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Northern Analyses Using Single-Stranded Probes Do Not Support a Role for GATA/GACA Repeats in Sex Determination in Mice and Men

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ABSTRACT The possible role of GATA/GACA repeated sequences in mammalian sex determination was investigated using Northern analyses of mouse and human RNA. Brain, liver, and gonadal RNA from three developmental stages of mice of both sexes and also human fetal RNA from various tissues were hybridized to both sense and antisense Bkm riboprobes as well as to the synthetic oligonucleotide $(GATA)_5$. At low levels of stringency, putative transcripts of various sizes were observed in all tissue samples with all probes. At high stringency, only a putative transcript of approximately 12 kb was observed, but this was later shown to consist of contaminating DNA. No sex-specific differences were observed in any tissue or developmental stage. Thus, we find no evidence that the GATA/GACA repeated sequences are specifically expressed in quantities detectable by Northern analyses in a manner important to mammalian sex determination.

Key Words: Bkm sequences, Gonad differentiation, Riboprobes

INTRODUCTION

The sex-specific Banded krait minor (Bkm) satellite sequences have attracted considerable attention since they were first reported (Singh et al., 1976). This satellite DNA cross-hybridizes to DNA from a variety of organisms, from *Drosophila* to humans, which indicates either sequence conservation or independent evolution (Singh et al., 1980, 1981; Levinson et al., 1985). In addition, the sex-specific hybridization patterns observed in some of these organisms suggested a possible role in sex determination. Bkm sequences contain many GATA tetranucleotide repeats as well as some GACA repeats (Epplen et al., 1982; Singh et al., 1984). They have been found in open reading frames and if translated could encode a very hydrophobic protein. Singh and coworkers (1984) found Bkm sequences to be transcribed with preferential transcription of the GATA strand in different tissues of mice and *Drosophila*. In addition, these investigators found transcription to be male specific and developmentally regulated. However, the sex specificity of these transcripts has not been confirmed by other investigators (Epplen et al., 1982; Schafer et al., 1986). None of these studies used fetal tissue except liver, which was not distinguished by sex.

If Bkm sequences are involved in primary sex determination, we postulated that they might be expressed in a sex-specific or tissue-specific pattern nearer to the time of gonadal differentiation. Using both the sense and antisense Bkm riboprobes (Erickson et al., 1987), as well as the synthetic oligonucleotide $(GATA)_5$, we were unable to detect age, sex, or tissue-specific transcripts in these human or mouse samples. From these results, we suggest that there is little evidence that Bkm sequences are transcribed and translated to effect sex determination.

MATERIALS AND METHODS

Total cellular RNA was isolated from brain, liver, and gonads of 14 day postcoitum, newborn, and adult random bred mice of each sex by the guanidinium isothiocyanate-cesium chloride method (Chirgwin et al., 1979). Total human RNA was isolated using guanidinium-HCl (Chirgwin et al., 1979) from 13.5 to 21 week fetuses aborted by dilation and extraction in 1984. Since the fetal remnants were fragmented and most tissues unidentifiable, fetal sex was established using the human Y chromosomal centromeric probe Y97 (Stalvey and Erickson, 1987). Kidneys, lung, heart, and placenta were positively identified tissues, whereas several "glands" might have included gonads. For some samples, poly(A)$^+$ RNA was isolated from

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total RNA using one pass over oligo(dT) cellulose (Maniatis et al., 1982).

Approximately 10 μm of each total mouse and 5 μg of each total human RNA sample was run on 1.5% agarose-formaldehyde denaturing gels (Maniatis et al., 1982) and transferred to Gene Screen Plus. For Northern hybridization with either the sense or antisense Bkm riboprobes, filters were prehybridized at 65°C in 6 × SSC, 5 × Denhardt's solution, and 0.5% SDS. Bkm riboprobes derived from PCS316 (Singh et al., 1981) were synthesized using the pGEM vector system (Promega Biotec, Madison, WI) and either the SP6 (sense) or T7 (antisense) polymerase (Erickson et al., 1987). The probes were labeled with ^{32}P-UTP to 1×10^9 cpm/μg and added to a final concentration of 6×10^5 cpm/ml and 50 μg/ml denatured *Escherichia coli* DNA (which does not contain Bkm sequences). After hybridization for 16 hours at 65°C with constant agitation, filters were washed to 0.1 × SSC–0.1% SDS at 70°C for 30 minutes. Filters were exposed to Kodak XAR film with an intensifying screen at −70°C for up to 10 days before developing.

