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Microinjection of Bkm-related male-specific mouse DNA into autologous zygotes

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Abstract. GATA-rich Bkm-related DNA in the mouse Y chromosome is closely associated with the sex determining locus in the Sxr (sex reversed) mutation (Singh L and Jones K W, *Cell* 28: 205–216, 1982). Bkm-related male-specific DNA was gel separated from male mouse DNA as a > 23 kb band after *Hae* III and *Bst* NI digestions and microinjected into mouse zygotes to determine its effects on sexual differentiation. We observed that 1 out of 7 XX embryos and 3 out of 35 weaned female mice possessed a > 23 kb male-specific band, though the hybridization intensity was very low in comparison to the normal control male. Two presumptive male carriers of the injected Bkm DNA were identified by progeny tests. Various sporadic anomalies in sexual phenotypes were noted but no obvious connection was established between these and the injected Bkm-related DNA. No significant effects on sex ratio were found.

Keywords. Bkm; microinjection; sex determination.

1. Introduction

Bkm probes identify a sex specific > 23 kb *Hae* III band on Southern blots of male mouse DNA (Singh *et al* 1981) and the sex-determining locus has been shown to map to a small paracentromeric portion of the Y chromosome (Singh and Jones 1982). This region is enriched in a conserved component of Bkm DNA comprising repeats of the tetranucleotide GATA (Singh *et al* 1984). In humans, related sequences are comparatively concentrated in the proximal region of Yp (Singh and Jones 1986), which is also supposedly involved in sex determination. Male-specific mouse DNA sequences covalently linked to GATA repeats therefore may include the sex determining genes which cause sex reversal in the mouse mutant Sxr (Singh and Jones 1982). We have microinjected the > 23 kb male-specific Bkm-positive *Hae* III restriction band DNA into mouse zygotes to determine whether it can be stably transfected and to observe its effects on various sex-related phenotypes.

2. Materials and methods

2.1 *DNA*

High molecular weight DNA was isolated from the liver of normal male and sex reversed (XX/Sxr) male mice. Tissue homogenates were lysed with 2% sarcosyl in TES (30 mM Tris, pH 8·0, 5 mM EDTA, 50 mM NaCl) and centrifuged isopycnically in the presence of CsCl (1 g/ml) and EtBr (260 μg/ml). DNA was isolated, treated with butan-2-ol to remove EtBr, and after dialysis in TE (10 mM Tris, pH 7·4, 1 mM EDTA), extracted with phenol/chloroform/isoamyl alcohol (50 : 48 : 2). After precipitation with ethanol, the DNA was suspended in TE at appropriate concentrations.

NTS (non-transcribed spacer) of *Xenopus laevis* ribosomal DNA (pXlrsl) was a gift from Dr. A Bird. PCS–754 is a subclone of the Bkm-positive Eco RI fragment of *Drosophila melanogaster* clone CS316 (Singh *et al* 1981). 2(8) is a subclone of the Bkm-positive *Drosophila melanogaster* clone CS314, consisting substantially (48%) of GATA repeats (Singh *et al* 1984). M34 is a Y specific mouse clone (Singh and Jones 1984).

2.2 *Double digestion of DNA*

DNA was cleaved with the first restriction enzyme and the reaction stopped by heat inactivation. The digest was precipitated, dissolved and cleaved a second time with the desired enzyme in an appropriate buffer.

2.3 *Microinjection of DNA into zygotes*

CBA or CBAC57BLF1 female mice 3–6 weeks old were superovulated with 5 i.u. pregnant mare serum (PMS, Sigma) followed 20 hrs later by 5 i.u. human chorionic gonadotrophin (HCG, Sigma). The HCG injected mice were mated with *Ta* male mice. Fertilized eggs were isolated surgically from oviducts, treated with hyaluronidase (Sigma) to remove the cumulus layer and cultured in Whitten's medium at 37° C with 5% CO_2 in air under paraffin oil (Biggers *et al* 1971). Glass microneedles of 0·1–0·2 μm bore size were back-filled with the aid of small bore capillaries and used for microinjection of DNA (5000–10000 molecules/pl) into the pronuclei of the zygotes. Injection was stopped when the nucleus was judged to have increased two-fold in volume (Gordon *et al* 1980). After the operation, the zygotes were incubated for 1–5 hrs before transplantation into the oviducts of pseudopregnant CF1 surrogates synchronized to the developmental stage of the treated zygotes.

The X-linked coat colour mutation tabby (*Ta*) in the fathers of the microinjected zygotes was used as a genetic marker to differentiate the mice of female genotype (XX, *Ta*/+) from those of male genotype (XY, +) (Lyon 1963).

2.4 *DNA analysis*

The distal half of the tail tissue was cut off from weaned mice older than 3 weeks and used for extraction of DNA (Palmiter *et al* 1982). Whole embryos (10–13 days) removed from placenta and amnion were also used for preparation of DNA. DNA was extracted as described above, digested with *Hae* III, *Alu* I or *Eco* RI, separated

on 0·8% agarose gel and, after electrotransfer (Bittner *et al* 1980) to nitrocellulose, hybridized with the radiolabelled probes (Singh and Jones 1984).

