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Sex- and Tissue-specific Bkm(GATA)-binding Protein in the Germ Cells of Heterogametic Sex*

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The ZZ male/ZW female system of sex determination (female heterogamety) is found in snakes and birds whereas XY male/XX female system of sex determination (male heterogamety) operates in mammals including humans. The W and Y chromosomes are largely heterochromatic and undergo cycles of condensation and decondensation in the germ cells of ovary and testis, respectively, whereas they remain highly condensed and transcriptionally inactive in all somatic cells. Both chromosomes have enriched stretches of GATA repeats along their entire length (which is identified as banded krait minor satellite DNA and called Bkm) that are highly conserved through widely separated orders of eukaryotes. Here we report the existence of a factor, which specifically binds to Bkm, in the germ cells of the heterogametic sex (ovary in female heterogamety and testis in male heterogamety) where decondensation (activation) of the W and Y chromosomes, respectively, occurs; it has been purified as a polypeptide of 57.5 kDa from the rat snake ovary and designated as Bkm-binding protein (BBP) by virtue of its binding to GATA repeats of Bkm. Such a sex- and tissue-specific BBP is also present in the ovary of other species of snakes and in the testis of mouse and human where the Y chromosome is highly decondensed. We suggest that GATA repeats of Bkm brings about a coordinated decondensation of the W and Y sex chromosomes in the germ cells of the heterogametic sex in response to BBP which may serve as a "switch" for the activation of the genes present on the W and Y chromosomes.

Chromosomal sex determination systems of ZZ male/ZW female in snakes and birds and XY male/XX female in mammals and other higher eukaryotes have evolved on many separate occasions (Jones and Singh, 1985). In every case it has led to the same consequence of heterochromatinization of the entire sex-determining chromosome. To understand the mechanism and evolutionary significance of chromosomal sex determination, Singh *et al.* (1976, 1979, 1980) have studied the DNA of the W chromosome and have shown that, in snakes, the W chromosome is highly rich in repeated DNA which can be recovered as a satellite fraction, the Bkm. This DNA is present in high copy number in W chromosome of all snake families with differentiated sex chromosomes (Singh *et al.*, 1980), and in the species where the W chromosome, though heterochromatic, is indistinguishable morphologically from the Z chromosome (Singh, 1972). In addition, the Bkm sequences are conserved in widely separated orders of eukaryotes (Singh *et al.*, 1981; Singh and Jones, 1982). The major component of Bkm is GATA repeats (Epplen *et al.*, 1982; Singh *et al.*, 1984). Although GATA repeats are preferentially associated with the sex-determining chromosomes of a wide range of vertebrates including the sex-determining region of the mouse Y chromosome which is necessary and sufficient to convert a female into a male (Singh and Jones, 1982), their function is yet to be discerned.

Singh *et al.* (1979) observed that W chromosome in snakes remains condensed in interphase nuclei of somatic tissues but decondenses extensively in developing oocytes. This phenomenon has led to the speculation that the Bkm sequences might be involved in bringing about coordinated decondensation of the entire W chromosome in response to a specific signal. The present work shows the existence of such a signal which has been identified, purified, and partially characterized as a protein (57.5 kDa). This sex- and tissue-specific protein, as predicted, is present in the ovary of various species of snakes and in the testis of mouse and human where the W and Y chromosomes, respectively, are highly decondensed (Singh *et al.*, 1979, 1994). We demonstrate here the capacity of BBP to bind specifically to GATA repeats of Bkm. The protein has, therefore, been named as the Bkm-binding protein (BBP).[1] We propose that BBP is involved in bringing about the coordinated decondensation and, therefore, activation of the W and Y sex chromosomes in the germ cells of the heterogametic sex.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Extracts—Rat snakes (*Ptyas mucosus*) were killed during the breeding season (July to September) to collect various tissues. The tissues were frozen in liquid nitrogen and stored at −70 °C till further use. Ovaries were cleared off from the surrounding tissues and homogenized (10% w/v) in phosphocellulose buffer (PCB: 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.2 mM PMSF, 0.2 mM DTT, 2% glycerol) at 4 °C in a Polytron homogenizer. The sample was centrifuged at 400 × *g* at 4 °C for 20 min. Triton X-100 was then added in the supernatant to a final concentration of 0.5%, and the mixture was incubated at 4 °C for 30 min with intermittent gentle shaking. It was centrifuged at 10,000 × *g* at 4 °C for 60 min, and the resulting supernatant was checked for the presence of BBP by Southwestern and slot-binding assays. The positive samples were processed for purification. The same procedure was used to prepare extracts from other tissues.

Isolation of Nuclei—For the preparation of ultrapure nuclei, the procedure of Grace and Blobel (1980) was used. Approximately 3 g of the tissue was minced in STM (0.25 M sucrose, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF). The supernatant was discarded, and the tissues were homogenized in a glass homogenizer (25 strokes)

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This work is dedicated to Dr. P. M. Bhargava, Former and Founder Director of Centre for Cellular and Molecular Biology, Hyderabad, India, who has been a constant source of inspiration to us.

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[1] The abbreviations used are: BBP, Bkm-binding protein; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; cpm, counts/minute; HPLC, high performance liquid chromatography.

in homogenization buffer (0.25 M sucrose, 50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 25 mM KCl, 0.5 mM PMSF, 2 mM DTT). The homogenate was filtered through six layers of cheesecloth to remove cellular debris and connective tissue. The filtrate was centrifuged in Sorvall SS 34 rotor at 4000 revolutions/min for another 10 min. The pellet was then resuspended in 8 ml of DS (50 mM Tris, pH 7.4, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM PMSF) containing 2 M sucrose. This was layered on 4 ml of DS solution in 13.2-ml SW 41 tubes and centrifuged at 21,000 revolutions/min in an Beckman ultracentrifuge for 1 h at 4 °C. The nuclei pelleted at the bottom of the tube were resuspended in 1 ml of STM, counted by using a hemocytometer slide, and stored at 10^7 nuclei/ml at 4 °C.

