not been elucidated how such highly repetitive DNA sequences participate in the formation of heterochromatin. Nearly the entire W chromosome, a female-specific sex chromosome of chicken, *Gallus gallus domesticus*, is stained dark by the Giemsa C-banding technique in the metaphase set and forms a heterochromatic body in the interphase nucleus. We have shown that a highly repetitive DNA family, designated the XhoI family, constitutes about 50% of the W chromosomal DNA of the chicken (Tone et al. 1984) and that its major repetitive unit, i.e. an about 0.7 kb sequence produced by XhoI digestion, consists of tandem repeats of about 21 bp, the majority of which contain $(A)_{3-5}$ and $(T)_{3-5}$ clusters separated by six or seven relatively G+C-rich base pairs (Kodama et al. 1987). Both cloned and genomic sequences of the 0.7 kb XhoI repetitive unit show unusually slow mobilities on polyacrylamide gel electrophoresis at room temperature, which is most likely caused by sequence-directed DNA bending (Kodama et al. 1987). We have also shown that a non-histone nuclear protein, designated the W-protein, binds with high affinity to both cloned and genomic sequences of the XhoI family repetitive unit (Harata et al. 1988).

In this study, female-specific repetitive DNA sequences of turkey and pheasant were detected by hybridization with the XhoI family repetitive unit under conditions of low stringency and were cloned and sequenced. The cloned repetitive units were shown to share the internal repeat structure and DNA bending with the XhoI family repetitive unit. It was also shown that W-protein could interact with these heterologous repetitive units in the same manner as with the chicken XhoI family repetitive unit.

Materials and methods

Preparation of DNA. High molecular weight DNA was prepared from the blood of the following species as described by Tone et al. (1982); male and female White Leghorn chickens (*G. g. domesticus*), male and female turkeys (*Meleagris gallopavo*), male and female Japanese common pheasants (*Phasianus versicolor*), and male and female Bobwhite quails (*Colinus virginiatus*).

Southern blot hybridization under different conditions of stringency. DNA samples digested with appropriate restriction enzymes (4 units enzyme per 1 μg DNA, 37° C, 12 h) were separated by 1.0% agarose gel electrophoresis, visualized by staining with ethidium bromide, denatured and transferred by capillary blotting to a nylon membrane filter (Biodyne A BNNG222S, Pall Corp. or Gene Screen Plus, NEN). Cloned repetitive units isolated from the recombinant plasmids were labeled with [α-^{32}P]dCTP (~3,000 Ci/mmol, Amersham) by the mixed-primer extension method of Feinberg and Vogelstein (1983) and used as probes. The DNA filter was pre-incubated in a hybridization buffer [5 × SSPE (1 × SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA, pH 7.5), 0.1% sodium dodecyl sulphate (SDS), 5 × Denhardt's solution, 50 μg/ml sheared, denatured salmon sperm DNA] at 65° C for 6 h. The heat-denatured ^{32}P-labeled probe was then added and the reaction was carried out at either 65° or 37° C for 18–20 h.

After the reaction, the DNA filter was washed first in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) at room temperature for 15 min and then in 6 × or 0.3 × SSC for 15 min, three times, at the same temperature as for the reaction. The extent of base pair mismatch allowed in the DNA-DNA hybrid was estimated according to an empirical formula (Meinkoth and Wahl 1984) and assuming that the T_m of the hybrid decreases by 1° C for 1% base pair mismatch present (Bonner et al. 1973). The washed filter was subjected to autoradiography at −70° C using X-Omat S film (Kodak) and X-Omatic regular intensifying screen (Kodak).

Slot blot hybridization. A sheet of nylon membrane filter (Gene Screen Plus) was soaked in 2 × SSC and placed on a filtration manifold (Minifold II, Schleicher & Schuell). High molecular weight DNA samples were heat-denatured, applied into the wells of the manifold with gentle suction, and rinsed with 6 × SSC. The DNA filter was dried at 60° C for 30 min and subjected to hybridization with the ^{32}P-labeled probe in the hybridization buffer at 65° C. After the reaction, the filter was washed in 0.3 × SSC at 65° C, dried and autoradiographed. The intensity of autoradiographic signals was determined by densitometry using a Chromato-scanner (CS-930, Shimadzu Corp.)

Molecular cloning of female-specific repetitive DNA units of turkey, pheasant and Bobwhite quail. DNA from the female turkey was digested with PstI and separated by 1.0% agarose gel electrophoresis using a low melting temperature agarose (Agarose EP, Takara Shuzo, Kyoto). An ethidium bromide-stained band of about 0.4 kb was recovered according to the method of Wieslander (1979). The 0.4 kb fragments were ligated to PstI-digested pUC119 and used to transform *Escherichia coli* MV1304. One thousand colonies were screened by hybridization with the ^{32}P-labeled 0.4 kb PstI fragment recovered from the gel under stringent conditions (reaction in the hybridization buffer at 65° C and final washing in 0.3 × SSC at 65° C). One of the positive clones, pUMG0401, containing one copy of the 0.4 kb fragment, was selected. DNA from the female pheasant was digested with TthHB8I(TaqI) and subjected to 1.0% agarose gel electrophoresis. DNA fragments were recovered as described above from the region a little lower than the ethidium bromide-staining 0.5 kb band. These fragments were ligated to AccI-digested pUC119 and used to transform *E. coli* MC1061. Two thousand colonies were screened by hybridization with the ^{32}P-labeled 0.4 kb PstI unit from the turkey under less stringent conditions (reaction in the hybridization buffer at 45° C and final washing in 6 × SSC at 45° C). One of the positive clones, pUPV0501, containing one copy of the approximately 0.5 kb fragment was selected. A fragment containing an AT region (shown in Fig. 3B) was recovered from pUPV0501 by digestion with AluI and was subcloned into the SmaI site of pUC119 to yield pUPV-AT. DNA from the female Bobwhite quail was digested with BamHI and an ethidium bromide-stained band of about 0.4 kb was recovered, ligated to BamHI-digested pUC119 and used to transform *E. coli* JM101. Three hundred colonies were screened by hybridization with the ^{32}P-labeled 0.4 kb BamHI fragment recovered from the gel under stringent conditions as described above. One of the positive clones, pUCV0401, containing one copy of the 0.4 kb fragment, was selected.

