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Bkm sequences from the human X chromosome contain large clusters of GATA/GACA repeats

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SUMMARY

In order to determine whether the regional localizations of Bkm repeats detected on the human X chromosome consisted of typical GATA/GACA repeats, clones were isolated, mapped, and sequenced. Nine Bkm-hybridizing clones from Kunkel's fluorescent-activated, cell-sorted X-chromosome library were all unique. Five were mapped in detail with restriction enzymes and the Bkm-hybridizing segments were localized. Confirmation of X chromosomal homology was obtained for 2 of the clones and Bkm segments from these 2 clones were sequenced. Seventeen contiguous GATA repeats were found in each clone and the overall repeat arrangement showed relatively few differences from previously sequenced Bkm sequences. These are the first sequences of human Bkm repeats. The results, when compared with previously published results, suggest that there may be significant differences between the organization of Bkm repeats on the human X and on the human Y chromosome.

INTRODUCTION

A satellite DNA sequence that appeared to be exclusive to the female, heterogametic sex was previously isolated from the venomous snake, the banded krait (*Bungarus fasciatus*) (Singh *et al.* 1980*a*). When the banded krait minor satellite DNA (Bkm) was hybridized to DNA of a range of snake species representing a wide spectrum of sex-chromosome evolution, it hybridized strongly to the W chromosome, the heterogametic chromosome. Bkm DNA was also found in a variety of other organisms. In *Drosophila*, Bkm is localized near the base of the X chromosome (Singh, *et al.* 1980*b*), while in the mouse, Bkm sequences were found to be located near the centromere of the Y chromosome (Jones & Singh, 1981), and in studies with XX,*Sxr* mice, this region was shown to be strongly male determining (Singh & Jones, 1982).

Restriction enzymes have shown little sex difference in Bkm hybridization patterns in humans (Jones & Singh, 1981). *In situ* studies have shown that Bkm is concentrated on two regions of the X chromosome and on two autosomal chromosomes but relatively little was found on the Y chromosome (Kiel-Metzger *et al.* 1985). This result was unexpected due to the presence of Bkm in the heterogametic chromosome of other mammals, such as mice. We were unable to find Bkm-positive human Y chromosomal cosmids when using the *Drosophila*-derived pCS316 Bkm-containing clone, either using salmon sperm DNA (Wolfe *et al.* 1984) or *E. coli* DNA (Erickson *et al.* 1987) as the non-specific competitor. In contrast, Arnemann *et al.* (1986) detected Bkm-positive human Y cosmids using a synthetic oligodeoxynucleotide probe. However, these cosmids seem to have only relatively short runs of GATA repeats, as

determined by a non-sequencing technique, compared with mouse control clones. We have isolated Bkm-hybridizing clones from a fluorescent-activated, cell-sorted human X-chromosome library, characterized them by restriction mapping and sequencing, and shown that they contain long repeats and higher order structures as have been found in other mammalian Bkm-containing clones.

MATERIALS AND METHODS

A library of the human X-chromosome, prepared by fluorescent-activated sorting, in the lambda bacteriophage Charon 21A vector was obtained from Dr Louis Kunkel (Kunkel *et al.* 1982). *E. coli* K802 was infected by the bacteriophage and about 10000 plaques were screened for the presence of Bkm with the Bkm-containing plasmid pCS316 obtained from a *Drosophila* library (provided by Dr Kenneth Jones) by standard techniques. Thirty-three plaques showed strong hybridization to the Bkm probe. Twenty were chosen at random of which nine were analysed. These nine were unique, as shown by comparing their restriction patterns with a variety of restriction enzymes (data not shown). Five were characterized in more detail.

The human inserts were separated from the bacteriophage DNA by digestion with *Hin*dIII and subsequent electrophoresis (about 2·5 μg DNA per well) through a 0.9% agarose gel with recovery of the insert DNA on Schleicher & Schuell NA45 paper per manufacturer's suggested protocols. Restriction enzymes were obtained commercially from Boehringer Mannheim, Bethesda Research Laboratories, International Biotechnologies, and P.L. Biochemicals and used per manufacturer's protocols. Location of restriction sites was performed by analysis of appropriate double digestions. Identification of Bkm-containing fragments was performed after Southern transfer (Southern, 1975). Prehybridization was in 50% formamide, 1% SDS, and 1 M-NaCl at 42 °C. Nick-translated pCS316 (10^7 cpm/μg) and sheared salmon sperm DNA (final concentration 50 μg/ml) were denatured by boiling and added to the prehybridization mixture. After overnight hybridization, the filters were washed to 0·5 × SSC in 0·1% SDS at 65 °C and exposed to Kodak X-Omat AR at −70 °C with intensifying screens.