2.5 *Probe labelling*

Double stranded DNA was labelled by nick translation according to the method of Maniatis *et al* (1976), using ^{32}P-dCTP (specific activity 3000 Ci/mM, Radiochemical Centre, Amersham). Single strand specific DNA probes were prepared according to the method of Hu and Messing (1982).

2.6 *HY antigen test*

HY antigen of spleen cells was assayed by Dr. E Simpson by the cytotoxicity test and T cell clone proliferation test (McLaren *et al* 1984).

3. Results

3.1 *Isolation of Bkm-related male-specific DNA*

DNA from male mice was digested with *Hae* III at 2 unit/μg at 37°C overnight and separated by preparative gel electrophoresis on 0·8% agarose gels. The top ethidium-stained fluorescent DNA band (> 23 kb), as shown in figure 1a, lane 1, was cut out and electro-eluted into a dialysis bag. This band, which is common to both sexes, includes Bkm-related male-specific DNA (figure 1b, lane 1). DNA common to both sexes can be eliminated by subsequent *Bst* NI digestion (figure 1a, lanes 3 and 4) leaving a Bkm-positive male-specific hybridization band (figure 1b, lanes 3 and 4) which was cut out and eluted as described above. The male specific DNA was concentrated to 0·25–1 μg/μl and used for microinjection. The final preparation represented a $2–3 \times 10^4$ fold reduction of the > 23 kb DNA originally eluted.

Alu I digested total male DNA shows a number of Bkm-positive bands above 4 kb which are absent in similarly treated female DNA (figure 1b, lanes 5 and 6). This band pattern remains the same after double digestion with *Hae* III (figure 1b, lanes 5 and 9), indicating that most of the *Alu* I male-specific Bkm bands are included in the *Hae* III male-specific Bkm band. The combined molecular weight of the Bkm-positive *Alu* I bands amounts to more than 100 kb, indicating the complexity of the > 23 kb *Hae* III male-specific DNA. The *Alu* I male-specific band pattern was not changed by the subsequent *Bst* NI digestion (figure 1b, lanes 5 and 7) used for the elimination of the DNA, including mouse satellite DNA, common to both sexes.

Mouse satellite DNA is extensively cut by *Bst* NI (Southern 1975) and was undetectable in the > 23 kb region by hybridization after *Hae* III and *Bst* NI double digestion (data not shown). However, a large preparation of *Hae* III/*Bst* NI resistant fragments of male-specific DNA contained a minor contamination of mouse satellite DNA. The extent of other non-Bkm DNA sequences in our preparation was not known.