DNA sequencing. Inserts in pUMG0401 and pUPV0501 were recovered and recloned into pUC118. Five kinds of subfragments from these inserts, as indicated in Figure 3, were also recloned into pUC118 and pUC119. The insert in pUCV0401 was digested with EcoRI and the resulting two subfragments were recloned into pUC118 and pUC119. Single-stranded forms of these recombinant plasmids were prepared according to Vieira and Messing (1987) and sequences of the inserts were determined by the dideoxy chain termination method (Sanger et al. 1977) using the 17-mer M13 sequencing primer (Amersham) and [α-^{32}P]dCTP as a radioactive substrate.

Detection of DNA bending by polyacrylamide and agarose gel electrophoresis. DNA fragments (0.2 μg/lane) were electrophoresed in a 4.0% polyacrylamide gel (30:0.5 (w/w), monomer:bisacrylamide) or in a 1.5% agarose gel at 5 V/cm in TAE buffer (0.008 M Tris acetate, 0.001 M sodium acetate, 0.4 mM EDTA, pH 8.0). During electrophoresis, the temperature was maintained either at 16° or 55° C in an air incubator. After electrophoresis, DNA fragments were detected by staining with 0.5 μg/ml ethidium bromide and their mobility in different gel systems was compared.

Purification of W-protein. W-protein was purified from a 0.35 M NaCl extract of nuclei from female chicken liver as described by Harata et al. (1988).

Gel retardation assay. Cloned repetitive units were recovered from the recombinant plasmids and labeled at their 5′ ends with [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Takara Shuzo) according to Maxam and Gilbert (1980). The following reaction mixture (25 μl) was incubated at 15° C for 30 min: 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA, 0.01 M 2-mercaptoethanol, 0.1% Triton X-100, 4% glycerol, 0.08 M NaCl, 0–7,000 ng of sheared *E. coli* DNA, 2.5 ng (about 10000 cpm) of the ^{32}P-labeled repeating unit, 1–20 μl (about 10 ng protein/μl) of the purified W-protein fraction. After incubation, 5 μl of 25% glycerol, 0.05 M EDTA, 0.05% bromophenol blue was added and the mixture was subjected to 1.5% agarose gel electrophoresis as described by Harata et al. (1988). The gel was dried partially in a gel drier without heating and autoradiographed.

DNase I footprint analysis. pUMG0401 containing the 0.4 kb PstI repetitive unit was linearized with EcoRI (HindIII) or HindIII (XbaI) and its 5′ or 3′ ends were ^{32}P-labeled. Labeling of the 5′ end was performed as described above. The 3′ end was labeled with [α-^{32}P]dCTP (Amersham) and Klenow fragment (Takara Shuzo). The end-labeled plasmid was digested with HindIII(EcoRI) or XbaI-(HindIII), to yield a DNA insert which was ^{32}P-labeled at one end. pUPV-AT containing the AT fragment was linearized with HindIII(XbaI), end-labeled as above and cleaved with XbaI(HindIII). The labeled fragments were separated by 1.0% agarose gel electrophoresis and recovered from the gel using DE81 paper according to Dretzen et al. (1981). About 2.5 ng of the labeled DNA fragment and 40 ng of sheared *E. coli* DNA were incubated for 30 min at 22° C in the presence or absence of W-protein in 25 μl of the mixture as used for the gel retardation assay, except that 0.004 M $MgCl_2$ was added. DNA was then partially digested with 5 μg/ml DNase I (Takara Shuzo) for

40 s at 22° C and the digestion was terminated by addition of 0.02 M EDTA and 10 μg of tRNA. Preparation of the A/G-cleaved sequence ladder and analysis by denaturing polyacrylamide gel electrophoresis and autoradiography were carried out as described by Harata et al. (1988).

Results

Molecular cloning of female-specific repetitive DNA units from the genomic DNA of turkey and pheasant

When genomic DNA from male and female turkeys was digested with PstI and separated by agarose gel electrophoresis, a female-specific band of about 0.4 kb was visible by staining with ethidium bromide (Fig. 1A, lane 2). This 0.4 kb fragment was hybridizable with the chicken XhoI family 0.7 kb repetitive unit under conditions allowing about 60% base pair mismatches (Fig. 1B, lane 2). Similarly, when genomic DNA from male and female pheasants was digested with TaqI and subjected to electrophoresis and Southern blot hybridization with the XhoI family repetitive unit under conditions of low stringency as above, female-specific bands of about 0.5 and 0.9 kb were detected (Fig. 1D, lane 2). The 0.5 kb fragment moved a little faster on electrophoresis than the ethidium bromide-stained fragments shown in lanes 1 and 2 of Figure 1C.

The XhoI family repetitive unit did not hybridize with the 0.4 kb PstI and the 0.5 kb TaqI fragments under conditions allowing about 40% base pair mismatches (data not shown), indicating that these two fragments have limited sequence similarity with the chicken XhoI family repetitive unit.

The 0.4 kb PstI and the 0.5 kb TaqI fragments were cloned into the PstI and AccI sites of pUC119, respectively. Two recombinant clones, pUMG0401 containing the 0.4 kb sequence of turkey and pUPV0501 containing the 0.5 kb sequence of pheasant, were used in the following experiments.

Female specificity and copy numbers of the cloned repetitive units

Genomic DNA of male and female turkeys was digested with PstI (Fig. 2A, lanes 1 and 2) or HinfI (Fig. 2A, lanes 3 and 4), electrophoresed and subjected to Southern blot hybridization with the ^{32}P-labeled 0.4 kb insert of pUMG0401 under stringent reaction conditions. The cloned repetitive unit hybridized only with several female-derived restriction fragments produced with either enzyme. Similar experiments were carried out on TaqI or HinfI digests of DNA of male and female pheasants using the ^{32}P-labeled 0.5 kb insert of pUPV0501 as a probe. As shown in Figure 2B, several female-specific hybrid bands (lanes 2 and 4) were detected in the digests with either enzyme, but, in contrast to the results with the turkey DNA, common bands of hybridization between the DNA of males and females were also present.