Isolation of Nuclear Proteins—The nuclear protein was isolated as described by Harata *et al.* (1988). Maximum Bkm binding activity was found in the nuclear fraction but, for convenience, we mostly used total protein extracts.

Protein Purification—Ammonium sulfate (Sigma) was gradually added to the ovary extracts to achieve 60% saturation, stirred for 20 min, and left for precipitation for 1 h at 4 °C. The mixture was centrifuged at 8000 × *g* for 30 min to separate 60% ammonium sulfate-precipitated proteins. The precipitate was dissolved in PCB and checked for Bkm binding activity after dialysis in 0.1 × PCB.

A 60-ml column (45 × 1.5 cm) was packed with Bio-Gel P-200 which was equilibrated with PCB containing 0.1 M NaCl. Ammonium sulfate-precipitated proteins (2.0 mg) was loaded onto the column and eluted with PCB containing 0.1 M NaCl, at the rate of 1 ml/10 min until the A_{280} dropped to zero. Alternate fractions were checked for the presence of BBP by slot-binding assay. The positive fractions (the Bio-Gel sample) were pooled, lyophilized, dissolved in PCB, and dialyzed against 0.1 × PCB at 4 °C for 24 h.

Phosphocellulose (Whatman P11) was equilibrated to pH 7.6 with PCB for 36 h at 4 °C with frequent changes of the buffer. This was packed in a 20-ml syringe. The Bio-Gel sample was loaded on the column and step eluted with PCB containing 0.2, 0.4, and 0.6 M NaCl sequentially. Fractions were pooled for each step eluant and analyzed for BBP. The BBP-positive fractions which eluted in 0.4 M NaCl were extensively dialyzed against PCB and loaded onto the preswollen and equilibrated DEAE-cellulose matrix packed in a 5.0-ml syringe. The column was step eluted with PCB containing 0.4 M NaCl at the rate of 1 ml/10 min. Fractions of each step were pooled and analyzed for BBP.

BBP-positive fraction from the DEAE-cellulose column was further fractionated on a 25 × 4 mm ET 250/8/4 NUCLEOSIL/300–7 C4 (7-µm particle size)-reverse phase (Macherey-Nagel) column in HP 1090 liquid chromatography apparatus, using an acetonitrile gradient of 0–100% (buffer A: 0.1% trifluoroacetic acid in H_2O; buffer B: 90% acetonitrile with 0.1% trifluoroacetic acid in H_2O) at a flow rate of 1 ml/min. Protein peaks were collected and assayed for BBP.

SDS-PAGE—SDS-PAGE was carried out as described by Laemmli (1970). Gels were normally stained with Coomassie Brilliant Blue except when high sensitivity was required, as in the case of HPLC fractions, where silver staining was performed. Duplicates of the gels were processed for Southwestern assay.

Southwestern Assay—Gels were incubated in the renaturation buffer (50 mM NaCl, 2.0 mM EDTA, and 100 mM Tris, pH 7.0) for a minimum of 3 h to overnight with constant gentle shaking, and subsequently equilibrated with transfer buffer (20 mM Tris, pH 8.0, and 192 mM glycine in 20% methanol) followed by electrophoretic transfer to nitrocellulose membrane (0.45-µm pore size; Schleicher & Schuell) at room temperature in an electroblot apparatus (Towbin *et al.*, 1979).

Slot-blot Binding Assay—Nitrocellulose membrane (Schleicher & Schuell, Germany) was soaked in double-distilled water and rinsed in standard binding buffer (SBB: 10 mM Tris-HCl, pH 7.0, 1 mM EDTA, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.05 M NaCl). The protein extract 1–1000 µl or more (if the preparation is too dilute) was loaded onto the membrane by using slot-blot apparatus (Schleicher & Schuell). For column fractions showing A_{280} = 0.5, 10 µl of sample was loaded. 500 µl of SBB was used to transfer the contents onto the slot under vacuum for 30 min followed by air-drying. For the detection of Bkm-binding fractions, Bkm-binding assay was performed as below. The above assay procedure was standardized using 1–20 µg of bovine serumn albumin, 10 ng to 1 µg of histones, and 1–10 µg of crude protein from ovary, testis, and other tissues and was found to be comparable to gel mobility shift assay and Southwestern assay.

Bkm-binding Assay—The assay was performed by the method of Bowen *et al.* (1980) with some modifications. DNA fragments 2(8) or synthetic $GATA_{16}$ were end-filled by Klenow reaction using [^{32}P]dATP (3000 Ci/mmol) to a specific activity of 10^6 cpm/µg DNA. Labeled fragments were separated from free nucleotides through a Sephadex G-50 (Pharmacia) column. Blots were preincubated in standard binding buffer (SBB) for 1 h at room temperature in the presence of sheared *Escherichia coli* DNA (100 µg/ml) as competitor. ^{32}P-labeled probe (10^5 cpm/ml) was added to the fresh SBB, and filters were incubated at room temperature for 2 to 3 h and washed in SBB with an increasing amount of NaCl (50 mM to 500 mM) at room temperature until an optimum signal was obtained. Blots were exposed to x-ray films (Konica) overnight at −70 °C.

Oligonucleotide Synthesis—$GATA_{10}$ and $TATC_{10}$ were synthesized using phosphoramidite chemistry and a Pharmacia gene assembler. Synthetic oligonucleotides were annealed, kinased, ligated, and cloned into the *SmaI* site of pUC18. $GAGA_{12}$ and $TCTC_{12}$ and $GGTA_{12}$ and $TACC_{12}$ were also synthesized, annealed, and used directly without cloning.

2(8) Probe—The 2(8) probe is a Bkm-positive subclone of the *Drosophila* clone CS314 (Singh *et al.*, 1981, 1984) containing a 545-base pair insert. The main feature of this clone is the presence of 66 copies of the tetranucleotide GATA, a highly conserved component of Bkm.