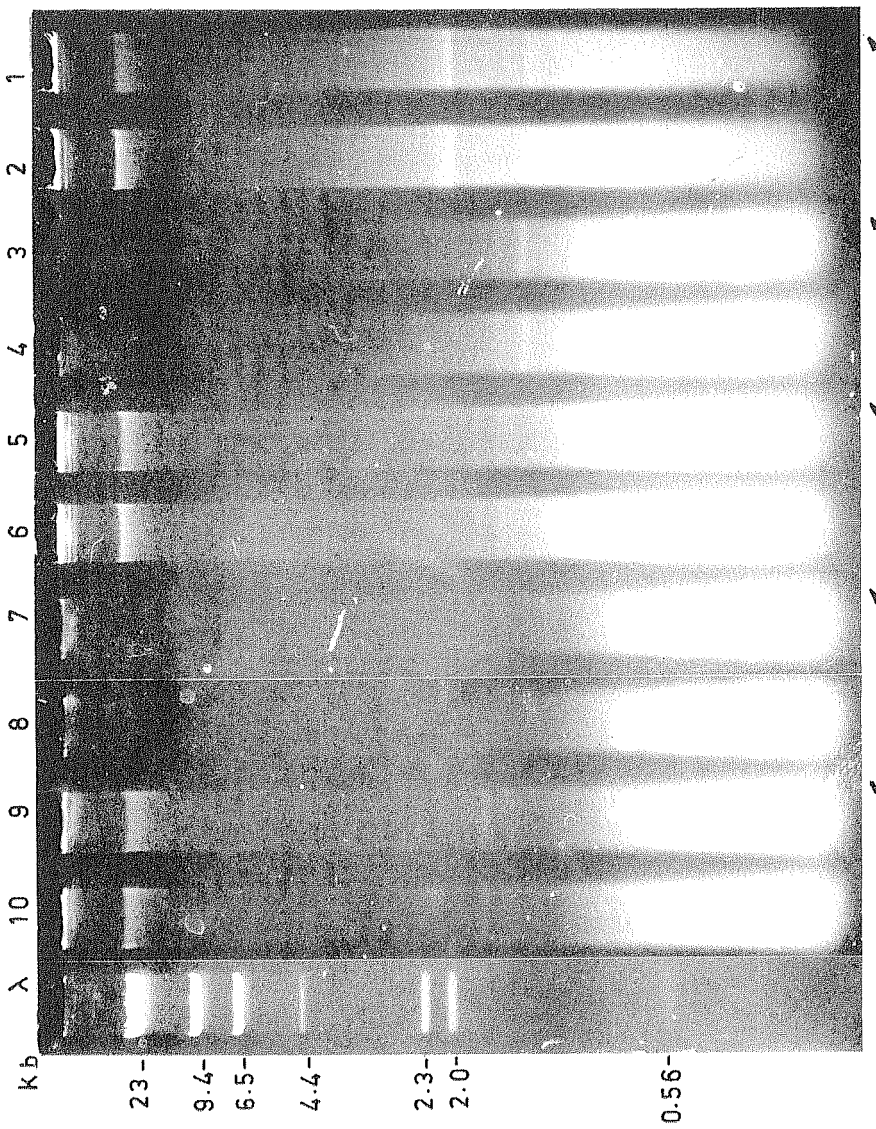

Figure 1a. An EtBr-stained gel after electrophoresis of male (lanes 1, 3, 5, 7, 9) and female (2, 4, 6, 8, 10) DNA (10 μg) digested with *Hae* III (lanes 1, 2), *Hae* III and *Bst* NI (lanes 3, 4), *Alu* I (lanes 5, 6), *Alu* I and *Bst* NI (lanes 7, 8), *Alu* I and *Hae* III (lanes 9, 10). The size marker is lambda DNA cut with *Hind* III. Note the top fluorescent band after single digestion and its absence after double digestion (*Hae* III/*Bst* NI, *Alu* I/*Bst* NI).