The repetition frequency of the 0.4 kb PstI and 0.5 kb TaqI units in the genomic DNA of turkey and pheasant, respectively, was estimated by probe-excess Southern blot and slot blot hybridization procedures under stringent conditions and assuming that the diploid genome size of turkey and pheasant was 1.9 and 1.7 pg, respectively (Shapiro 1968). These results are summarized in Table 1A, B. The

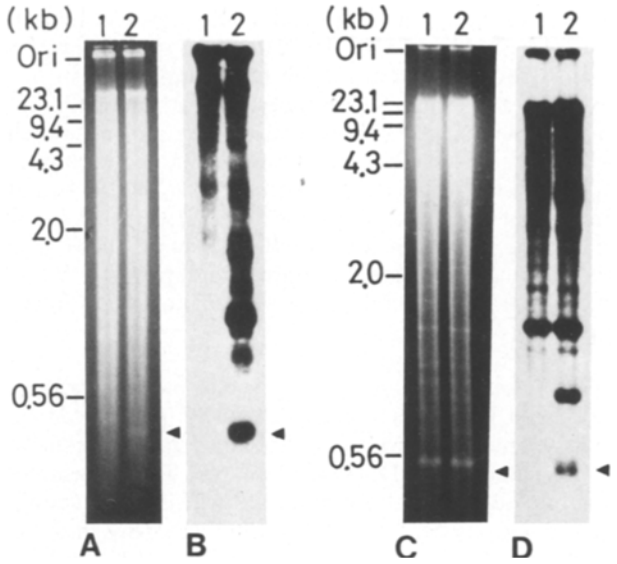

Fig. 1A–D. Detection of female-specific restriction fragments hybridizable with the XhoI family sequence of chicken in the genomic DNA of turkey (**A** and **B**) and pheasant (**C** and **D**). PstI-digested DNAs from male (lane 1) and female (lane 2) turkey were electrophoresed (4 μg/lane) in a 1.0% agarose gel, stained with ethidium bromide (**A**) and subjected to Southern blot hybridization with the ^{32}P-labeled 0.7 kb XhoI fragment from pAGD0601 (Tone et al. 1984) and autoradiography (**B**). TaqI digested DNAs from male (*lane 1*) and female (*lane 2*) pheasant were subjected to electrophoresis (**C**) and blot hybridization (**D**) as for turkey DNA. DNA filters were incubated with the ^{32}P-labeled probe in hybridization buffer at 37° C and washed in 6 × SSC at 37° C. *Arrowheads* indicate fragments that were recovered from gels for molecular cloning

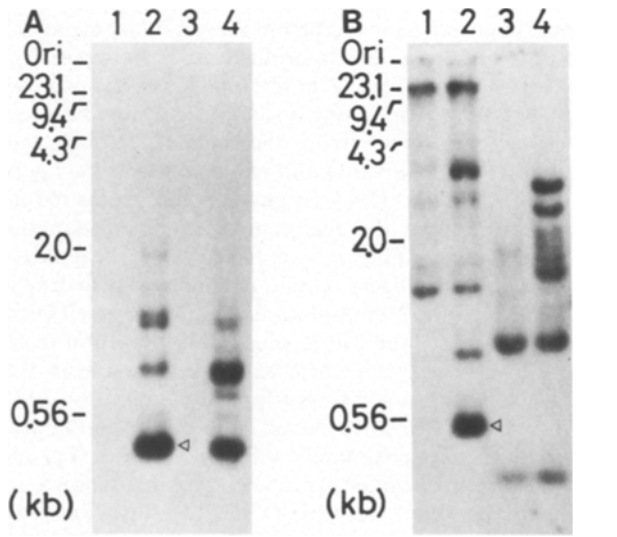

Fig. 2A, B. Female specificity of the cloned repetitive units. Genomic DNA (4 μg/lane) from male (*lanes 1* and *3*) and female (*lanes 2* and *4*) turkey (**A**) or pheasant (**B**) were digested with PstI (*lanes 1* and *2* in **A**), TaqI (*lanes 1* and *2* in **B**) or HinfI (*lanes 3* and *4* in **A** and **B**), electrophoresed, blotted and probed with the ^{32}P-labeled 0.4 kb PstI fragment from pUMG0401 (**A**) or the ^{32}P-labeled 0.5 kb TaqI fragment from pUPV0501 (**B**). Reactions were carried out in hybridization buffer at 65° C and DNA filters were finally washed in 0.3 × SSC at 65° C. *Arrowheads* indicate the fragments corresponding to those which were cloned

Table 1. Repetition frequency of the 0.4 kb PstI and 0.5 kb TaqI repetitive units and their related sequences in the diploid genomes of turkey and pheasant

A

Genome	0.4 kb PstI unit[a]	0.4 kb PstI-related sequences[b]
Female turkey	9300–11000	30000–33000
Male turkey	UD[c]	<600

B

Genome	0.5 kb TaqI unit[a]	0.5 kb TaqI-related sequences[b]
Female pheasant	3700– 4500	18000–21000
Male pheasant	UD	7500– 9000

[a] Determined by Southern blot hybridization
[b] Determined by slot blot hybridization and expressed as multiples of the repetitive unit
[c] Undetectable

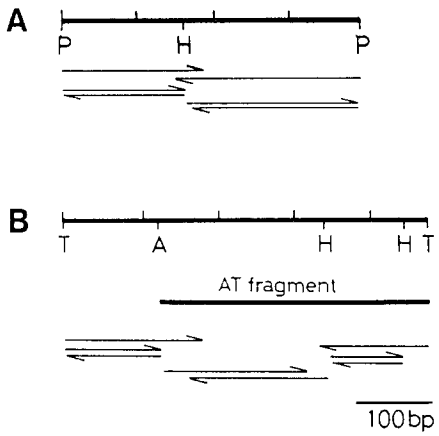

Fig. 3A, B. Sequencing strategy for the inserts in pUMG0401 (0.4 kb PstI repetitive unit of turkey) (**A**) and pUPV0501 (0.5 kb TaqI repetitive unit of pheasant) (**B**). In **B**, an AluI-TaqI subfragment, designated the AT fragment and used in the DNase I footprint analysis, is shown. Restriction sites shown are: *P* PstI; *H* HinfI; *T* TaqI; *A* AluI. *Arrows* indicate direction and extent of sequencing