Electrophoretic Mobility Shift Assays—Assay mixtures (30 µl) contained 1 ng (30,000 cpm) of labeled $GATA_{16}$ fragment, 3 µg of *E. coli* DNA, and 20 µg of nuclear protein extract in 10 mM Tris, pH 7.5, containing 50 mM NaCl, 1 mM $MgCl_2$, and 5% glycerol. In competition assays, unlabeled double-stranded 2(8) insert along with 3 µg of unlabeled *E. coli* DNA were allowed to interact with nuclear extracts for 10 min at 4 °C before the addition of labeled probe DNA. The mixtures were incubated at room temperature for an additional 45 min and separated on 5% polyacrylamide gel run in TBE (0.089 M Tris, pH 8.0, 0.089 M boric acid, 0.002 M EDTA) at 60 V at room temperature (24–25 °C). Gels were dried before autoradiography.

RESULTS

BBP Is Sex-, Tissue-, and Stage-specific—Crude protein extracts (50–100 µg) from various tissues of banded krait, water snake, and rat snake were separated on 10% SDS-PAGE. The gels were incubated in renaturation buffer, and the protein was electrophoretically transferred onto nitrocellulose membrane which was subjected to protein-DNA binding assay by using [^{32}P]dATP-labeled 2(8) DNA as a probe. Presence of a 57.5-kDa BBP was detected specifically in the small ovary at an early stage of oocyte development (Fig. 1*A*, *lane 3*) and its absence in later stage ovary-containing eggs full of yolk (Fig. 1*A*, *lanes 4* and *5*; Fig. 1*C*, *lane 6*) and in other tissues of female as well as male snakes (Fig. 1*A*, *lanes 1*, *2*, *6*, and *7*). The sex and tissue specificity of BBP was further confirmed by electrophoretic mobility shift assay using ^{32}P end-labeled $GATA_{16}$ fragment as a probe. One specific DNA·protein complex was observed only with BBP-positive Bio-Gel column fractions of ovary extract (Fig. 1*B*, *lane 3*). Male gonads (testis) and female liver did not exhibit the presence of BBP even during the breeding season (Fig. 1*B*, *lanes 1* and *2*, respectively).

From a number of independent experiments, using three different species of snakes (water snake, *Natrix piscator*; banded krait, *Bungarus fasciatus*; and rat snake, *P. mucosus*), we deduced the existence of a sex-, tissue-, and stage-specific ovarian protein of 57.5 kDa in all the species analyzed (Fig. 1, *C* and *D*, *lanes 1–5*), which has high binding affinity for GATA-rich Bkm (the assays described above were performed in the presence of a 1000-fold excess of *E. coli* DNA as competitor). Significantly, sand boa (*Eryx johni johni*), which is evolutionarily a primitive snake and shows no differentiation of sex chromosomes, also showed the presence of such a BBP although quantitatively much less than the highly evolved snake species (data not shown).

Sequential Purification of BBP—After demonstrating the presence of BBP exclusively in the ovary of various species of snakes at a specific stage of developing oocytes during the breeding season, we decided to work on rat snake *P. mucosus*, a nonpoisonous species of snake found in abundance in the neighboring areas of Hyderabad. Immediately after the onset of monsoon a large number of adult females of rat snake were collected, and ovaries at the right stage were dissected out and pooled together.

The crude protein extract of pooled ovaries of rat snake was

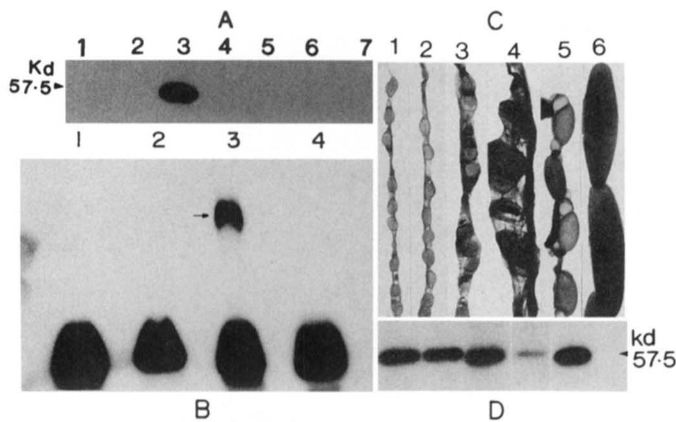

FIG. 1. **Sex and tissue specificity of BBP.** *A*, Southwestern assay with ^{32}P end-labeled 2(8) probe containing mainly GATA repeats. *Lanes 1–5*: female banded-krait kidney, liver, small ovary; water snake large ovary; rat snake large ovary, respectively. *Lanes 6* and *7*: male banded-krait liver and heart, respectively. BBP is detected only in ovary at an early stage of developing oocytes. *B*, gel mobility shift assay showing sex and tissue specificity of BBP using ^{32}P end-labeled oligonucleotide $GATA_{16}$ probe in the presence of 20 μg of Bkm-positive Bio-Gel fraction of rat snake ovary and corresponding fractions of rat snake female liver and male testis protein extracts, 20 μg *E. coli* DNA, 500 ng of poly(dI-dC), 3–4 ng (40,000 cpm) of probe in binding buffer. The mixture was incubated at 37 °C for 20 min and run on a 6% polyacrylamide gel. *Lane 1*, rat snake testis; *lane 2*, rat snake female liver; *lane 3*, rat snake female ovary; *lane 4*, ^{32}P-labeled $GATA_{16}$ probe. *C*, part of the ovary containing developing oocytes of different species of snake from which protein was extracted, run on SDS-PAGE, and subjected to Southwestern assay (*D*). *D*, Southwestern assay with ^{32}P end-labeled 2(8) probe. Note the presence of a BBP of similar molecular weight in the ovary of water snake (*lanes 1* and *2*), banded krait (*lanes 3* and *4*), and rat snake (*lanes 5* and *6*). There is reduced signal in *lane 4* and no signal at all in *lane 6*, containing large oocytes in banded-krait and rat snake, respectively.