Mouse cell lines 1R (Nabholz *et al.* 1969) and HORL9X (1R with the addition of an intact human X chromosome (Goodfellow, 1975)) were the generous gift of Dr Peter N. Goodfellow. DNA was restricted to completion with *Eco*RI, transferred by the Southern technique and hybridized (as described above or in 1% SDS, 1 M-NaCl at 65 °C) with nick-translated intact lambda or random oligodeoxynucleotide-primed probes (Feinberg & Vogelstein, 1984) to the isolated lambda *Hin*dIII inserts. Those lambda clones which showed repetitive patterns with both cell lines' DNA were prehybridized to C_0T 100 following the procedure of Litt & White (1985). Briefly, freshly precipitated probe was added to 100 μg/ml of sonicated (300–500 bp) human DNA in 5 × SSC, boiled for 10 min, cooled at 0 °C for 1 min, and incubated at 68 °C for 10 min before its addition to the filter and prehybridization mixture.

The 0·6 kb *Pst*I-*Hin*dIII fragment of λX13 and the 1·1 kb *Bam*HI-*Hin*dIII fragment of λX81 were cloned into M13 vectors mp18 and mp19 in both orientations and sequenced by the method of Sanger *et al.* (1977), using T7 polymerase from U.S. Biochemical as recommended (Taber & Richardson, 1987), and using sequentially loaded wedged polyacrylamide gels. Dideoxynucleotide triphosphates and deoxynucleotide triphosphates were purchased from P.L.

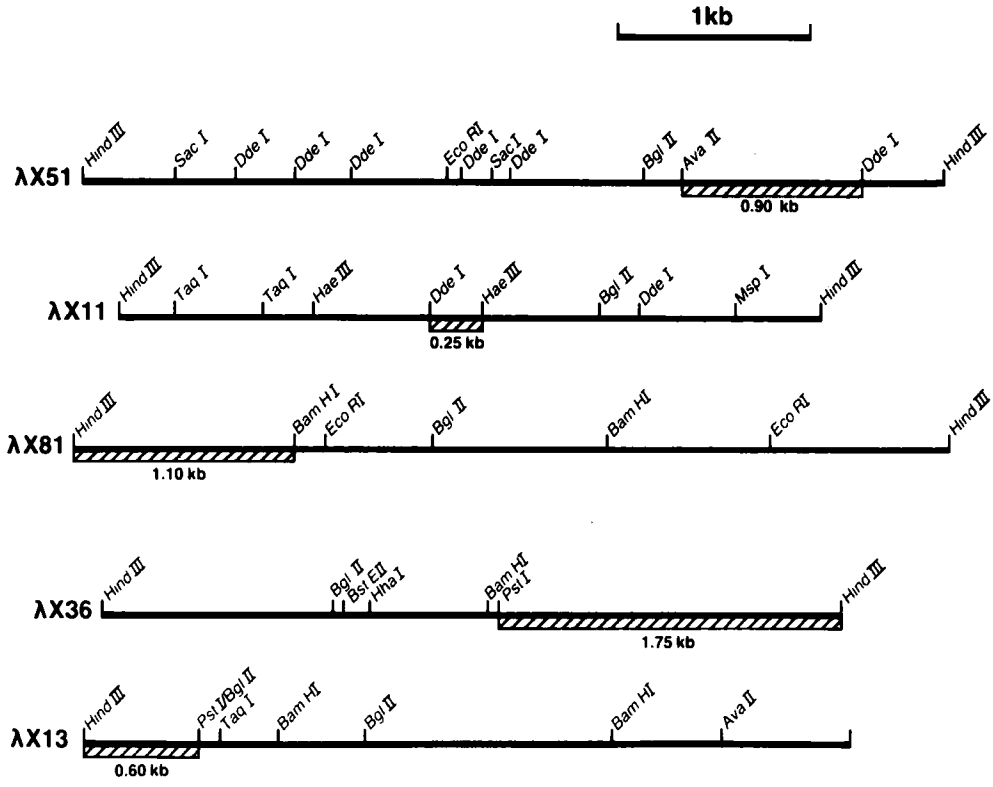

Fig. 1. Restriction maps and Bkm-hybridizing fragments of λ clones isolated with the *Drosophila* Bkm plasmid, pCS316.