Internal Repeat No.	Position	Sequence	Number of Nucleotides
	1	GTTTCCTCCC	
1	11	ACAAATACCATTTTTTCAACC	21
2	32	AGAAATAGGACGTTTTTCTCCC	22
3	54	AGAAATACCGGATTTTTGCCCC	22
4	76	CAAAACATGACATTTTCTCCC	21
5	97	AGAAATACGAGTTTTCTCCC	20
6	117	AAAATATGATATTTTGCACC	20
7	137	AGAAATTCCAGTTTTATCACC	21
8	158	GAAGACTCTACGTTTTCTACC	21
9	179	AGAAATACCAATTATCTCC	19
10	198	GCAAAAATTACATTTTCTCC	20
11	218	AGAAATACCAGATTTCTTCCC	21
12	239	TTAAATATGACACCTTTTCC	20
13	259	AAGAAATAGTAGATTTTTCCCC	22
14	281	AAAAATATGACATTTTCTCC	20
15	301	AGGAAATGCCAGTTTTATCGT	21
16	322	ATAAATATGACATTTTATACC	21
17	343	GCAAATATCCGCTTTCTCCC	20
18	363	AAAAATATGCCATTTTCTGCC	21
	384	AGGAACTGCA	
A Consensus		AGAAATA$^{TG}_{CC}$NCATTTTCTCCC	21

Internal Repeat No.	Position	Sequence	Number of Nucleotides
	1	C	
1	2	GACAAAATACCACCATTCTCCC	22
2	24	ACAGAGATGGCATTTCATCCC	21
3	45	ACAAGTACTACTTCACACTCC	21
4	66	ACACGATGATACTTTCCATC	20
5	86	AAGAATAGGGCATTGGACCAC	21
6	107	AGAAATACCAGCTTTCTGCCT	21
7	128	AAGAGATGACATTTTCTCCC	20
8	148	AGAAATACCACTTTTCTCCC	20
9	168	AGAAATACAGGAACTTTTCTGC	22
10	190	CAGAAACACCATGCTCATCCTC	22
11	212	TCCAGATGTTGTTTTCACCCC	21
12	233	AAACACTAGGACCTTTCCTC	20
13	253	TCACTACTCCCTGCTTTTTC	20
14	273	AAAACTAGATGGTCTTCTCTCCC	23
15	296	AGAAATACTAGCATTCTCTGC	21
16	317	AAGATGGGACCTTTTCCAA	19
17	336	CCAAAGATGGTAGAGTCTCCC	21
18	357	AGAAATAGCACTTTTCTTC	19
19	376	ATCAGAACTGTCATTTTCTCC	21
20	397	AAAAAAATACTACTTTCAACT	21
21	418	GTTCCAGGTGACATTTGCAGCC	22
22	440	AAACATAGGAGAGATTCTCC	20
23	460	ACAAAGTAACCACTTTTCTCTC	22
	482	ACGT	
B Consensus		AGAAATANNNN$^{A}_{C}$TTTTCTCCC	21

Fig. 4A, B. Nucleotide sequence and internal repeats of the inserts in pUMG0401 (**A**) and pUPV0501 (**B**). *Underlining* indicates A_n and T_n clusters ($n \geqq 3$). Nucleotide position 1 in **A** is the G after the cleavage site in the PstI recognition sequence and that in **B** is the C after the cleavage site in the TaqI recognition sequence. N in the consensus sequence means any one of four bases

0.4 kb PstI unit is repeated about 10000 times in the diploid genome of the female turkey. Closely related sequences, about twofold more abundant than the 0.4 kb repeats, are also present almost exclusively in the genome of the female (Table 1A). On the other hand, although the 0.5 kb TaqI repeats are female specific, about twofold more closely related sequences are present in the genome of the male pheasant (Table 1B).

Nucleotide sequences and internal repeat structures

The nucleotide sequence of the inserts in pUMG0401 and pUPV0501 was determined using the strategies shown in Figure 3. It was revealed that the cloned 0.4 kb PstI and 0.5 kb TaqI units comprised 393 and 485 bp, respectively, and that both units consisted of internal repeats similar to those constituting the W chromosome-sepcific XhoI family repeats of the chicken (Kodema et al. 1987). Figure 4 shows that the 0.4 kb PstI and 0.5 kb TaqI units consist of 18 and 23 tandem repeats of 19 to 23 bp sequences, respectiely, most of which contain $(A)_{3-5}$ and $(T)_{3-5}$ clusters. These internal repeats were clearly noticeable by Harr plot analysis (Harr et al. 1982; data not shown). The unique feature of the chicken XhoI family sequence, that is, alternate appearances of $(A)_{3-5}$ and $(T)_{3-5}$ clusters possibly at every pitch of a DNA helix, is also well applicable to the 0.4 kb PstI sequence of turkey, but, to a lesser degree, to the 0.5 kb TaqI sequence of pheasant.