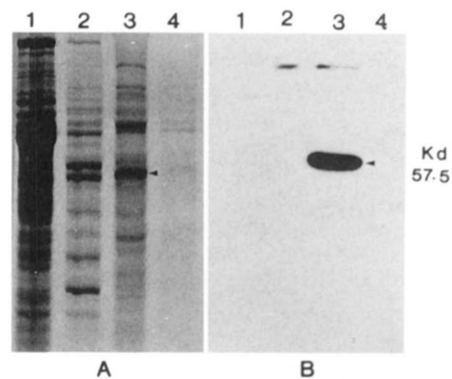

FIG. 2. **Southwestern assay of BBP in step-eluted phosphocellulose column fractions.** *A*, Coomassie Brilliant Blue-stained SDS-PAGE protein gel; *B*, Southwestern assay of the other half of the same gel having identical lanes, with ^{32}P end-labeled 2(8) probe. *Lanes 1–4*: unadsorbed, 0.2, 0.4, and 0.6 M NaCl eluted fractions, respectively. BBP is present only in the 0.4 M fraction (*lane 3*).

fractionated by ammonium sulfate and the fractions assayed for BBP either by Southwestern assay or by slot-blot-binding assay. The Southwestern assay showed that BBP was completely precipitated out at 60% ammonium sulfate saturation with no detectable binding capacity left in the supernatant. This fraction was subjected to gel filtration on Bio-Gel P-200 where the BBP activity was concentrated in one peak which was then subjected to phosphocellulose (PC) column chromatography.

It was possible to separate BBP from many other proteins by specific elution with 0.4 M NaCl (Fig. 2, *A* and *B*). The latter signifies its basic nature as expected for DNA-binding proteins. The 0.4 M PC column BBP-containing fraction was loaded onto a DEAE-cellulose column after extensive dialysis. It bound to the column and was eluted with 0.4 M NaCl which signifies the presence of acidic domains in the BBP. The 0.4 M DEAE eluant was fractionated in a reverse-phase C4 HPLC column. The sample was resolved into nine peaks which were checked for Bkm binding activity. The peak eluting at 25.63 min showed the presence of BBP (Fig. 3, *a* and *b*); on SDS-PAGE it showed only one band after silver staining (Fig. 3*c*).

BBP Binds to GATA Repeats—Since the probe 2(8) used for Southwestern assay also contains sequences other than GATA repeats, we synthesized $GATA_{16}$, annealed and cloned in the *Sma*I site of pUC18. The sequences of such clones in pUC18 were confirmed by DNA sequencing. The other sequences, apart from GATA repeats, present in the insert are 2 residues (CC) at 5′ and 3 residues (GGG) at the 3′ end. Insert cleaved by *Kpn*I and *Bam*HI double digestion of the clone was used for gel retardation assay. The gel retardation assay with undigested clone pT7–1/2(8) (containing 2(8) insert cloned in the *Pst*I/*Bam*HI site of the vector pT7) and the clone containing synthetic $GATA_{16}$ in pUC18 showed mobility shift with the purified BBP (Fig. 4*A*, *lanes 1* and *2*, and *5* and *6*, respectively), whereas, vectors alone did not show such shift (Fig. 4*A*, *lanes 3* and *4*, and *7* and *8*, respectively). The linearized clone pT7–1/2(8) also showed retardation with BBP (Fig. 4*A*, *lanes 9* and *10*). The 545-base pair long 2(8) insert alone showed the mobility shift with the purified BBP (Fig. 4*A*, *lanes 11* and *12*). The shift was more pronounced in supercoiled forms than in linear forms. Sequence specificity of BBP was further confirmed by electrophoretic mobility shift assay using a ^{32}P end-labeled $GATA_{16}$ fragment as a probe. One specific DNA·protein complex was observed with BBP-positive Bio-Gel column fraction of ovary extract (Fig. 4*B*, *lane 2*). The formation of the DNA protein complex was completely abolished by the addition of a 40–100-fold excess of unlabeled $GATA_{16}$ as competitor DNA (Fig. 4*B*, *lanes 4* and *5*) or unlabeled 2(8) insert (data not shown). The synthetic $GAGA_{12}$ and $GGTA_{12}$ single base variants of GATA repeat did not show any DNA·protein complex with BBP-positive Bio-Gel fractions of the ovary extract. These results confirm the binding of BBP to GATA repeats.

Sex- and Tissue-specific BBP in Mouse, Rat, and Human Testis—A BBP was also detected by electrophoretic mobility-shift assay in mouse, which has a distribution of GATA repeats along the Y chromosome that is similar to that in the W chromosome of snakes (Singh *et al.*, 1994). The $GATA_{16}$ probe detected two specific DNA·protein complexes (C_1 and C_2) with testis nuclear extracts (Fig. 5*A*, *lane 2*), which were not detected with the nuclear extracts of other tissues of male and female mice (Fig. 5*A*, *lanes 3–6*). However, when 2–4-fold excess of nuclear extracts of other tissues were used, a signal of C_1 and C_2 complexes that was faint when compared to testis nuclear extract was detected in other tissues as well suggesting that the DNA protein complexes are not formed below a certain level of BBP concentration. The C_1 and C_2 complexes were abolished by 300–900-fold excess of unlabeled 2(8) insert containing mainly GATA repeats (Fig. 5*B*, *lanes 3–5*), confirming GATA as the recognition sequence. C_1 and C_2 DNA protein complexes were also detected with nuclear extract of rat testis under conditions used for mouse (data not shown). Nuclear extract of human testis under similar conditions also showed C_1 and C_2 complexes by gel retardation assay using $GATA_{16}$ probe, but these complexes were different in size from the complexes from mouse (Fig. 6*A*, *lane 3*).