For hybridization with the synthetic oligonucleotide $(GATA)_5$, conditions similar to those of Schafer et al. (1986) were used except that the hybridization temperature was 42°C. This probe was end labeled with γ-^{32}P-ATP and T4 polynucleotide kinase (Maniatis et al., 1982). Molecular weight determinations were made by isolation of the lane containing DNA size markers and staining it with ethidium bromide or by staining the filters in a solution of 0.04% methylene blue–0.5 M sodium acetate to localize 28S and 18S ribosomal RNAs (Maniatis et al., 1982).

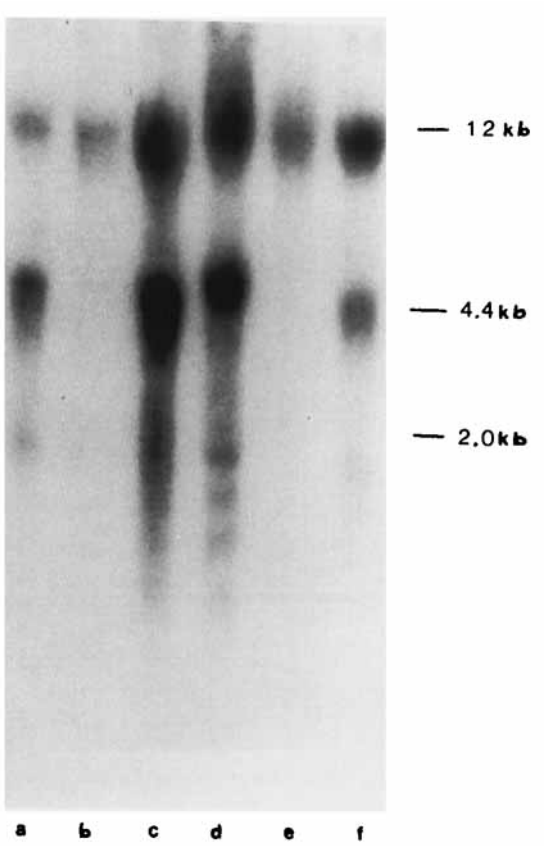

Fig. 1. Northern hybridization of Bkm antisense riboprobe to adult mouse RNA. Results with female (**a–c**) and male (**d–f**) brain (a,d), liver (b,e), ovary (c), and testes (f) samples are shown.

RESULTS

We used Bkm riboprobes, both sense and antisense, for Northern hybridizations because RNA–RNA hybrids have a higher Tm than do DNA–RNA or DNA–DNA hybrids and, therefore, are more stable. In addition, very high specific activities can be obtained. When the antisense Bkm riboprobe (i.e., 5′ → 3′, CUAU) was hybridized to adult mouse total RNA, all tissues of both sexes gave positive signals. At least three different sized signals were observed in all tissues except liver (Fig. 1). One signal was approximately 12 kb and was observed in all samples. A 4.5–5.0 kb band and a 1.8–2.0 kb band were visible in all tissues except in the liver of both males and females. Additional smaller bands (<1.8 kb) were observed in ovary and male brain RNA samples. The 4.5–5.0 kb band was present in all tissue RNAs from both sexes of fetal (14 days postcoitum) and newborn mouse samples, whereas the 1.8–2.0 kb band was present in all but male liver (results not shown).

The sense riboprobe (i.e., 5′ → 3′, GAUA) hybridized only to a 1.8–2.0 kb band in all fetal and newborn samples (data not shown); whereas in adults this band, and also the 12 kb band, were observed (Fig. 2). All of these results are similar to those of Schafer et al. (1986) who found that both strands of these repeats are expressed differently but no sex differences for each organ were observed. This stands in contrast to the sex-specific differences observed by Singh and coworkers (1984).