Biochemicals. α-^{32}P-dATP of 400 Ci/μmole was purchased from Amersham. The 615 bp *Pst*I-*Hin*dIII fragment of λX13 was completely sequenced; 30% was sequenced off both strands. 206 bp, starting 73 bp from the 5′ *Hin*dIII site and including the GATA repeats, were sequenced from the *Bam*HI-*Hin*dIII fragment of λX81.

RESULTS

Of the 10000 plaques screened for Bkm, 33 were chosen that showed strong hybridization. Nine of these – λX11, λX13, λX35, λX36, λX51, λX54, λX57, λX81, λX101 – have been plaque purified and shown to be unique by comparing their restriction patterns with specific enzymes (data not shown). Five bacteriophage have been mapped by restriction analysis and Bkm-hybridizing fragments identified. The results of the λX11, λX13, λX36, λX51 and λX81 bacteriophage insert characterizations are illustrated in Fig. 1. The λX11 insert is 3·6 kb in length with its 250 bp Bkm-hybridizing fragment located near the centre of the insert and bordered by *Dde*I and *Hae*III restriction sites. The λX13 insert is 4.0 kb in length with a 600 bp Bkm-hybridizing fragment bounded by a *Pst*I site and one of the *Hin*dIII cloning sites. The 3·8 kb λX36 insert was restriction mapped and found to have a 1·75 kb Bkm-hybridizing region which is located between a *Hin*dIII cloning site and a *Pst*I restriction site. The λX51 insert is 4·4 kb long and contains a Bkm-hybridizing fragment of 900 bp which is bounded by

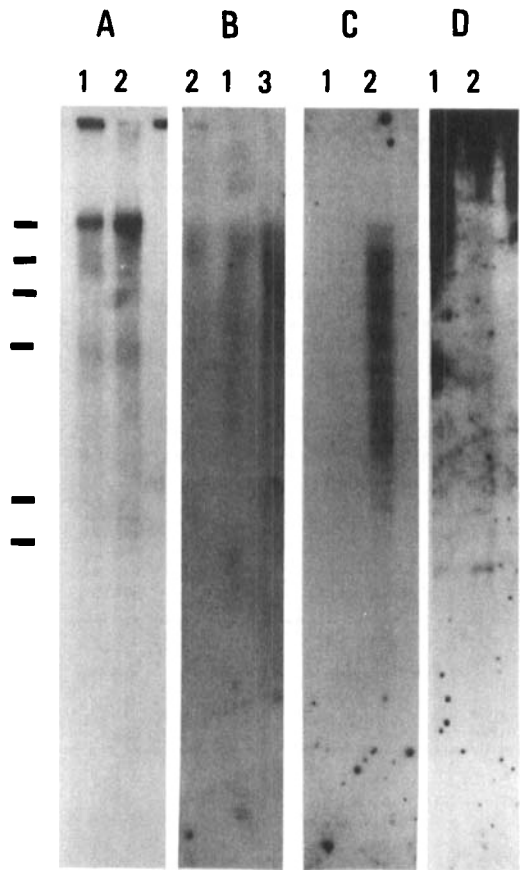

Fig. 2. Hybridization of λX Bkm clones to mouse DNA or mouse + human X DNA. DNA was restricted with *Eco*RI, electrophoresed, transferred to Genescreen Plus™ by the Southern technique, and hybridized with or without preassociation to sonicated human DNA, (A) λX51, 0·5 × SSC, without preassociation, (B) λX51, 0·5 × SSC, with preassociation, (C) λX13, 0·2 × SSC, without preassociation and (D) λX81, 0·2 × SSC, without preassociation. 1, 1R; 2, HORL9X; 3, control human female DNA.

*Ava*II and *Dde*I restriction sites. The λX81 insert is 4·5 kb long with its 1·10 kb Bkm-hybridizing fragment located between the *Hin*dIII cloning site and a *Bam*HI restriction site.