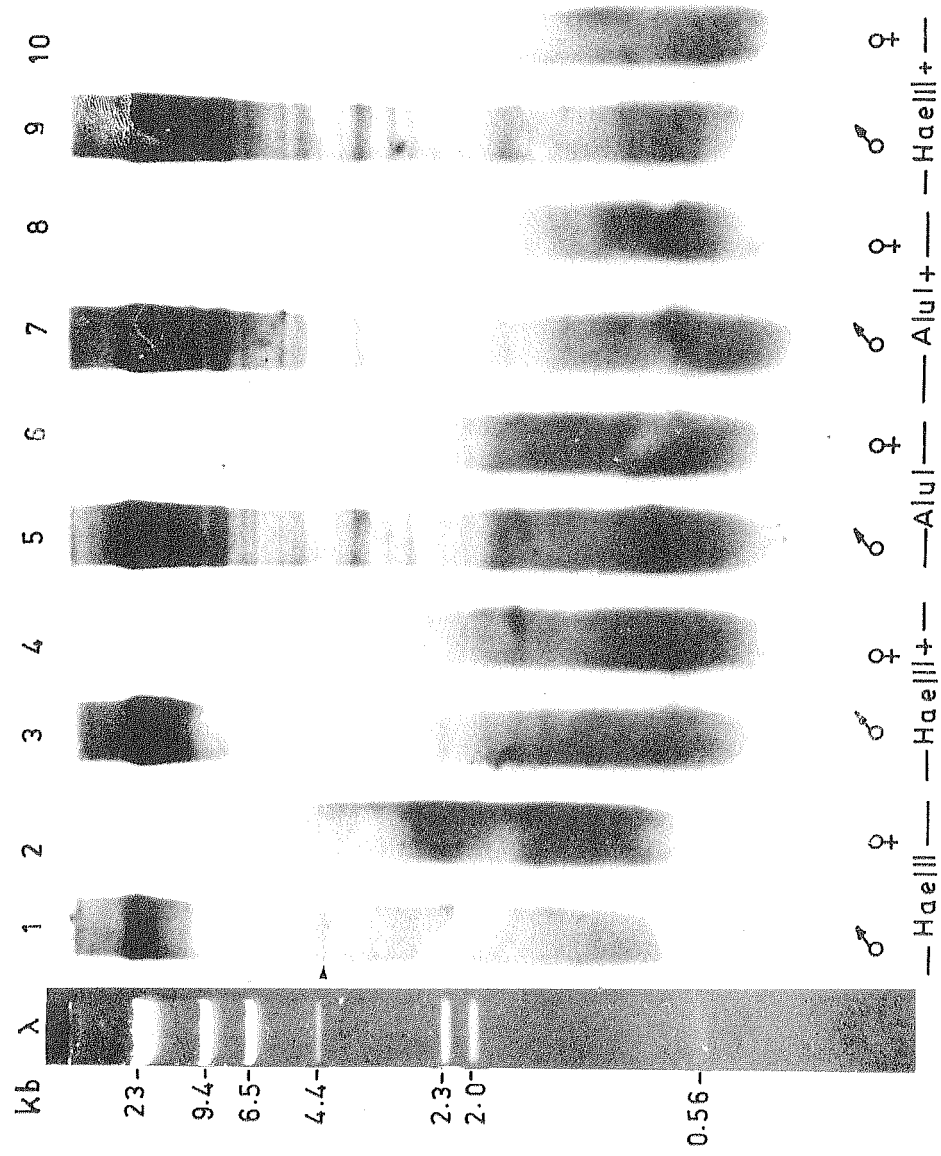

Figure 1b. Bkm-positive hybridization patterns of DNA in the gel shown in figure 1a, after transfer to nitrocellulose and hybridization with single stranded ^{32}P GATA probe 2(8). Note the striking sex difference (male: lanes 1, 3, 5, 7, 9; female: lanes 2, 4, 6, 8, 10). The male-specific patterns of the *Hae* III digest (lane 1) and of the *Alu* I digest (lane 5) remain unaltered by the subsequent *Bst* NI digestion (lanes 3 and 7, respectively). The male-specific hybridization pattern of DNA after *Alu* I digestion (lane 5) is almost the same as that after *Hae* III/*Alu* I double digestion (lane 9) implying the presence of *Alu* I sites within the male-specific large *Hae* III fragments. A series of Bkm bands between 4 kb and 10 kb in the *Hae* III/*Alu* I digest reflect the heterogeneity and complexity of the *Hae* III resistant male-specific high molecular weight DNA.

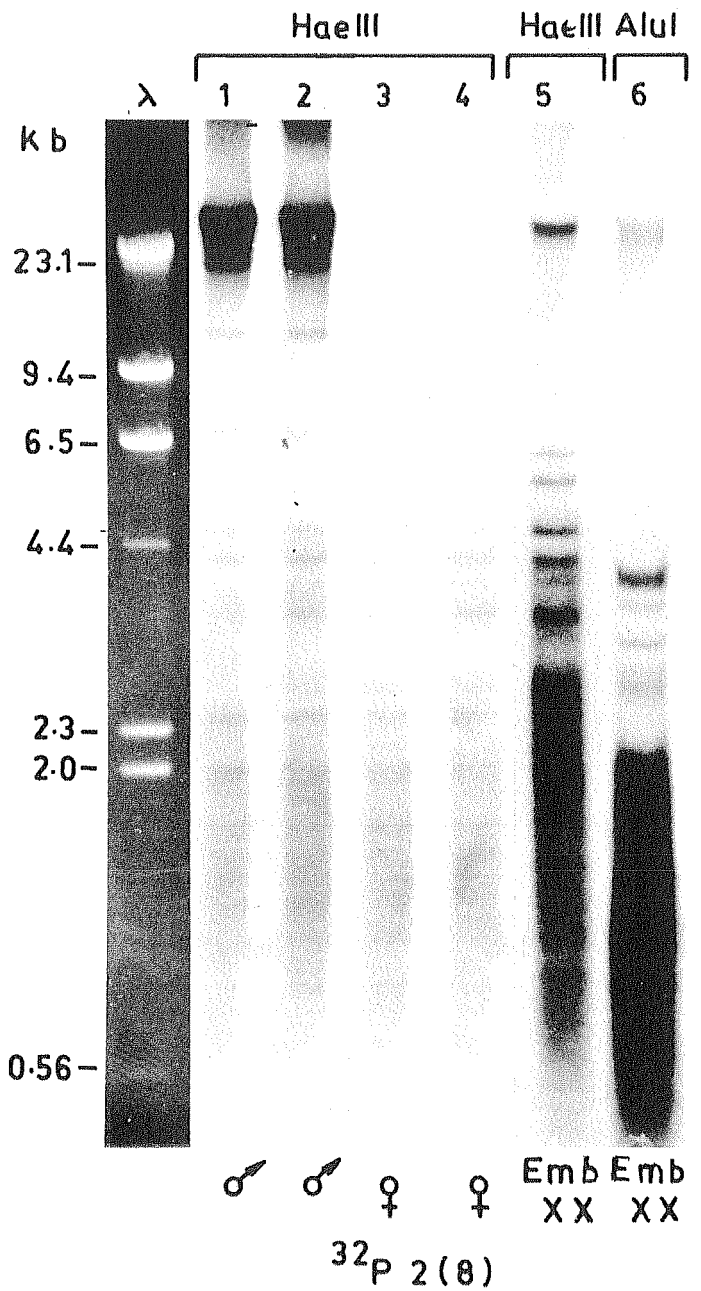

Figure 2. An electro-transferred blot of restriction enzyme digested and gel separated DNA (10 μg) hybridized with ^{32}P labelled single stranded GATA probe 2(8). Lanes 1 and 2: *Hae* III digested normal male mouse DNA, lanes 3 and 4: *Hae* III digested female mouse DNA, lanes 5 and 6: XX embryo DNA digested with *Hae* III (lane 5) and *Alu* I (lane 6) showing integration of the Bkm-positive male-specific large *Hae* III fragment microinjected at the zygote stage. Note a band of more than 23 kb in *Hae* III digested XX embryo DNA and its absence in normal female DNA (lanes 3, 4). The reduced intensity of the 23 kb band and the exceptionally strong 4 kb male-specific band in the *Alu* I digest (lane 6) compared to the single strong high molecular weight band in the *Hae* III digest (lane 5) indicates the heterogeneity of the injected Bkm-positive male-specific *Hae* III fragment (see text).