Developmental Expression of BBP in Mouse—A progressively increasing signal of C_1 and C_2 complexes with $GATA_{16}$ probe was detected in mouse testis during development from 1 week (after birth) onward; the signal reached the maximum level at the onset of spermatogenesis (Fig. 7). This is in agreement with the expression of Y-linked genes (Page *et al.*, 1987; Burgoyne,

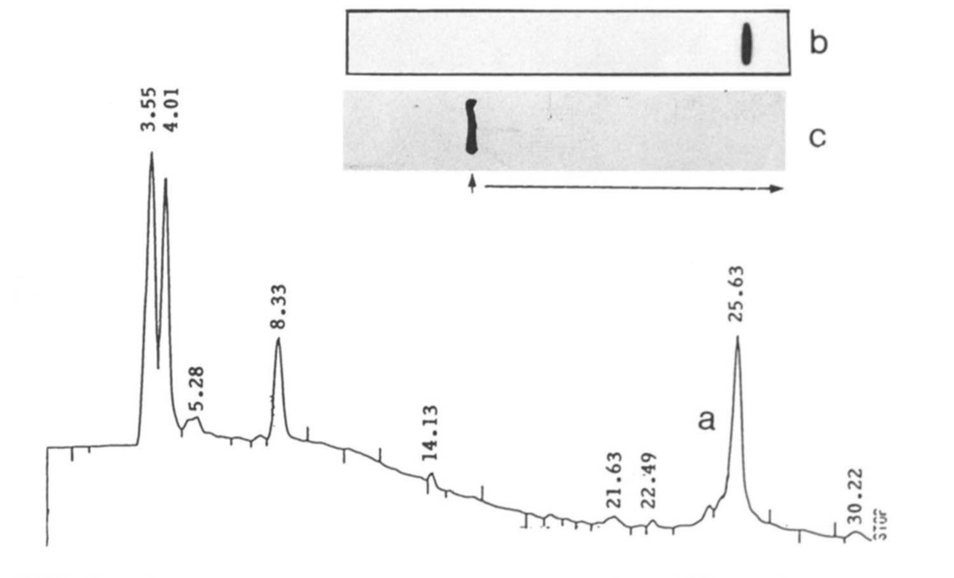

FIG. 3. **Final purification of BBP by using HPLC-reverse phase column chromatography.** Only the peak eluting at 25.63 min (*a*) showed the presence of BBP by slot-blot-binding assay (*b*). A finally purified fraction of BBP run on SDS-PAGE and silver stained showing a single band (*c*). There is no detectable contamination with any other protein.

1987; Gubbay *et al.*, 1990; Kay *et al.*, 1991; Prado *et al.*, 1992). This prompted us to look for BBP in the testis of XXSxr′ mice (McLaren *et al.*, 1984) which lack spermatocytes and spermatids due to total block of spermatogenesis (Burgoyne *et al.*, 1986). The nuclear protein extract of testis of XXSxr′ mice revealed the presence of only a C_2 complex by gel retardation assay with $GATA_{16}$ probe even when 50 µg of the nuclear extract was used compared with 20 µg in the case of a normal male (Fig. 6*B*, *lane 2*).

Since XXSxr′ mouse testis contains predominantly Sertoli cells, it is probable that BBP-forming C_2 complex is expressed in somatic cells of the gonad (Sertoli cells or Leydig cells). This suggests that C_1 and C_2 may be two different BBPs, C_1 specifically and exclusively expressed in the germ cells and C_2 expressed poorly in the somatic cells of the gonad. Although the function of C_2 in somatic cells is not clear at present, it is possible that its quantitative insufficiency may be the cause of its ineffectiveness in bringing about decondensation of the Y chromosome in somatic cells.

BBP Binds to W Chromosome in Snake and Y Chromosome in Mouse—Although GATA repeats are predominantly concentrated on the W and the Y chromosomes, they are also present on other chromosomes. This raises the question whether BBP binds only the W and Y chromosomes or to the GATA repeats present on other chromosomes as well. To resolve this, 50 ng of purified BBP was slot-blotted on nitrocellulose membrane in quadruplicate and subjected to DNA-protein-binding assay using *Hin*fI-digested, ^{32}P end-labeled male and female genomic DNA probes of snake and mouse separately, in the presence of a 1000-fold excess of *E. coli* DNA as a competitor. Predominant binding was observed with the female snake and male mouse DNAs (Fig. 8*A*, *slot b*; Fig. 8*B*, *slot a*, respectively) compared with that of the male snake and the female mouse DNAs (Fig. 8*A*, *slot a*; Fig. 8*B*, *slot b*). When a 1000-fold excess of the snake male or mouse female DNAs were used as the competitor, along with a 1000-fold excess of *E. coli* DNA, binding with the female snake and male mouse DNAs was only slightly reduced but was clearly detectable (Fig. 8*A*, *slot d*; Fig. 8*B*, *slot c*, respectively), whereas binding with the male snake and female mouse DNAs was completely abolished (Fig. 8*A*, *slot c*; Fig. 8*B*, *slot d*). When a 1000-fold excess of the snake female- and mouse male-unlabeled DNAs were used as the competitor, the binding with the male as well as with the female snake and mouse DNAs was completely abolished (Fig. 8*A*, *slots e* and *f*; Fig. 8*B*, *slots e* and *f*; respectively). This is consistent with the predominant concentration of GATA repeats on the snake W and the mouse Y chromosome (Singh *et al.*, 1979; Jones and Singh, 1985; Singh and Jones, 1982).