Chromosomal assignment of clones

Even though the flow-sorted library has shown a preponderance of X-derived clones (Kunkel *et al.* 1982), we examined the chromosomal assignments of the clones we characterized. The clones showed a variety of patterns of hybridization to the DNAs of a mouse cell line (1R) and a mouse-human somatic cell hybrid with only the human X chromosome (HORL9X)(Fig. 2). Inasmuch as Bkm sequences are also abundant in mice, some clones gave similar patterns with these two DNAs. λX51 hybridized to a large band and also gave a smear and a faint repeat ladder with the mouse DNA whether or not the human X was present (Fig. 2A). Prehybridization to sonicated human DNA eliminated the large band in both cell lines, leaving faint smears with the mouse DNA and mouse with human X DNA and a stronger smear with control human DNA (Fig. 2B). These results suggest that the large MW band detected by λX51

Sequence of humanXBkm13

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  1  AGCTTTGGTT CAATTTTTAT GGTTTTTATG GAAGGTACAA AGTTACTAAT GCACCACCCC

 61  ACAGTAAGCA CCAGCCCCAC ATGGTTTCAT AGAGAAATAT TAGAAAATCT TCAAAGATTG

121  GGTAGTTGTG ATGCTACGAA AACTTTTCCA AAACAAACTC CTACTGAAGT TTTATGAAAT

181  ACTAAAATAT TGATACTTTA ACCTAATAGA TAGTACAATA AAACCTACAT ATAATTGCAC

241  TTATGGATAT AGATGTACTA ATTAAAATAC TAGCAAACAT AACCCAACAC TGCATTCAGA

301  TATAACATAC CATGACCAAA AGAACTTCAT TCTATTAATG TAAAAAAAAT CACATTAATA

361  GAATTAAGGG GAAAACATAT GATTATCTAC AGTGATGCTA AATGAAACCA ACAATATTTA

421  AAATTATTCT GATAAGACCC TCAAGAACTT GAATTGATGG AGATTCAACT ATATATATAT

481  AAATATATGT TTATAAATAT AGATAGACAG ATAGATAGAT AGACAGATAG ATAGATAGAT

541  AGATAGATAG ATAGATAGAT AGATAGATAT AGATATAGAT ACTTTAATCC TAAAGGCAAG

601  ATCTTACCTA CTGCA
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Sequence of humanXBkm81

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  1  AGTCTACAGA GCTGCAAGAA CACCACATCA TAAATCTTAC GATTTGAATG ACATCGATAG

 61  ATAGATAGAT AGATAGATAG ATAGATAGAT AGATAGATAG ATAGATAGAT AGATAGATAG

121  ATATAGATAG ATACATACAT ACATACATAC ACACATAGAA ATAGAGATAT CCTGATGGAA

181  AGAATAAACC ATGAGTCTGG TAATAG
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Fig. 3. Sequences of λX13 and λX81 fragments with GATA repeats. Sequences read from both strands are underlined, GATA/GACA repeats are overlined.

is a repeated element other than Bkm, while the diffuse smear could be due to Bkm. At high stringency, λX13 hybridized as a smear, with faint bands suggestive of unique sequences, only with the somatic cell hybrid (containing the human X) DNA (Fig. 2C). Thus, λX13 contains repeat sequences that hybridize to the human X, but not to mouse DNA, at this stringency. λX81 demonstrated unique sequence bands with the somatic cell hybrid DNA (containing the human DNA) after high stringency washing (0·2 × SSC) but not with the parental mouse line (Fig. 2D). Thus, λX13 and λX81 contain human X homologous sequences, as predicted for members of this library, although they still could be derived from other human chromosomes. A definite determination could not be made for λX51 (Fig. 2A, B) and λX11 and λX36 (data not shown).

The 615 bp λX13 Bkm-hybridizing fragment contained 23 GATA/GACA repeats in a span

of 390 bp (Fig. 3). Repeats 21, 22, and 23 were separated by the dinucleotide, TA, and abundant TATA repeats preceded the major block of GATA/GACA repeats. The 68 bp of contiguous repeats were preceded by ATA. Reading 5′ to 3′, an open reading frame of 150 bp at 28–177 precedes the GATA/GACA repeats but does not have consensus codons flanking the initiation site to suggest likely usage. There are other shorter open reading frames (131–193, 243–305, 457–522). An in-frame start codon for the 3′–5′ complementary strand which would lead into the tyr leu ser leu hydrophobic repeat potentially coded by the complement to the GATA repeats was not seen, but the fragment only extended 29 bp further in this direction. The 206 bp of the λX81 Bkm-hybridizing fragment contained 21 GATA/GACA repeats, again with a separation by the dinucleotide, TA. A start codon in the complementary strand immediately precedes the 20th repeat complement and remains in frame through the 20 repeats to bp 37. Thus, although the *Drosophila* Bkm clone used to find these clones might have contained other repeats (two *Drosophila* clones closely related to the one we used have been sequenced and did not contain other repeats; Singh *et al.* 1984), the sequenced human clones did not have detectable repeats other than the GATA/GACA, Bkm-related repeats.