Table 1. Microinjection of Bkm-related male-specific DNA into mouse zygotes.

Injected DNA	Number of microinjected zygotes	Number liveborn		Number of dead newborn[e]	Number positive for extra DNA
		male	female		
Uninjected control	184	14	9	1	—
Bkm (CBA)[a]	612	14	16	12	1
Bkm (Danish)[b]	155	6	4	2	0
Bkm (Sxr)[c]	182	3	7	3	2
Bkm (Sxr) + NTS[d]	182	13	10	3	3 (NTS only)

[a]Bkm male-specific DNA isolated from CBA male DNA; [b]Bkm male-specific DNA isolated from Danish mouse male DNA; [c]Bkm male-specific DNA isolated from XXSxr male DNA; [d]NTS non-transcribed spacer DNA from *X. laevis* ribosomal DNA; [e]found dead mostly within 24 hrs after birth.

3.2 *Identification of the injected DNA in weaned mice*

In the first series of experiments, 1131 zygotes injected with various DNA, as shown in table 1, were transplanted into the reproductive tracts of surrogates. Of 93 pups born, 20 died before hair growth. Of 73 weaned pups, 37 were female and 36 male. X chromosome linked *Ta* varigated coats were found only in females, indicating no sex reversal in this population.

DNA was prepared from the tail tissue of these weaned mice, digested with *Hae* III, separated by agarose gel electrophoresis and hybridized with radiolabelled GATA probe 2(8). Male DNA showed a typical intense > 23 kb hybridization band which would have obscured any additional bands arising from transfection (figure 2, lanes 1 and 2). In female DNA., however, it is possible to detect extraneous DNA bands in this region (figure 2, lanes 3 and 4). Among 35 female DNA examined in the experimental group, 3 were found (table 1) which exhibited a > 23 kb Bkm hybridization band, absent in unoperated female controls. However, the intensity of this band was very low in comparison to male control DNA (not shown). An extra Bkm-positive 4 kb band was seen in some male and some female DNA. However, this is also found in the parental strains and we interpret it to be a polymorphic band, irrelevant to the injected DNA.

In 182 embryos, the Bkm male-specific > 23 kb DNA from the sex reversed male mice was co-injected together with repetitive NTS DNA of the *Xenopus laevis* ribosomal gene as an additional means of determining the efficiency of the injection procedure. Among 23 mice weaned, three females were shown to carry only this foreign DNA (table 1). Although this series produced the highest number of mice with apparent integration of foreign DNA, the efficiency of integration of the co-injected male-specific > 23 kb DNA remained differentially low.

3.3 *Identification of injected DNA in embryos*

A high incidence of early death in the new-born mice from injected embryos led us to examine the possibility of selective loss of embryos carrying extraneous > 23 kb DNA. Mouse zygotes were injected either with male-specific DNA or with another

cloned DNA, PCS 754, and transplanted into surrogates. Embryos at 10–13 days of gestation were recovered from the gravid surrogates and their DNA examined.

23 embryos (38%) developed normally from the male-specific > 23 kb DNA-injected zygotes. 15 DNA samples from these embryos were examined with a Y specific repeated DNA probe (M34) to distinguish XX from XY genomes. 7 embryo DNA samples were judged XX and were digested with *Hae* III and hybridized with GATA probe 2(8). One of them showed a clear > 23 kb band which was distinctly less intense than the equivalent band in the male control DNA (figure 2, lane 5). The digestion of this 23 kb-positive embryo DNA with *Alu* I significantly reduced the > 23 kb hybridization signal and gave rise to an intensely hybridizing band at 4 kb (figure 2, lane 6, compared to figure 1b, lanes 5, 7, 9 for the male control and lanes 6, 8, 10 for the female control). This comparatively simplified *Alu* I band pattern suggested the integration of only a few component copies of the male specific > 23 kb *Hae* III fragments in this embryo DNA. It also indicated that > 23 kb DNA was composed of a small GATA-rich domain flanking a large GATA-sparse domain.

21 embryos (33%) developed normally from the PCS-754 injected zygotes, and 1 of 7 DNA samples tested showed hybridization to the PCS 754 DNA. Apparently the integration rate in embryos of Bkm-related male-specific DNA (1/7) was comparable to that of plasmid DNA (1/7) and to that of the NTS DNA in weaned mice (3/23). In contrast, the integration rate of the injected > 23 kb male-specific DNA in the weaned pups (3/35) appeared to be lower than that in the embryos.

3.4 *Transmission of transfected male-specific > 23 kb DNA to female progeny*

Progeny tests were carried out to determine (1) whether the > 23 kb bkm bands found in three transfected female mice would be sexually transmitted, and (2) whether transfected male mice carry cryptic > 23 kb DNA of extraneous origin on chromosomes other than the Y.