Since these hybridizations/washes were carried out at low stringency, they were repeated at high stringency. Under these conditions only the 12 kb band was observed in all tissues of both sexes (Fig. 3). We were concerned about the possibility that the 1.8–2.0 and 4.5–5.0 kb bands were merely nonspecific binding to 18S and 28S ribosomal RNAs since they are the most abundant species in total RNA preparations. Preparations of poly(A)$^+$ RNA were hybridized with the antisense Bkm riboprobe (Fig. 4). All lanes containing total RNA showed all three bands observed previously, but the poly(A)$^+$ lanes showed only hybridization to 4.5–5.0 and 1.8–2.0 kb bands, which probably represent nonspecific hybridization to 28S and 18S rRNA since contamination with rRNA can occur even after two passes over oligo(dT) columns (Maniatis et al.,

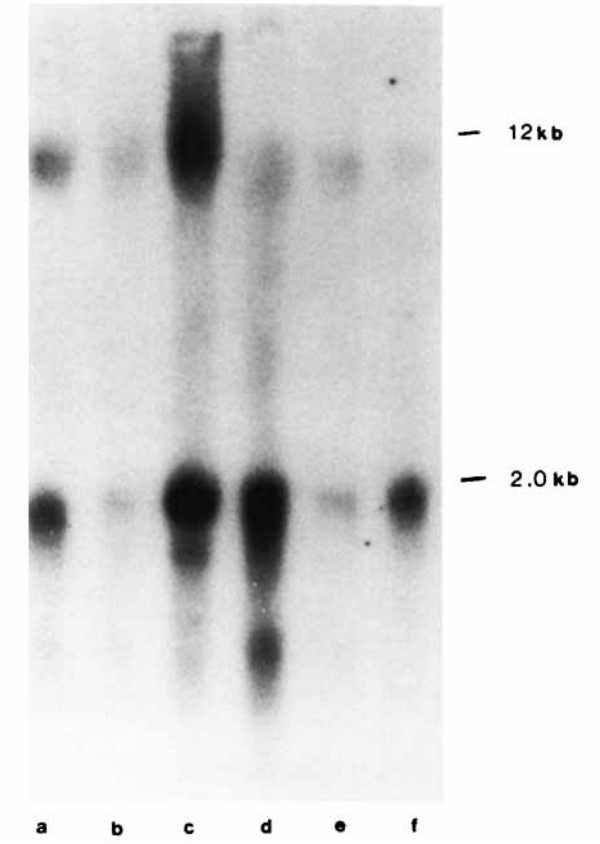

Fig. 2. Northern hybridization of Bkm sense riboprobe to adult mouse RNA. Results with male **(a–c)** and female **(d–f)** testes (a), liver (b,e), brain (c,f), and ovary (d) samples are shown.

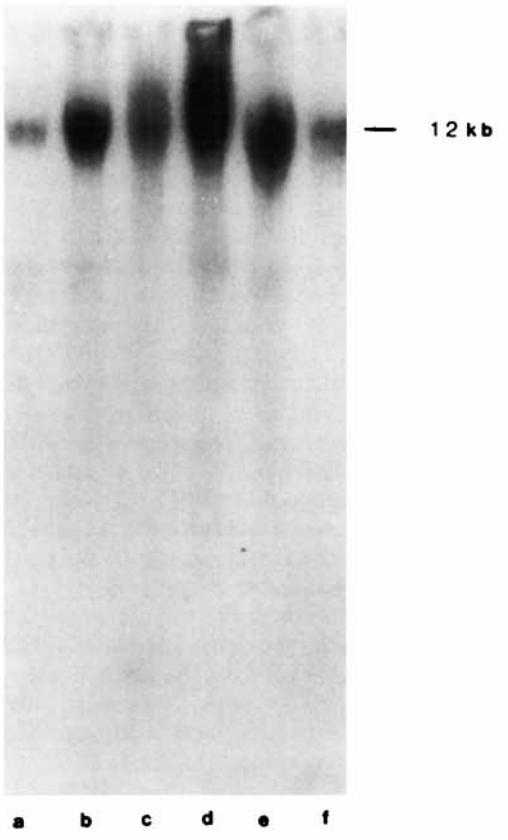

Fig. 3. Northern hybridization of Bkm antisense riboprobe to adult mouse RNA under stringent conditions. Results with female (a–c) and male **(d–f)** brain (a,d), liver (b,e), ovary (c), and testes (f) samples are shown.

1982). The absence of the 12 kb band in poly(A)$^+$ preparations was puzzling. It was possible that the large "transcript" size prohibited binding to the oligo(dT) cellulose column or that there was DNA present in the total RNA preparations that would not bind to the oligo(dT) column. The possibility of DNA contamination was explored. We treated RNA samples with RNase and observed a band at about 12 kb (results not shown). We also treated RNA samples with RNase-free DNase and observed no hybridization at the 12 kb putative transcript size (results not shown). Therefore, we conclude that small amounts of DNA contamination running at an unresolved, about 12 kb mobility hybridized to our probe since repetitive Bkm sequences in DNA will hybridize strongly. Thus we were unable to see any age-, sex-, or tissue-specific expression of Bkm sequences in mouse samples.