DISCUSSION

The hypothesis that Bkm is involved in sex differentiation originated in work by Singh *et al.* (1976). Analytical equilibrium centrifugation was done on the venomous banded krait which has heteromorphic sex chromosomes. A minor satellite unique to the heterogametic, female sex was discovered (Singh *et al.* 1976). Radioactive banded Krait minor satellite DNA (Bkm) was then hybridized to DNA of ten species of snakes. These snakes represented all stages of sex-chromosome evolution. With the exception of the evolutionarily primitive boa constrictor, significantly more Bkm hybridized to female DNA than to male DNA. *In situ* studies showed that the Bkm homologous regions were located throughout the genome, but they were especially abundant on part or all of the W chromosome.

Mammals broke away from the evolutionary line long before the lineages of birds and reptiles appeared. Therefore, the presence of Bkm in the heterogametic sex in mammals could support the hypothesis that Bkm is universally involved in sex determination. In mice, Singh *et al.* (1980*b*) found by *in situ* hybridization that Bkm was localized proximal to the centromere on the telomeric Y. Also, a sex specific pattern emerged when genomic DNA was digested with *Hae*III, electrophoresed, blot transferred, and hybridized with Bkm: male DNA had a large molecular weight Bkm fragment which contained many *Alu*I restriction sites that was absent in the female. When DNA from XX phenotypic males (XX,*Sxr*) was restricted, blot transferred, and hybridized with Bkm, the pattern was nearly identical to that of the normal male mouse. Using *in situ* studies, Singh & Jones (1982) demonstrated that the XY,*Sxr* male carrier possesses an inherited aberrant Y chromosome with two Bkm homologous regions, one region proximal to the centromere as in a normal Y chromosome plus an unexpected region at the tip of the Y chromosome. This second region was shown to be the result of translocation (Evans *et al.* 1982). Singh and Jones (1982) concluded that the XX,*Sxr* male is the result of a cross-over between the distal ends of the X and the Y chromosomes which transfers a male determining Bkm region to the X chromosome, an interpretation of *Sxr* which was also suggested by electron microscopical and immunological studies (Shapiro *et al.* 1982).

This information on the morphologic location of Bkm in various organisms led to the assertion that Bkm was evolutionarily conserved. Many investigators then turned to sequencing Bkm and its flanking regions in the hope of confirming the hypothesis. Sequencing experiments found the highly repetitive Bkm to consist of multiple copies of GATA or GACA. Sequences of Bkm containing clones of *Elaphe radiata* (Epplen *et al.* 1982), *Mus musculus* (Epplen *et al.* 1983; Singh *et al.* 1984) and *Drosophila melanogaster* (Singh *et al.* 1980*a*, *b*) were determined individually and Levinson *et al.* (1985) pooled these data to analyse them. Long clusters of GATA and GACA are common to *M. musculus* and *D. melanogaster* but the similarities between the two organisms' clusters ends there. The number of tandem GATA or GACA repeats per cluster varies between the two organisms and the disruptions between these tandem repeats are not similar. Also, the mouse Bkm has GATA and GACA regions intermixed while *Drosophila* has distinct tandem arrays of GATA and GACA. This evidence suggests that Bkm sequences are not evolutionarily conserved regions, but rather are developed by convergent evolution in which there could be selection favouring Bkm's evolution (Levinson *et al.* 1985; Platt & Dewey, 1987). However, in the Mediterranean meal moth, *Ephestia kuehniella*, Bkm sequences form autosomal hypervariable DNA loci which, it was suggested, may rapidly vary by transposition of mobile elements (Traut, 1987). Thus, rapid evolution of Bkm sequences without selection for function is also possible.