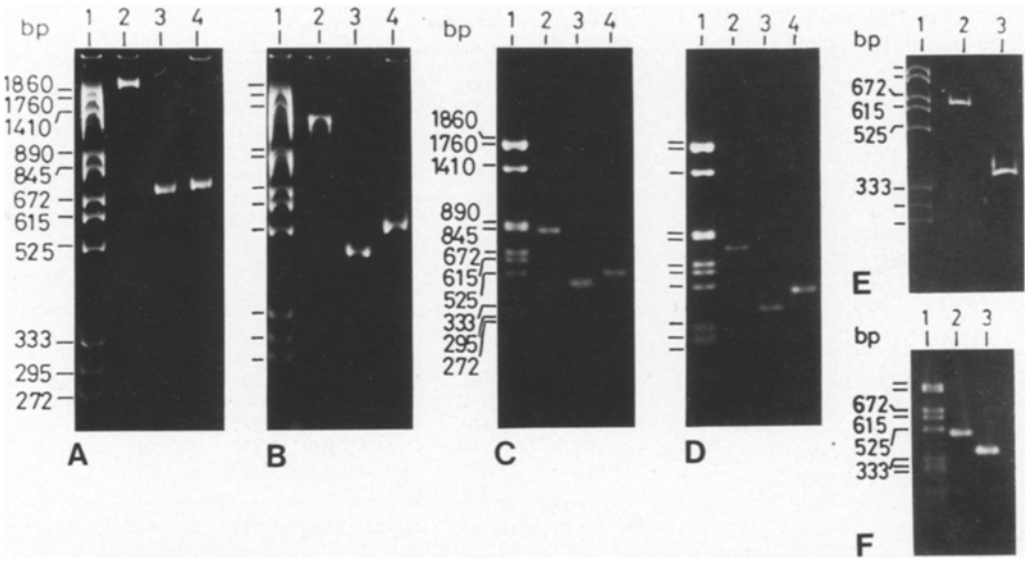

Fig. 5A–F. Electrophoretic behavior of the cloned repetitive units. 4% polyacrylamide gel electrophoresis (**A, B** and **E**) and 1.5% agarose gel electrophoresis (**C, D** and **F**) were carried out at 16° C (**A, C, E** and **F**) or 55° C (**B** and **D**). In **A** to **D**, DNA samples applied were as follows: HaeIII digest of PM2 DNA as size markers (*lane 1*), 0.7 kb XhoI unit (chicken) from pAGD0601 (*lane 2*), 0.4 kb PstI unit (turkey) from pUMG0401 (*lane 3*) and 0.5 kb TaqI unit (pheasant) from pUPV0501 (*lane 4*). In **E** and **F**, DNA samples were: HaeIII digest of PM2 DNA (*lane 1*), AT fragment from pUPV0501 (*lane 2*) and 0.4 kb BamHI unit (Bobwhite quail) from pUCV0401 (*lane 3*)

Table 2. Fragment size of cloned repetitive units determined by nucleotide sequencing or deduced from electrophoretic mobility

Repetitive unit	Fragment size determined by nucleotide sequencing (bp)	Fragment size deduced from electrophoretic mobility (bp)			
		1.5% agarose gel		4% polyacrylamide gel	
		16° C	55° C	16° C	55° C
0.7 kb XhoI (chicken)[a]	717	800 (1.12)[b]	730 (1.02)	>1860 (>2.59)	1200 (1.16)
0.4 kb PstI (turkey)	399	490 (1.23)	400 (1.00)	700 (1.75)	450 (1.13)
0.5 kb TaqI (pheasant)	489	540 (1.10)	490 (1.00)	720 (1.47)	530 (1.08)
AT fragment (pheasant)	451[c]	480 (1.06)	–[d]	650 (1.44)	–
0.4 kb BamHI (Bobwhite quail)	389	380 (0.98)	–	350 (0.90)	–

[a] Origin of the repeating unit
[b] Size relative to that determined by nucleotide sequencing
[c] Including 83 bp of vector sequences
[d] Not determined

Unusual electrophoretic mobility of the repetitive units suggesting DNA bending

The chicken XhoI family repetitive units migrate much more slowly than expected from their fragment sizes on polyacrylamide gel electrophoresis at room temperature (Kodama et al. 1987). It has been shown that anomalously slow electrophoretic mobility of a DNA molecule on polyacrylamide gel electrophoresis at relatively low temperature is caused by DNA bending and the bending is generally considered to be sequence directed (Wu and Crothers 1984; Hagerman 1985; Koo et al. 1986; Diekman 1987). Because of the common features of the 0.7 kb XhoI, 0.4 kb PstI and 0.5 kb TaqI sequences, both the latter are expected to behave as bent DNA molecules.

As shown in Figure 5A and summarized in Table 2, the 0.7 kb XhoI, 0.4 kb PstI and 0.5 kb TaqI fragments moved much more slowly than expected from their actual fragment sizes on 4% polyacrylamide gel electrophoresis at 16° C. On the other hand, nearly normal mobility was observed for all of these repetitive units when 4% polyacrylamide gel electrophoresis was carried out at 55° C (Fig. 5B) or

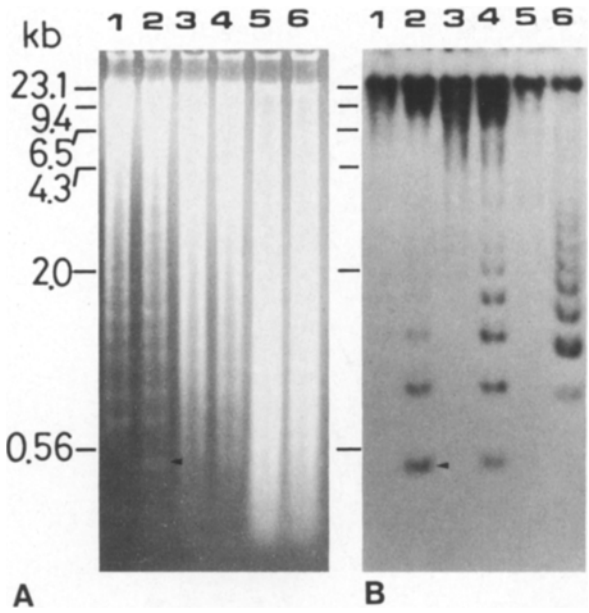

Fig. 6A, B. Detection of female-specific restriction fragments in the genomic DNA of Bobwhite quail. DNA from male (*lanes 1, 3* and *5*) and female (*lanes 2, 4* and *6*) Bobwhite quail was digested with BamHI (*lanes 1* and *2*), EcoRI (*lanes 3* and *4*) or PstI (*lanes 5* and *6*), electrophoresed (4 μg/lane) in a 1% agarose gel, stained with ethidium bromide (**A**) and subjected to Southern blot hybridization with the ^{32}P-labeled 0.4 kb BamHI fragment from pUCV0401 and autoradiography (**B**). *Arrowheads* indicate the 0.4 kb BamHI fragment which corresponds to the fragment used for the cloning of pUCV0401