Female progeny DNA from 23 pairs of transfected male and female embryos which had developed to sexual maturity were mated and yielded 185 male and 222 female progeny. The females of these were examined for the Bkm-positive extra *Hae* III bands. One female mouse, which carried a > 23 kb Bkm band produced 10 female progeny. DNA from 7 of these females was probed with 2(8) but no corresponding Bkm-positive > 23 kb *Hae* III band was found. The remaining two Bkm-positive females produced only a small number of progeny and subsequently became infertile. 3 female DNAs examined from these progeny also showed no > 23 kb Bkm-related *Hae* III band (table 2).

18% and 25% respectively, of female progeny sired out of normal females by two transfected male mice exhibited an unequivocal > 23 kb *Hae* III band positive to Bkm (table 2, figure 3, lane 7 left panel and lane 1 right panel). This band was not found in the mothers of these positive females, indicating a paternal origin. The intensity of the > 23 kb male-specific bands in these daughters was comparable to that of the equivalent bands in three other females described in §3.2. As expected, the male progeny DNA from these matings showed a normal male pattern (figure 3, right panel, lanes 2–8).

The two females with a male-specific > 23 kb *Hae* III band, which developed from injected zygotes, and two of the daughters who received this component from

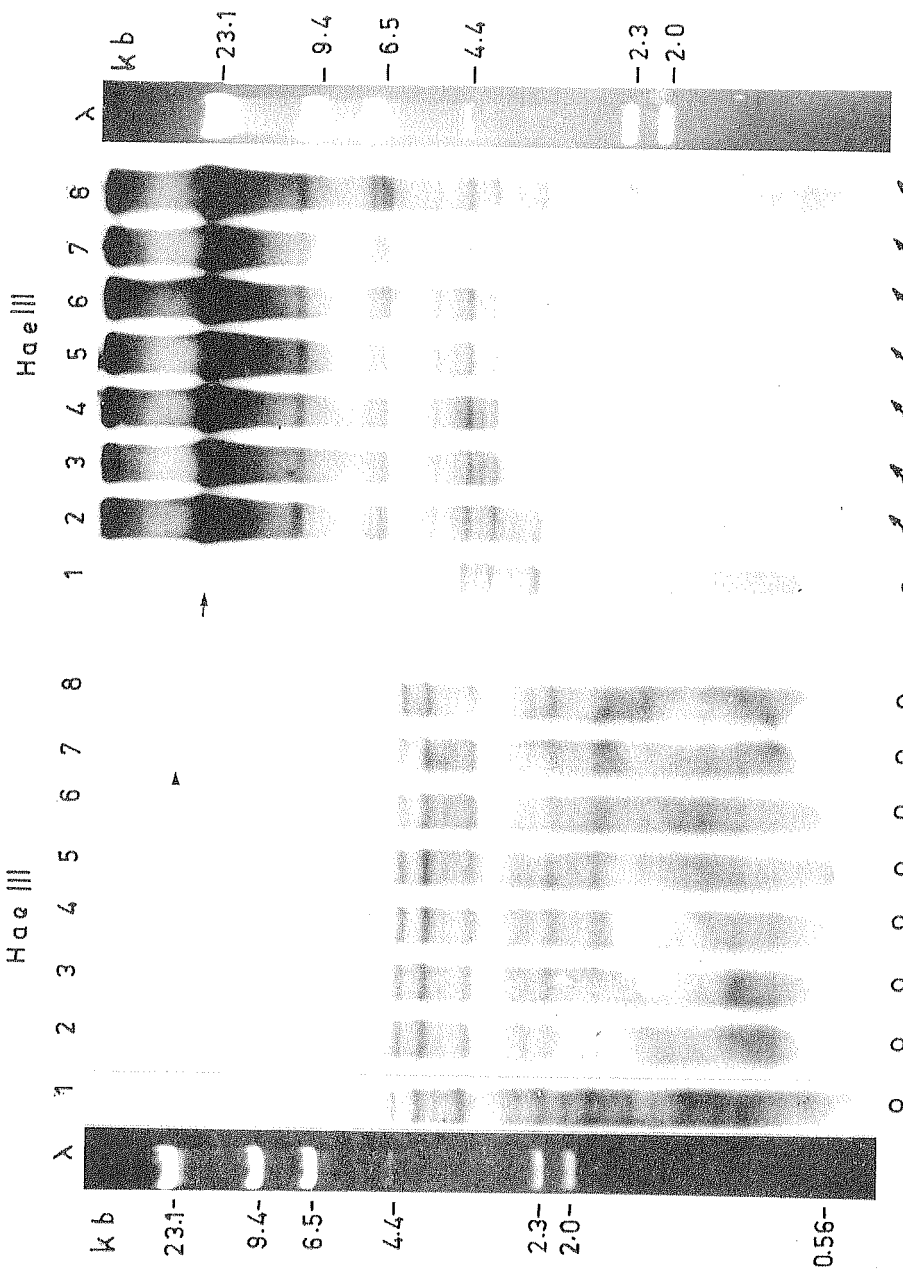

Figure 3. DNA of male (right panels 2–8) and female (right panel 1, left panel) progeny sired by a male mouse (83-6) which developed from a DNA microinjected zygote. The DNA (10 μg), digested with *Hae* III, was transferred to nitrocellulose and hybridized with a single stranded ^{32}P GATA probe 2(8). The arrows indicate the presence of a Bkm-positive male-specific *Hae* III fragment in two female DNAs (lane 7, left panel, lane 1, right panel). Some polymorphic lower molecular weight bands are also evident in several tracks and are derived from the parental strains.