The hypothesis that Bkm sequences play a role in sex determination was a generalization from the occurrence of high concentrations of these GATA/GACA repeats on heterogametic chromosomes in snakes and mice. We found much lower relative concentrations of Bkm sequences in primate DNA than in mouse DNA (Erickson *et al.* 1987) and a relative paucity of *in situ* hybridization to the human Y (Kiel-Metzger *et al.* 1985). Using a different *Drosophila* Bkm clone for *in situ* hybridization, Singh & Jones (1986) reported relatively more grains on the human Y chromosome than we had but no quantitation was given. We had screened over 100 human Y-chromosome-derived cosmids with the same clone used for our *in situ* studies without finding a positive cosmid (Wolfe *et al.* 1984; Erickson *et al.* 1987). Arnemann *et al.* (1986) used $(GATA)_4$ and $(GACA)_4$ synthetic oligodeoxynucleotides to identify a number of hybridizing human Y cosmids. The maximum tandem repeats they found in the human Y cosmids were much shorter ($n = 12$) than those characteristic of mice ($n = 32$ and $n = 80$ in the controls used). We asked whether human X-chromosome Bkm clones would be more typical of other mammalian Bkm clones than those found on the human Y.

Our studies have characterized Bkm-hybridizing DNA from a flow-sorted human X-chromosome library. Table 1 shows a summary of previously published characterizations of Bkm homologous DNA in *E. radiata*, *D. melanogaster* and *M. musculus* (Epplen *et al.* 1982, 1983; Singh *et al.* 1984) and our results. The lengths of Bkm homologous regions that we have isolated (0·25–1·75 kb) are similar to the lengths of GATA/GACA regions in other organisms. Although the lengths of repeats are similar, the contents of contiguous regions appear to be somewhat different in humans than in other organisms. Epplen *et al.* (1982, 1983) obtained very fine restriction maps of Bkm clones from *E. radiata* and *M. musculus* (the largest fragments in the map were about 0·2 kb) and restriction sites were found intrinsic to the GATA/GACA repeat regions. An extreme example of this is in *E. radiata* which has a GATA/GACA repeat spanning 189 base pairs containing only two short non-repeat regions. Despite this, there still exists an *Alu*I restriction site in the middle of the 80·4% GATA/GACA region. In our experiments, we

Table 1. *Sequenced Bkm homologous regions*

	*Elaphe radiata**	*Drosophila melanogaster*†	*Drosophila melanogaster*†	*Mus musculus*‡	*Mus musculus*†	*Homo sapiens*§	*Homo sapiens*§
Clone	pErs5	pCS3142(8)	pCS319	pMC14	pM3.1	X13	X81
Length of clone	2·5 kb	1·5 kb	N/G	2·5 kb	N/G	4·0 kb	4·5 kb
Length of region sequenced	2487 bp	545 bp	499 bp	2461 bp	420 bp	615 bp	206 bp
Total number of GATA/GACA repeats	152 bp (38 repeats)	264 bp (66 repeats)	148 bp (37 repeats)	544 bp (133 repeats)	88 bp (22 repeats)	92 bp (23 repeats)	84 bp (21 repeats)
Longest stretch of GATA/GACA repeats	80 bp (20 repeats)	52 bp (13 repeats)	60 bp (15 repeats)	56 bp (14 repeats)	32 bp (8 repeats)	68 bp (17 repeats)	68 bp (17 repeats)
Span of GATA/GACA repeats	189 bp	421 bp	364 bp	2244 bp	362 bp	390 bp	125 bp
GATA/GACA in span (%)	80.4%	62.7%	40.7%	24.2%	24.3%	23.6%	67.2%

N/G, Information not given. * Epplen *et al.* 1982. † Singh *et al.* 1984. ‡ Epplen *et al.* 1983. § This work.

used a similar set of enzymes as Epplen *et al.* (1982, 1983) but obtained maps with fewer restriction sites (Fig. 1). Although the non-repetitive regions seem to be dissimilar, it appears that Bkm homologous regions in humans are about the same length as those in other organisms.

The sequence characterization of these clones revealed similarities to previously sequenced GATA/GACA repeats (Table 1). This is in apparent contrast to the results found by Arnemann *et al.* (1986) for Bkm-containing clones from the human Y chromosome which used an assay based on ligation of a synthetic oligodeoxynucleotide, $(GATA)_4$, hybridized to the cosmids. Since the conditions of hybridization were not given, it is possible that single base pair mismatches could have been tolerated and that the apparent maximum of 12 repeats was not perfect. Only sequence characterization of GATA/GACA repeats from the Y chromosome can clarify their nature. This first sequence characterization of human Bkm sequences, from the X chromosome, shows them to be similar to previously characterized mammalian, insect and snake Bkm sequences.

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