The use of the same sense and antisense Bkm riboprobes for Northern hybridizations to human fetal total RNA gave similar results. Variable degrees of degradation of these difficult to obtain RNAs had occurred so the ethidium bromide-stained gel is illustrated in Figure 5C for relative quantitation of amounts of RNA. With the antisense Bkm riboprobe, hybridization was at 4.5–5.0 kb in all samples except total fetal or 21 week female "gland" (Fig. 5A). These two lanes had lower amounts of RNA loaded than the other samples. The sense Bkm riboprobe hybridized strongly to a 1.8–2.0 kb band in all samples except total fetal RNA (Fig. 5B). In addition, fainter hybridization was observed at 4.5–5.0 kb in most tissues except total fetal RNA, 21 week female "gland," and another unidentified female tissue. These sizes again appear to correspond to the size of 18S and 28S ribosomal RNAs (Fig. 5C).

Hybridizations with the synthetic oligonucleotide $(GATA)_5$ gave results very similar to those observed with the antisense probe under stringent conditions (results not shown). The only hybridization was in the region of approximately 12 kb.

DISCUSSION

The role of Bkm sequence transcripts in sex determination appears to be minimal in mice and humans.

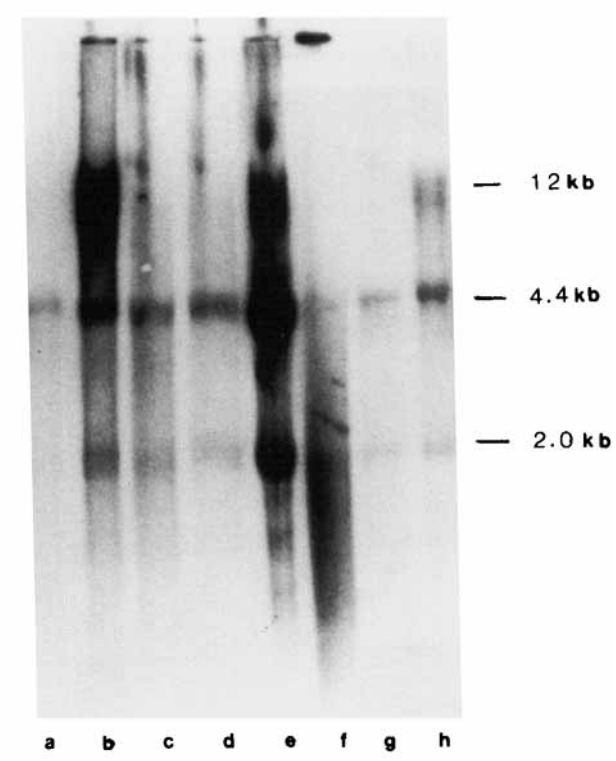

Fig. 4. Bkm antisense riboprobe to various mouse RNA samples. Results with liver poly(A^+) RNA (**a,g**), total mouse RNA (**b,h**), adult testes poly (A^+) RNA (**c,f**), immature testes poly (A^+) RNA (**d**), immature testes total RNA (**e**) are shown.

Although others have found transcripts in mice (see Introduction), we found no evidence using sensitive Bkm riboprobes at moderate to high stringency that either the sense or antisense sequences are expressed in human or mouse tissues. Singh and coworkers (1984) found sex-specific expression of Bkm-related sequences in mice. Using a *Drosophila* Bkm-containing clone as probe, three distinct mRNA bands were observed in adult male liver but not unsexed embryonic liver. However, the sex- and stage-specific expression has not been confirmed by others. Schafer et al. (1986) performed RNA hybridizations with adult mouse tissues using the synthetic oligonucleotides $(GATA)_4$, $(CTAT)_5$, and $(GACA)_4$ as probes. They found three distinct transcripts in total RNA from all tissues in hybridizations with $(GATA)_4$. The transcripts were 1.7 kb, 2.4 kb, and 3.0 kb in size. No sex specificity was observed. Their hybridization patterns and the size of at least one of these transcripts were similar to what we observed at low stringency in all tissues of both sexes for all stages studied with the antisense Bkm probe. However, at high stringency we were unable to detect any transcripts of this size.