Table 2. Transmission of integrated male-specific DNA to female progeny.

Mouse	Sex	Transfected DNA	Number of progeny		Female progeny with male/ specific band	HY antigen status
			male	female		
51–1	female	+	4	i.f.	0/3	negative
100–2	female	+	3	i.f.	–/–	negative
97–1	female	+	11	10	0/7	not determined
83–6	male	+?	31	37	5/20	not determined
83–6–4[a]	female	+	10	14	–/–	negative
90–7	male	+?	17	28	5/28	not determined
90–2–17[b]	female	+	2	6	–/–	negative

+ > 23 kb *Hae* III band; +? Presumed cryptic > 23 kb band; [a]daughter of 83-6 male; [b]daughter of 90-7 male; i.f. infertile.

transgenic fathers carrying cryptic exogenous 23 kb male-specific DNA, were subjected to tests for HY antigen (table 2). Reaction of the spleen cells from these

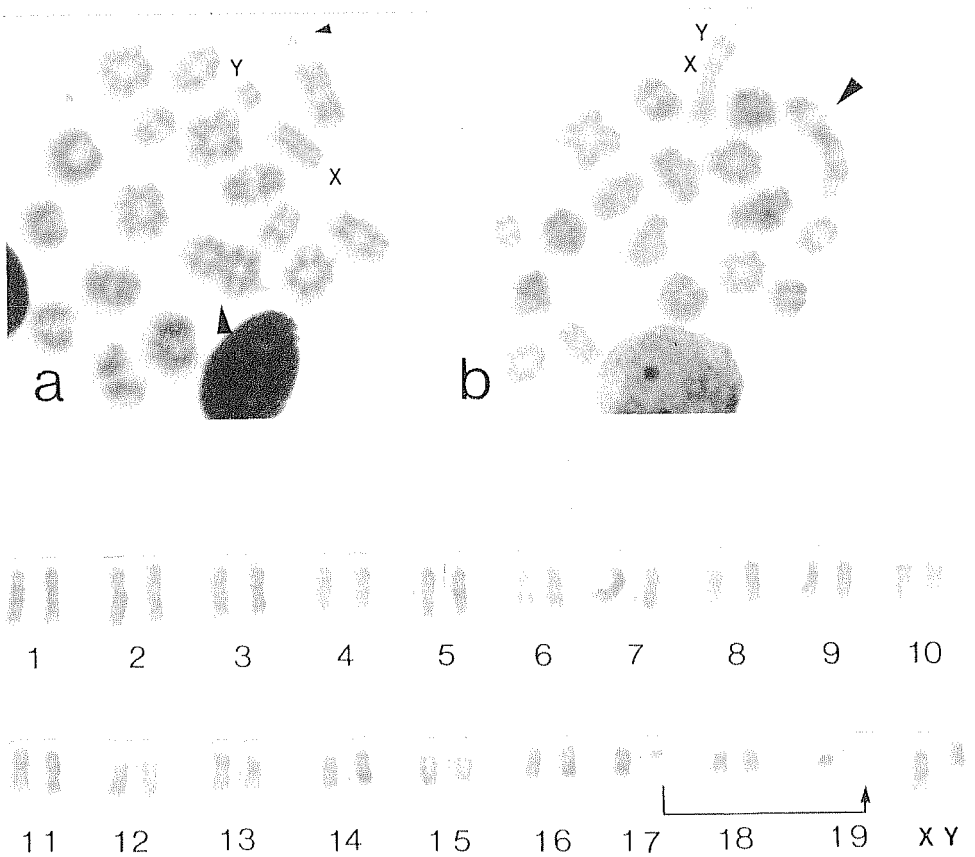

Figure 4. Giemsa-stained meiotic chromosomes in diakinesis and a karyotype of bone marrow chromosomes of a sterile male which developed from a DNA microinjected zygote. The karyotype shows a translocation involving chromosomes 17 and 19. At meiosis this gives rise to a trivalent (arrow head) and a small univalent (small arrow head) in (**a**) or a chain of 4 chromosomes (arrow head) in (**b**).

females was similar to the reaction of control CBA female spleen cells in both cytotoxicity and T cell clone proliferation assays.

3.5 *Phenotypic anomalies*

Among mice which developed to sexual maturity from DNA injected zygotes, three males and three females were infertile. Analysis of meiotic and bone marrow chromosome preparations from these male mice revealed the same chromosome aberration (der(17), t(19; 17)), shown in figure 4. Two infertile female mice also carried the same chromosome aberration. This aberration seemed to originate from one female of our CBA stock since we subsequently detected the same aberration in her infertile male progeny. As well-documented, reciprocal translocation causes sterility in males but not in females (Beechey *et al* 1980; de Boer and Searle 1980). Thus we cannot rule out the possibility that the presence of the transfected 23 kb *Hae* III band DNA in the two cases (table 2) produced adverse effects on female reproduction. In this regard, it may be worthwhile to mention that the fertile female, described above, with this male-specific band was found on autopsy to have developed an ovarian tumour.