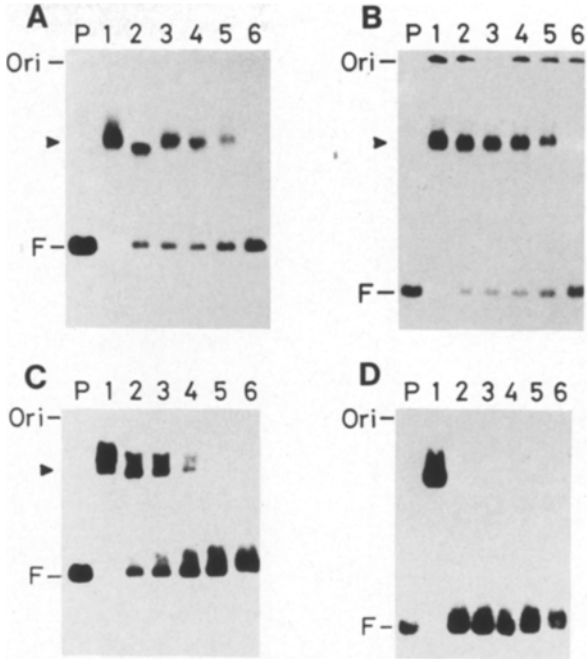

Fig. 8A–D. Gel retardation assays for the binding of W-protein with cloned repetitive units. One microliter from the purified W-protein fraction and 2.5 ng of one of the ^{32}P-labeled DNA fragments, as listed below, were incubated in the reaction mixture in the absence (*lane 1*) or presence of 150 ng (*lane 2*), 500 ng (*lane 3*), 1000 ng (*lane 4*), 3000 ng (*lane 5*) or 7000 ng (*lane 6*) of *Escherichia coli* DNA, and then the total reaction mixture was subjected to 1.5% agarose gel electrophoresis and autoradiography. The ^{32}P-labeled fragments used were: 0.7 kb XhoI unit from pAGD0601 (**A**), 0.4 kb PstI unit from pUMG0401 (**B**), 0.5 kb TaqI unit from pUPV0501 (**C**) and 0.4 kb BamHI unit from pUCV0401 (**D**). In *lane P*, a ^{32}P-labeled probe was electrophoresed with 2000 ng of *E. coli* DNA in the absence of protein. The position of the origin of electrophoresis (Ori), a free DNA probe (F) and specific DNA-protein complex (*arrowhead*), if present, is marked on the left of each panel

electrophoresis was carried out in a 1.5% agarose gel (Fig. 5C). In the latter case, mobility was affected only little at the two different temperatures (Fig. 5C, D). It is suggested from these results that the repetitive units from turkey and pheasant are also bent DNA molecules and the degree of bending is in the order of 0.7 kb XhoI > 0.4 kb PstI > 0.5 kb TaqI.

The mobility of the AT fragment (Fig. 3B) and of the 0.4 kb BamHI insert of pUCV0401, a female-specific repetitive unit of Bobwhite quail, in a 4% polyacrylamide gel (Fig. 5E) and a 1.5% agarose gel (Fig. 5F) at 16° C was also compared. As shown in Table 2, the AT fragment of pheasant moved more slowly than expected from its fragment size in the polyacrylamide gel, but the 0.4 kb BamHI fragment did not behave anomalously in either gel system. The latter fragment is thus suggested not to be a bent DNA molecule. The 0.4 kb BamHI fragment of Bobwhite quail is a female-specific repetitive unit (Fig. 6). Its nucleotide sequence contains several $(A)_{3-6}$ and $(T)_{3-5}$ clusters but their appearance is not in phase with the pitch of the DNA helix (Fig. 7).

Binding of chicken W-protein with the repetitive units from turkey and pheasant

W-protein, an M_r 72000 protein purified from an extract of nuclei of chicken liver, binds with high affinity to the XhoI family repetitive units of chicken (Harata et al. 1988). It has been suggested that the DNA wraps around W-protein or its multimeric form making contact through minor grooves consisting of A–T clusters and that the DNA bending facilitates stable interaction with the protein (Harata et al. 1988). If the DNA bending, caused by the appearance of A or T clusters at every turn of the DNA helix throughout the repetitive unit, is an important factor in the recognition and binding of W-protein, then the similar features of the 0.4 kb PstI sequence of turkey and the 0.5 kb TaqI