DISCUSSION

In somatic tissues of female snakes, the W chromosome, like the mammalian X chromosome, remains condensed, forms a W chromatin body, and is largely non-transcribed. However, the W chromosome decondenses during oogenesis in those oocytes which have received the W chromosome following meiosis (Singh *et al.*, 1979). Thus it seems that the W is reactivated only during early oogenesis, and the inactive and active phases involve the chromosome as a whole. It is worth noting that in the sea snake, *Enhydrina schistosa*, where W chromosome is in two separate parts, W1 and W2, both decondense simultaneously (Singh *et al.*, 1979), indicating their common regulation. An analogous system is present in the flour moth *Ephestia kuehniella* in which inactivation of the W chromosome occurs specifically in somatic cells. In the previtellogenic stages of oocyte, however, the W chromosome is transcriptionally active (Traut and Scholz, 1978). This shows that in growing oocytes decondensation of the W chromosome is associated with transcriptional activity.

Like the snake W chromosome, the mouse Y chromosome remains condensed and, therefore, largely transcriptionally inactive in the somatic cells but decondenses specifically in the germ cells (Singh *et al.*, 1994). This is the stage at which the X chromosome in all male heterogametic organisms gets inactivated and the Y chromosome becomes functional. This is in agreement with the expression of Y-linked genes in the testis (Koopman *et al.*, 1989; Page *et al.*, 1987; Burgoyne, 1987; Gubbay *et al.*, 1990; Kay *et al.*, 1991; Mitchel *et al.*, 1991). Prado *et al.* (1992) have reported the nucleotide sequence of a Y chromosomal repetitive sequence, 145SC5 of which there are about 200 copies distributed over the entire length of the Y chromosome including the sex-determining region. This is also transcribed in the testis. In this context it is interesting that a sex-specific DNA-binding protein, which recognizes GATA repeats of Bkm, is predominantly present in the germ cells of the heterogametic sex (testis in mouse and ovary in snake) in which the Y and W chromosomes are decondensed and transcriptionally active. It is very tempting to speculate that ordered organization of GATA repeats throughout the length of the Y and W chromosomes may be responsible for coordinated

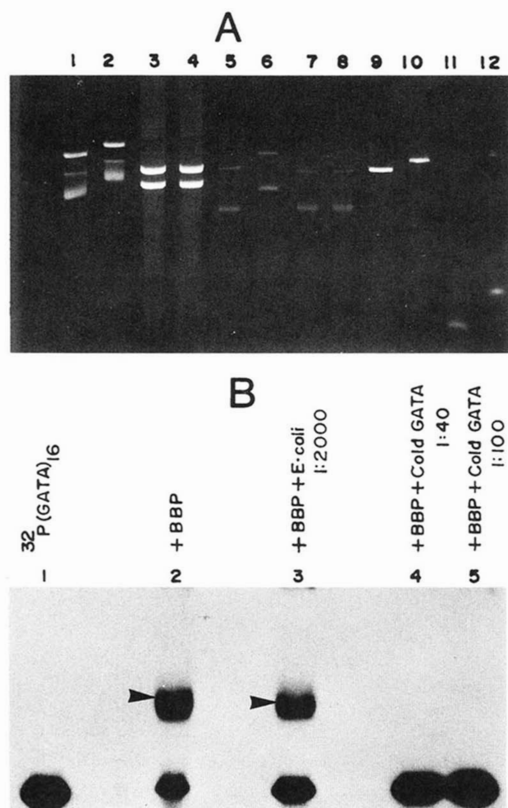

FIG. 4. **Gel retardation assay showing sequence specificity of BBP.** *A*: *lane 1*, undigested pT7–1/2(8) clone without BBP. *Lane 2*, the DNA used in *lane 1* with HPLC purified BBP; there is retardation in the mobility of all the bands (supercoiled, circular, and linear forms). *Lane 3*, undigested pT7 vector DNA without any insert and without BBP. *Lane 4*, DNA in *lane 3* but with BBP; there is no retardation. *Lane 5*, undigested clone of synthetic $GATA_{16}$ cloned in pUC18 vector without BBP. *Lane 6*, DNA as in *lane 5* but with BBP; note the shift in the mobility of all the bands. *Lane 7*, uncut plasmid pUC18 without any insert and without BBP. *Lane 8*, DNA in *lane 7* with BBP; there is no shift in the mobility of the bands. *Lane 9*, DNA in *lane 1* linearized with *Pst*I digestion. *Lane 10*, DNA as in *lane 9* but with BBP; note retardation in the mobility of the linear band. *Lane 11*, the 545-base pair long insert only, of 2(8) without BBP. *Lane 12*, DNA as in *lane 11* but with BBP; note the expected mobility shift. The BBP was HPLC purified and used in 3–4-fold molar excess of DNA. *B*, gel mobility shift assay using ^{32}P end-labeled oligonucleotide $GATA_{16}$ probe in the presence of 20 μg of Bkm-positive Bio-Gel fraction of rat snake ovary protein extract. *Lane 1*, ^{32}P-labeled $GATA_{16}$ probe. *Lane 2*, probe and BBP. *Lane 3*, probe with BBP, *E. coli* DNA, and poly(dI-dC). *Lane 4*, as in *lane 3* but with 40-fold excess of unlabeled GATA repeats as autologous competitor. *Lane 5*, as in *lane 4* but with 100-fold excess of unlabeled GATA repeats.