Similarly we also were unable to find any specific transcripts at moderate to high stringency using the sense Bkm riboprobe. This result was not surprising, since this probe would hybridize to the antisense or noncoding sequence. However, Schafer et al. (1986) found both sense and antisense Bkm repeat sequences expressed in their studies. In addition, Adelman et al. (1987) reported two mammalian genes transcribed from opposite strands of the same locus in the rat. This would argue for the possibility that the two Bkm strands are both coding for some function although the sequences for Bkm make this very unlikely: Translation of the (GATA) strand is not possible because stop codons occur repeatedly in each reading frame. Translation of the other (CTAT) strand would yield an extremely hydrophobic protein. In fact, expression seems very unlikely for this protein, since the synthetic dodecapeptide was soluble only in 50% formamide (Banting, Goodfellow, and Erickson, unpublished data). Since we cannot find any evidence that these sequences are expressed, it is possible that they function in some other capacity.

One possible function would be that the Bkm sequences serve as a control element, either expressed (a control RNA) or unexpressed (possibly as identifier sequences as Sutcliffe et al. [1982, 1984] reported for rat brain mRNA). Bkm identifier sequences would function in regulation of the genes involved in sex determination. The validity of the concept of identifier sequences has been challenged; two reports have presented evidence against such identifier sequences (Minarovits et al., 1984; Owens et al., 1985). However, Watson and Sutcliffe (1987) recently found expression of selected repetitive families of DNA to be conserved in monkey and human brain similar to the identifier sequences of rat brain.

Unexpressed Bkm sequences could also function in regulation in the model proposed by Chandra (1985). In this model the main element would be a multicopy, noncoding sequence, which could be Bkm. This sequence, probably Y chromosomal, would function to bind an autosomal repressor preferentially that otherwise would bind to a testis-determining gene on the X chromosome. The presence of Bkm would allow the male-determining pathway to proceed. However, evidence that Bkm sequences bind a protein is lacking, and Page et al. (1987) have cloned the testis-determining factor from the human Y and find it to be a member of the zinc finger, presumably regulatory, class. Recently Waring and Pollack (1987) reported the cloning and characterization of dispersed repeated DNA sequences concentrated on the *Drosophila* X chromosome that are postulated to play a role in dosage compensation. Proof for the function of these repeats is lacking, but the noncoding nature of the sequence, its sequence conservation at dispersed sites on the X, and unichromosomal location share similarities with Chandra's model (1985). However, the sequence conservation breaks down across species of *Drosophila* (G.