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1   GATCCAAAAAATTTCACTTCCAACACCATAAAATGAGTCCTTTTCACGTGTAAACCACGATTTCTTGGAATAGGACCGGT   80
81  ACCGGCTGATTATCGAAGTCGCGGTTTAGTGCACGGCTGTGTGCTGTCTAGCGACTTTTTCAGCGGCAATAGCAGCAGTA  160
161 ACCGCGATTGATGCGTTTCGAGCTGAATTCTCGGTTCAGCACAGTTCGAACATAAAACTCGTTGCCGTTTTGCTATGCAT  240
241 ATTATAAGGGTGCATTTGCAATGTTCCGATGTTAGAGTTTTTGGGGAATACTCAGAGCATGTAGAATCACACATGCATGA  320
321 GGATGTGGAAACATCCTAATGTAGAGCAGGATGAGAAGAAAAACGAACCCTCTCACTTACATTGG                385
```

Fig. 7. Nucleotide sequence of the insert in pUCV0401. *Underlining* indicates A_n and T_n clusters ($n \geq 3$). Nucleotide position 1 is the G after the cleavage site in the BamHI recognition sequence. The EcoRI site which was utilized for subcloning and sequencing is *boxed*

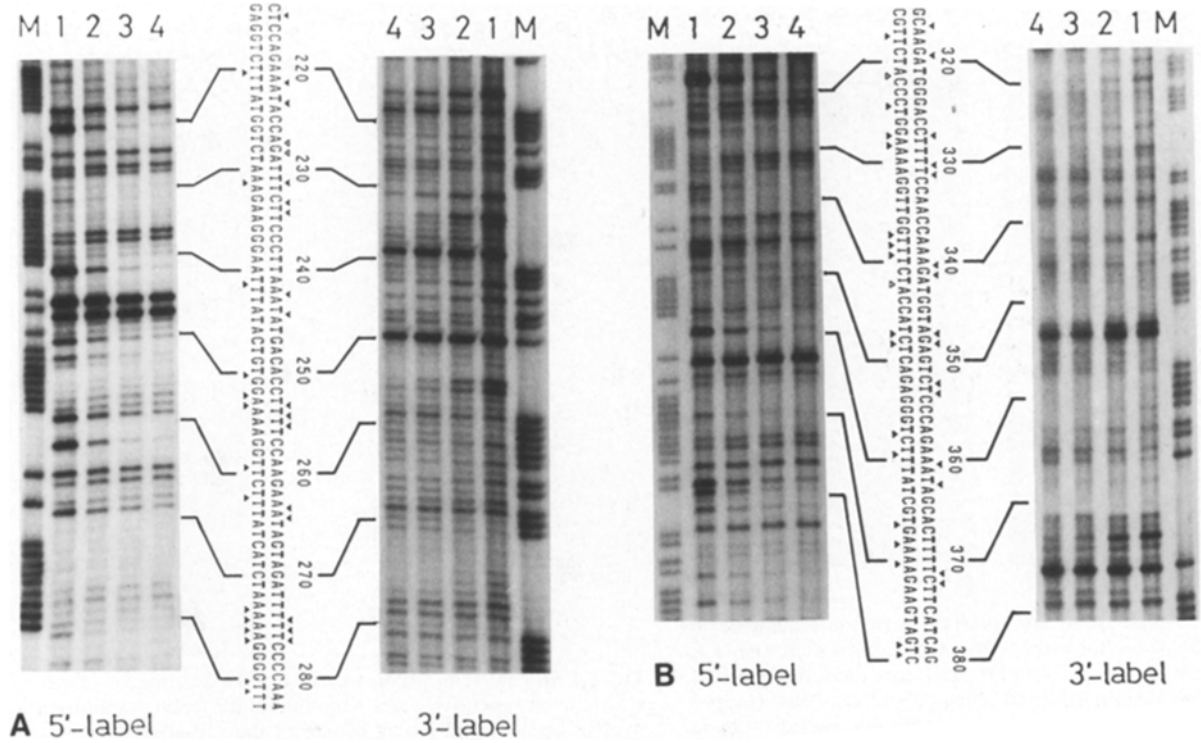
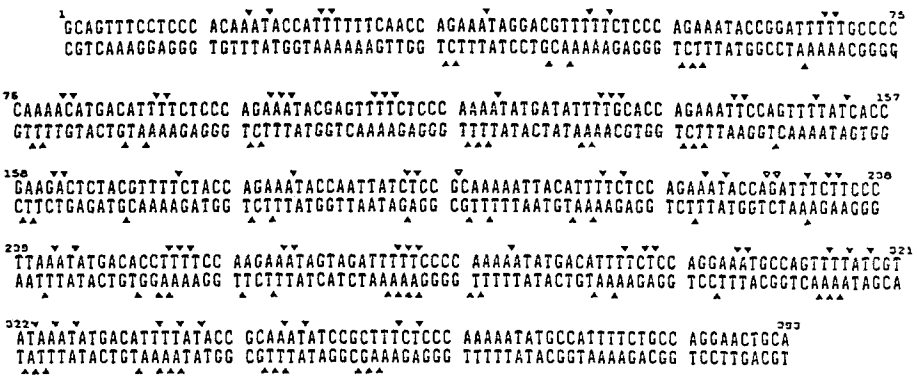


Fig. 9. Partial DNase I footprint patterns of the complex between W-protein and the 0.4 kb PstI unit (**A**) or AT fragment from the 0.5 kb TaqI unit (**B**). One of the 5′ or 3′ ends of the cloned repetitive unit was ^{32}P-labeled as indicated. A site protected against DNase I cleavage (▼) and a site with enhanced cleavage with DNase I (▽) are indicated. *Lane M*, the A+G cleavage reaction of Maxam and Gilbert (1980); *lanes 1–4*, 0, 5, 10 and 15 µl of the purified W-protein (approximately 10 ng/µl) were added, respectively, to the reaction mixture



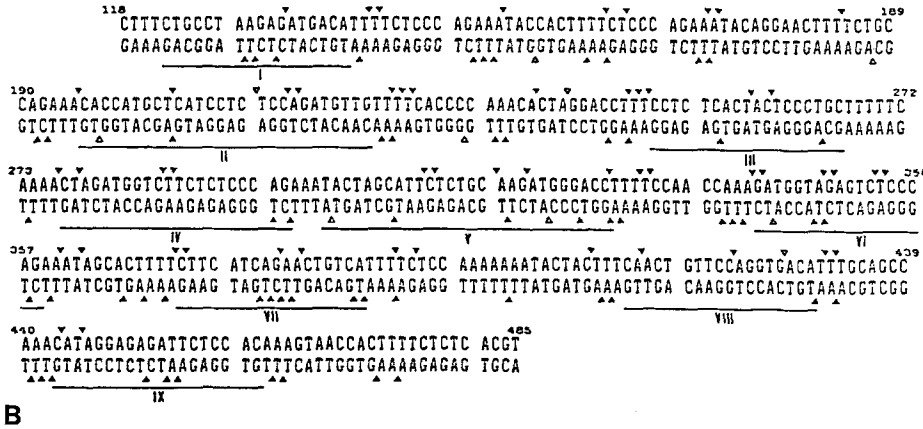


Fig. 10A, B. Periodic appearance of sites protected with W-protein (▼) against DNase I cleavage in each strand of the 0.4 kb PstI unit (**A**) and the AT fragment from the 0.5 kb TaqI unit (**B**). Sites of enhanced DNase I cleavage (▽) are also marked. Boundaries of internal repeating units of about 21 bp are shown with *spaces*. Protected sites in *underlined* regions (I–IX) in **B** appear with periodicity but without association with A_n or T_n ($n \geqq 3$) clusters

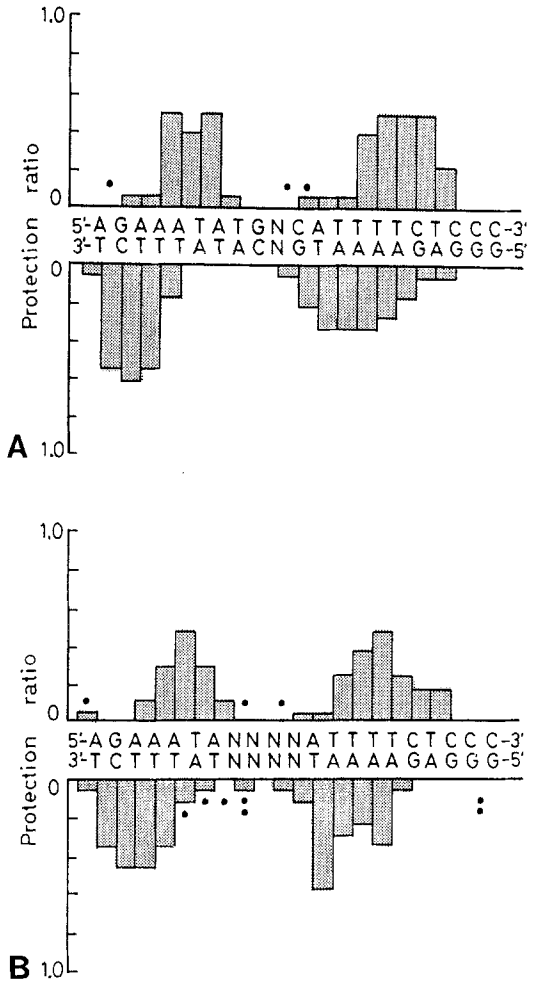


Fig. 11 A, B. Summary of the DNase I footprint analyses with respect to the 21 bp internal repeats in the 0.4 kb PstI unit (**A**) and the AT fragment from the 0.5 kb TaqI unit (**B**). The frequency of protection against DNase I cleavage calculated for the 18 (**A**) or 17 (**B**) internal repeats is indicated with *vertical bars* on each consensus sequence. When the fragment length of an internal repeat and that of the consensus sequence are different, the site was assigned according to the position from the first base pair of the internal repeat. Enhanced sites of DNase I cleavage are indicated by *dots*

sequence of pheasant may also be recognized by W-protein. This possibility was tested first by the gel retardation assay.

As shown in Figure 8 B, C, W-protein bound to the ^{32}P-labeled 0.4 kb PstI and 0.5 kb TaqI fragments in the presence of approximately 1200- and 400-fold excess, respectively, of *E. coli* DNA. The relative affinity of W-protein, as judged by the extent of competition with *E. coli* DNA, was shown to be about the same for the 0.7 kb XhoI (Fig. 8 A) and the 0.4 kb PstI (Fig. 8 B) sequences, but the affinity for the 0.5 kb TaqI sequence (Fig. 8 C) seemed to be a little lower. On the other hand, W-protein did not bind with the 0.4 kb BamHI fragment from Bobwhite quail in the presence of *E. coli* DNA (Fig. 8 D). These results are consistent with the notion that DNA bending facilitates the interaction with W-protein.

DNase I footprint analysis

The binding specificity of W-protein with the repetitive units from turkey and pheasant was further characterized by DNase I footprint analysis. In these experiments, the 0.4 kb PstI fragment or the AT fragment (an approximately 370 bp subfragment of the 0.5 kb TaqI repetitive unit) was labeled with ^{32}P at one end and subjected to binding with W-protein and partial digestion with DNase I.