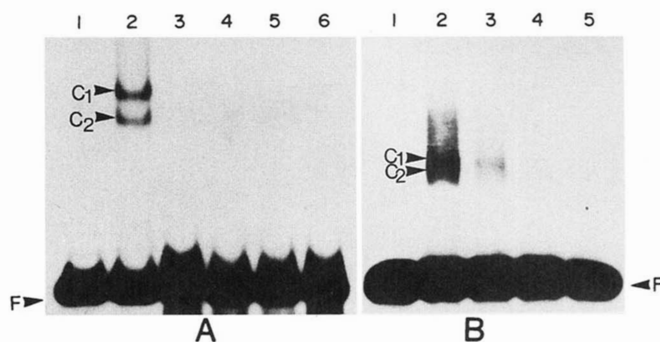

FIG. 5. **Autoradiograph of a gel retardation assay with ^{32}P end-labeled double-stranded oligonucleotide $GATA_{16}$ probe in the presence of 20 μg of nuclear extract.** *A*: *lane 1*, $GATA_{16}$ probe only. *Lanes 2–6*, nuclear extract from male mouse testis, liver, and kidney, and female mouse liver and kidney, respectively. *F*, free probe. Two DNA protein complexes (C_1 and C_2) were detected only with the nuclear extract of testis. *B*, gel mobility shift assay with nuclear extract of mouse testis and increasing concentrations of unlabeled 2(8) insert as autologous competitor. *Lane 1*, labeled probe only; *lane 2*, without cold competitor 2(8) showing two DNA protein complexes (C1 and C2); *lanes 3–5*, with 300, 600, and 900 ng of cold competitor 2(8) respectively. The C_1 and C_2 complexes were completely abolished with cold competitor 2(8), showing sequence specificity of the protein.

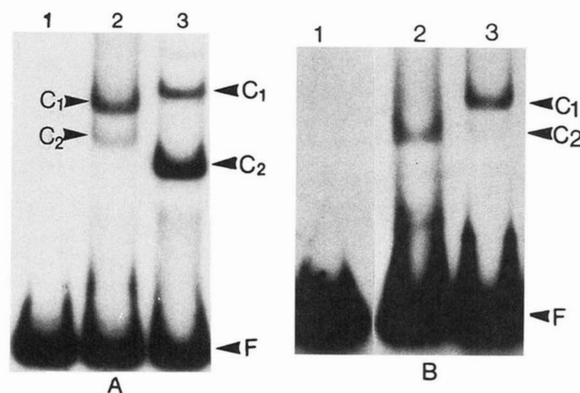

FIG. 6. **Gel mobility shift assay showing sex- and tissue-specific DNA-protein complexes in mouse and human.** *A*: *lane 1*, $GATA_{16}$ probe only; *lanes 2* and *3*, nuclear extract from testes of mouse and human, respectively. The C_1 and C_2 complexes in humans are of different molecular wieghts than the C_1 and C_2 complexes in mouse. *B*: *lane 1*, labeled $GATA_{16}$ probe only; *lane 2*, 50 μg of nuclear protein extract of XXSxr′ male mouse testis; *lane 3*, 20 μg of nuclear protein extract of normal male mouse testis. Other conditions of mobility shift assay were the same as described in the legend to Fig. 5.

decondensation of the W and Y chromosomes in response to BBP.

This is for the first time a sex- and tissue-specific protein has been implicated in bringing about coordinated decondensation of the entire W and Y chromosomes. A W protein with high affinity for the W chromosome-specific *Xho*I family of repetitive sequences in chicken has been reported to be involved in maintaining the stable condensed state of the W chromatin in somatic tissues (Harata *et al.*, 1988). This protein, however, does not bind to Bkm sequences (Harata *et al.*, 1988). A family of proteins, called the GATA family of transacting factors, that specifically interact with the GATA motif present in the regulatory elements has now been identified in vertebrates as lineage-specific transcriptional regulatory proteins (Martin and Orkin, 1990; Romeo *et al.*, 1990; Ho *et al.*, 1991). Targeted disruption of GATA-1 gene by homologous recombination in embryonic stem cells has revealed that this transcription factor is essential for normal erythroid differentiation (Pevny *et al.*, 1991) and can function as a regulator of megakaryocytic differentiation (Visvader *et al.*, 1992). Recently Arceci *et al.* (1993) have reported GATA-4 in mouse which is retinoic acid inducible and developmentally regulated transcription factor. Comparative sequence analysis has revealed strong conservation of the zinc-finger DNA-binding domain among vertebrate GATA-binding proteins (Yamamoto *et al.*, 1990; Romeo *et al.*, 1990; Martin *et al.*, 1990). Recently, in addition to its role in erythroid cell development, GATA-1 has also been shown to be expressed in mouse testis (Ito *et al.*, 1993).

The sex- and tissue-specific BBP is different from GATA-1 and GATA protein family discussed above in many respects. GATA-1 and other members of the GATA protein family have highly conserved zinc-finger DNA-binding domains. Addition of metal chelators such as EDTA and DTT leads to abolition of its binding to DNA (Evans and Hollenberg, 1988). Addition of 40 mM of EDTA or DTT or a combination of both, however, has no effect on DNA binding ability of BBP (Fig. 9, *lanes 1–3*). Immunofluorescence studies of GATA-1 suggest that it is predominantly expressed in spermatocytes, whereas mobility shift studies of testis nuclear extract of Sxr′ mice show that the C_2

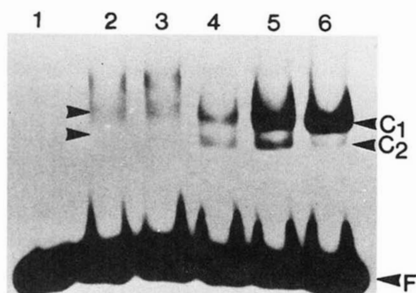

FIG. 7. **Detection of developmental expression of Bkm-binding protein by gel mobility shift assay using ^{32}P end-labeled oligonucleotide $GATA_{16}$ probe in the presence of 20 μg of testis nuclear protein extract of mouse.** *Lane 1*: labeled probe only; *lanes 2–6*, nuclear protein extract of 1–4 weeks (after birth) and adult mouse testis, respectively. Additional DNA protein complex in the higher molecular weight region (*lanes 2* and *3*) could not be detected consistently. Note the quantitative increase in BBP with the onset of spermatogenesis.