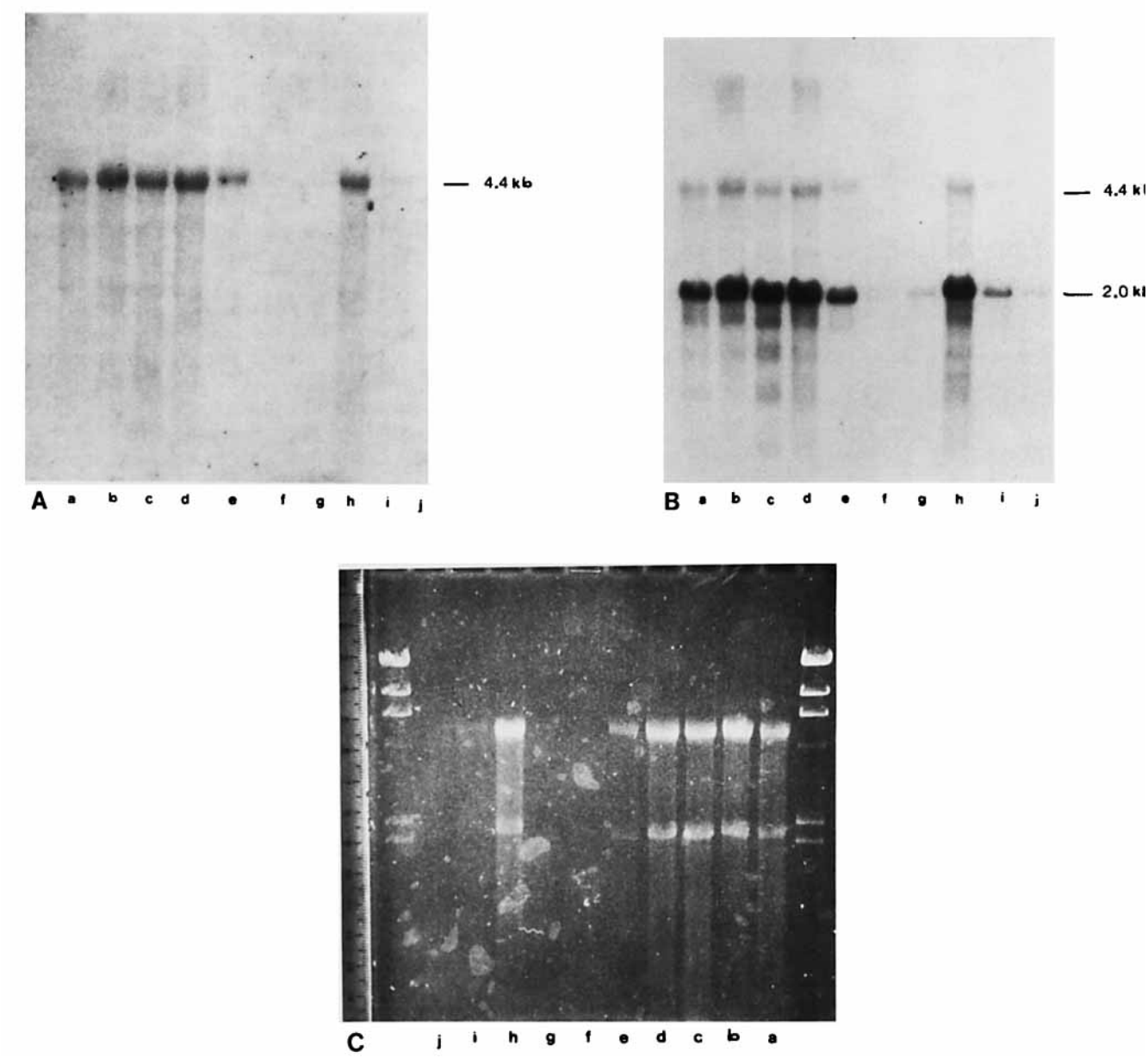

Fig. 5. Bkm riboprobe hybridization to human fetal RNA. **A** Antisense riboprobe. **B:** Sense riboprobe. **C:** Ethidium bromide stained. Results are from "gland" from XY, 13.5 week fetus **(a)**; kidneys from 13.5 week XY and 15 and 21 week XX fetuses **(b,d,h)**; "gland" from XX, 15 week fetus **(c)**; lung from 15 and 21 week XX fetuses **(e,i)**; total 16 week XY fetus **(f)**; "gland" from XX, 21 week fetus **(g)**; and another "gland" from XX fetus **(j)**.

Waring, personal communication), so its importance may be overstated.

Singh and Jones (1982) believed that Bkm sequences were probably more related to coordinate control than to a direct role in sex determination. They proposed that, at least in snakes, Bkm sequences most likely functioned in the cycle of condensation of the sex chromosomes. However, this is not true for the mealworm moth *Ephestia kuehniella* (Traut, 1987). This moth, although having sex chromosomes of the WZ type, contains Bkm sequences only on its autosomes. This autosomal localization of Bkm sequences does not rule out a relationship with sex determination, however, since the sequences could perform similar functions or could be marking other autosomal loci involved in the sex-determination pathway. The association of Bkm with mouse proximal chromosome 17 provides evidence for this. Through in situ chromosomal hybrid-

ization, Bkm sequences have been found to be present in an area of chromosome 17 related to sex determination (Kiel-Metzger and Erickson, 1984). Two deletions in this region of chromosome 17 cause hermaphroditism or sex reversal in mice on a C57BL/6 and Y Dom strain background (Eicher and Washburn, 1986). The possibility that these deletions delete Bkm sequences has been studied using in situ chromosomal hybridization. However, it appears that at least for one of the deletions, T^{hp}, the deletion does not significantly delete Bkm sequences (Durbin et al., 1988).

The question remains as to whether or not Bkm sequences serve any function. Perhaps they are merely nonfunctional accumulations on the Y chromosome. Phillips and coworkers (1982) found this for retroviral sequences on the mouse Y chromosome. This raises a point regarding the evolutionary conservation of these sequences. Perhaps, as Levinson et al. (1985) propose, the sequences are not conserved but have evolved independently in the wide variety of organisms studied. Differences have been found in the number and sequence of Bkm repeats in a number of organisms (Epplen et al., 1982; Singh et al., 1984; Erickson et al., 1987). These differences are not incompatible with independent evolution of the Bkm repeats. However, the role of Bkm repeat sequences in sex determination, if any, remains unknown.

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