Two male progeny had small testes but were found to be XYY and XY/XX mosaics (Singh *et al* 1987). One male progeny had one normal testis on the right side and one vestigial small testis on the left, and another male progeny had one vestigial left hind leg. Since these were isolated cases, we were not able to connect any of these findings to the DNA which was microinjected at the zygotic stage of the parents.

3.6 *Sex ratio after the male-specific DNA injection*

To exclude the chromosome aberration found in the CBA stock, we used CBAC57BLF1 females mated with the *Ta* males as a source of the zygotes to investigate possible effects on sex ratio of microinjected > 23 kb male-specific DNA. From a total of 811 zygotes injected, 106 survived to weaning (table 3). Again, the varigated coat correlated strictly with female development. Seven randomly chosen female DNA were examined but did not show any unexpected extra Bkm band. DNA analysis of these females was not extended further. Over a thousand progeny from 44 pairs of transfected mice were examined for developmental anomalies. Apart from one sterile male and two sterile females, all were fertile. There was only one case of anomaly (a vestigial testis on the left). When the sex ratio of the progeny in each pair was plotted on the cartesian coordinate system,

Table 3. Sex ratio of mice from male-specific DNA injected zygotes

Experiment number	Number of transplanted zygotes	Number weaned			Number sterile	Number of pairs bred	Number of progeny	
		male	female	total			male	female
1	1131	36	37	73	6	23	185	222
2	120	8[a]	7[a]	(sacrificed at 10–13 days gestation)				
3	812	53	53	106	3	42	600	532

[a]Data derived by hybridization with Y probe (M34)

there were some digressions, e.g. 29 male 15 female, 21 male 11 female, and 12 male 20 female, but otherwise no extreme ratios were found.

4. Discussion

The male-specific Bkm-related > 23 kb *Hae* III DNA used in our injection experiment was resistant to *Bst* NI digestion. However, mouse satellite DNA was not completely eliminated and other non-Bkm-associated DNA could still have remained and may have contributed to a reduction in the efficiency of integration of the male-specific DNA following microinjection. Nevertheless, the finding of three females and one XX embryo which contained a > 23 kb *Hae* III Bkm-positive male-specific DNA band indicated that this comprised a high proportion of our preparation.

The total length of the Bkm male-specific DNA was estimated to exceed 100 kb. Therefore, it is reasonable to assume that several different Bkm-related *Hae* III fragments comprise the visible > 23 kb *Hae* III fragment, which could account for the fact that the > 23 kb Bkm-positive DNA found in the transfected female mice hybridized faintly. However, other possible explanations can be offered, such as a loss of the short GATA-enriched segments, or that the mice were mosaics of transfected and untransfected cells (Palmiter *et al* 1984). However, the finding of transmission of the male-specific *Hae* III band from the presumptive carrier fathers to their female progeny suggests integration in the germ cells in reduced copies. The negative result after HY antigen tests also excluded the possibility that the results obtained reflected the presence of XY cells. This source of possible artefact was also ruled out by the use of another Y-specific DNA probe (M34) which is not present in the Bkm male-specific DNA fraction (in preparation).

Assuming 3/35 positive mice reflects the integration rate of the Bkm-related male-specific > 23 kb *Hae* III fragments, 16 carriers of such Bkm-related extra *Hae* III fragments might be expected in the mice which survived the injection operation. If the Bkm male-specific *Hae* III fragments consist of several different components, the present scale of experiments would therefore hardly be adequate to reveal possible effects on sexual or other development. Further problems would arise if different kinds of sequences are required to cooperate for effective gene expression or if multiple copies of Bkm-related male-specific sequences are required to achieve sex reversal in the genetic female. It is also possible that *Hae* III and *Bst* NI digestion destroys the putative functional sequences.

Tissue-specific expression of integrated genes in transgenic mice has been reported by several workers (Swift *et al* 1984; Grosschedl *et al* 1984; Selden *et al* 1986; Bucchini *et al* 1986) though identification of differentiation-controlling DNA sequences by this method has not yet been reported. The unique situation in sexual differentiation is the absence in females of a male-specific DNA region which controls testicular differentiation. Therefore introduction of the intact DNA sequence corresponding to such a chromosomal region into the female genome should give rise to sex reversal, as happens in the mouse Sxr mutation (Singh and Jones 1982) and presumably in various XX human males (Guellaen *et al* 1984; Vergnaud *et al* 1986). One approach to this would be to introduce into zygotes chromosome-sized DNA, obtained by microdissection (Rohme *et al* 1984; Fisher *et*

al 1985) and microextraction (Scalenghe *et al* 1981) of Y chromosomes. Bkm DNA can be used as a probe for identifying the injected DNA in the way we have described here.

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