Parts of the footprint patterns are shown in Figure 9 and overall results are shown in Figure 10. It is noteworthy that sites protected against DNase I cleavage are dispersed with a periodicity of about 10 nucleotides throughout the sequence of both repetitive units. When the frequency of protection against DNase I cleavage for each phosphodiester bond was plotted on the consensus sequence of the internal repeat, two preferential sites of protection on each strand were noted for both turkey and pheasant sequences (Fig. 11). These results are similar to those obtained for the 0.7 kb XhoI repetitive unit of chicken (Harata et al. 1988), i.e. there are about 10 nucleotides between the two centers of protection sites on each strand and about 3 bp between the adjacent centers on different strands, and occasional enhanced sites of DNase I cleavage on one strand are found between the two protection site centers.

Discussion

The present investigation, using the technique of hybridization under conditions of low stringency, revealed that sequences related to the XhoI family of repetitive sequences of chicken were present in the genome (most likely in the W chromosomes) of female turkey and pheasant. The two cloned, female-specific repetitive units, i.e. the 0.4 kb PstI unit of turkey and the 0.5 kb TaqI unit of pheasant, were shown to possess features in common with the chicken XhoI family repetitive units, although similarity between the nucleotide sequences is limited. These common features are the internal repeats of about 21 bp, the majority of which contain $(A)_{3-5}$ and $(T)_{3-5}$ clusters separated by 5–7 bp relatively G+C-rich sequences, the 5′-CTCC(C)-3′ elements present after the $(T)_{3-5}$ clusters, and the characteristics of bent DNA. Some of these features are, however, less noticeable for the 0.5 kb TaqI unit of pheasant.

W-protein from chicken binds with high affinity to all three repetitive units. DNase I footprint analyses suggested that the mode of interaction of W-protein with these repeating units was similar. However, one interesting aspect was noted from the interaction of W-protein with the AT fragment of pheasant. As shown in Figure 10 B, some protection sites appeared in association with AA or TT dinucleotides (in underlined regions I, IV, V, VII and IX) and some other protection sites were found without association with A or T stretches (in underlined regions II, III, VI and VIII). These results may suggest that when the $(A)_{3-5}$ and $(T)_{3-5}$ clusters, which are separated by two or three pitches of DNA helix, are bound with W-protein, then minor groove(s) between these clusters, irrespective of nucleotide sequence, can be situated close to the surface of the protein so that the phosphodiester bonds in the minor groove(s) become relatively inaccessible to DNase I.

Two interesting features of the W-protein-DNA interaction, suggested in the previous study (Harata et al. 1988) using the 0.7 kb XhoI repetitive unit, are that the DNA double helix wraps around the protein (or more likely its multimeric form) making two contacts through the A-T-rich minor grooves per strand per 21 bp internal repetitive unit and that more than 14 tandem repeats, or more than

about 300 bp, of the 21 bp unit are required to form a stable complex which can be recognized by the gel retardation assay. The present results add another feature, that is, W-protein can also bind with high affinity to sequences which have similar structural features, including DNA bending, to those of the chicken XhoI family repetitive unit but show only 40% to 60% similarity of overall nucleotide sequence. Thus, W-protein, unlike many sequence-specific DNA binding proteins, seems to recognize structural features and/or DNA bending over as much as a 300 bp stretch of DNA double helix and may be involved in the formation of the higher order structure of DNA or chromatin rather than having specific regulatory functions in the transcription of genes.

Recently, we have found that a protein which has a similar molecular mass to W-protein and crossreacts with monoclonal antibodies to W-protein is present in nuclear extracts of females and males of a variety of avian species including turkey (Mizuno et al. 1988, and to be published in more detail elsewhere). It is thus conceivable that interaction of W-protein is not restricted to the W-chromosome-specific repetitive DNA units but may be applicable more generally to genomic regions which show similar structural characteristics to those of the XhoI family repetitive units.

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