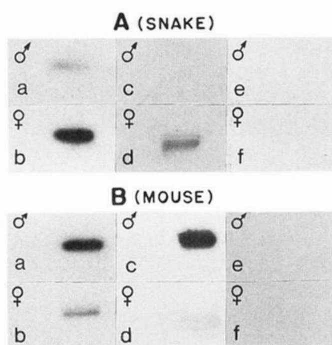

FIG. 8. A, slot-binding assay of BBP with ^{32}P end-labeled male and female DNA of common Indian krait showing preferential binding to female DNA (W chromosome). 50 ng of HPLC-purified BBP was slot-blotted on nitrocellulose, and DNA-protein-binding assay was carried out as described under "Experimental Procedures." Male and female DNAs were digested with *Hin*fI and end-filled with [^{32}P]dATP using Klenow fragment. *a* and *b*, 50 ng of BBP slot-blotted on nitrocellulose and bound to ^{32}P-labeled *B. caeruleus* male and female DNA 40,000 cpm (3 ng each), respectively, in the presence of a 1,000-fold excess of unlabeled *E. coli* DNA as a competitor. *c* and *d*, as in *a* and *b* but with a 1,000-fold excess of unlabeled *B. caeruleus* male DNA as a cold competitor. *e* and *f*, as in *a* and *b* but with a 1,000-fold excess of unlabeled *B. caeruleus* female DNA as a competitor. *B*, BBP slot-blot-binding assay with ^{32}P-labeled male and female mouse DNAs showing preferential binding to male DNA (Y chromosome). *a* and *b*, BBP bound to ^{32}P labeled male and female mouse DNAs 40,000 cpm (3 ng each), respectively, with a 1,000-fold excess of *E. coli* DNA as a nonspecific competitor. *c* and *d*, as in *a* and *b* but with a 1,000-fold excess of unlabeled female mouse DNA as a competitor. *e* and *f*, as in *a* and *b* but with a 1,000-fold excess of unlabeled male mouse DNA as a competitor.

complex is expressed in Sertoli cells (Fig. 6*B*, *lane 2*). Thus, C_2 DNA-protein complexes of Bkm and GATA-1 are expressed in different cell types. The mobility shift pattern of GATA-1 between human and mouse has been reported to be identical (Tsai *et al.*, 1989), whereas mobility shift patterns of BBP with testis nuclear extract of human and mouse are markedly different (Fig. 6, *lanes 2* and *3*). While GATA recognizes one copy of GATA the BBP recognizes larger stretches of GATA repeats ($GATA_{16}$). This is confirmed by the fact that 4000-fold excess of *E. coli* DNA, which contains single copy GATA sequences scattered in the genome, does not affect binding of BBP to $GATA_{16}$; 1000-fold excess of snake male and mouse female DNA, which contains large numbers of smaller stretches of GATA repeats, do not affect binding of BBP to snake female and mouse male DNA (Fig. 8) containing the W and Y sex chromosomes respectively, with longer stretches of GATA repeats (Singh *et al.*, 1980, 1981, 1984).

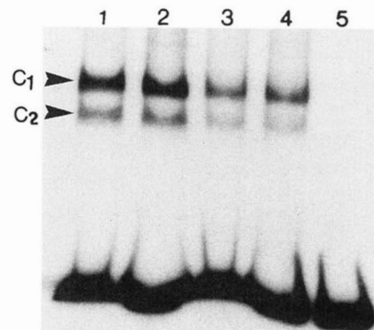

FIG. 9. **Effect of EDTA and DTT on the binding of BBP to $GATA_{16}$.** Mobility shift assays using 20 μg of mouse testis nuclear extracts; indicated amounts of EDTA and DTT were performed and resolved on 5% polyacrylamide gels. *Lane 1*, 20 μg of testis nuclear extract, 40 mM EDTA, and 40 mM DTT; *lane 2*, 20 μg of testis nuclear extract and 40 mM DTT; *lane 3*, 20 μg of testis nuclear extract and 40 mM EDTA; *lane 4*, 20 μg of testis nuclear extract; *lane 5*, labeled probe without nuclear protein. Note that the presence of EDTA and DTT have no effect on binding of BBP to $(GATA)_{16}$.

The fact that male and female DNAs can be distinguished on Southern blot indicates that Bkm sequences are differently organized on the sex chromosomes from that on the rest of the genome. On the basis of blot and *in situ* hybridization, it appears that larger stretches of GATA exist on the W and Y chromosomes. It is possible that, due to cooperative binding and a higher binding coefficient, BBP is able to recognize the W and the Y from the other chromosomes. However, Bkm sequences have also been localized in the proximal region of chromosome 17 containing the major histocompatibility complex (Kiel-Metzger and Erickson, 1984; Durbin *et al.*, 1989). Study of this region containing the inversion of the complex has revealed an impressively large number of genes, virtually all of which are expressed in testis (Yeom *et al.*, 1992). These findings have led to the suggestion that the proximal region of mouse chromosome 17 could play a central role in male germ cell differentiation. It is tempting to suggest that BBP present in the testis causing decondensation of the Y chromosome may also recognize GATA repeats present in chromosome 17 and bring about coordinated activation of genes in the proximal region of the chromosome.

Bkm-related sequences are scattered throughout the genome of all the species studied so far. It is possible that they are also involved in some aspect of generalized gene control. One could then envisage that chromosomal sex determination may have evolved by the extension and amplification of such individual gene controls to a point where the control would involve switching on and off of an entire chromosome. This view is supported by the fact that a Y chromosomal DNA sequence 145SC5, which has a potential open reading frame coding for a protein consisting of 227 amino acids, exists in 200 copies along the length of the Y chromosome and is specifically expressed in the testis (Prado *et al.*, 1992).

Although our demonstration of involvement of BBP in decondensation of the W and Y chromosomes is indirect, the coexistence of the BBP and the decondensed state of the W chromosome in the snake oocytes and Y chromosome in the mouse testis, and the preferential binding of BBP to the W and Y chromosomes, are compelling evidences in favor of its suggested role. Direct demonstration that binding of BBP causes extensive decondensation of the W and Y chromosomes, however, has to await the identification and isolation of the gene coding for the BBP.

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