

Nature of B chromosomes in the harvest mouse *Reithrodontomys megalotis* by fluorescence *in situ* hybridization (FISH)

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Received 7 May 1997; accepted for publication by D. Ward 4 July 1997

Using fluorescence *in situ* hybridization, we examined the characteristics of two types of B chromosomes in harvest mice of the genus *Reithrodontomys*. B chromosomes were interrogated with rDNA, telomeric repeat, LINE element and centromeric heterochromatin probes. The two types of B chromosomes share the following features: (a) telomeres present on the ends of both arms; (b) hybridization to LINE probes; (c) absence of hybridization to the ribosomal gene probes; (d) C-band-positive centromeric regions; and (e) euchromatic arms. They differ as follows: (a) the larger B element hybridizes to the centromeric heterochromatin (pMeg-1) probe whereas the smaller B element does not; (b) the amount of C-band-positive material is reduced in the smaller B chromosome relative to that present on the larger B chromosome; and (c) the smaller element is reduced in size by about a third. It is concluded that the larger B chromosome arose as a leftover centromere from centric fusion, whereas the smaller element has a different origin – perhaps as an intact fragment or as an amplified region from the A chromosomes. The presence of euchromatic regions on B chromosomes may account for their survival in the karyotype.

Key words: B chromosomes, centric fusion, fluorescence *in situ* hybridization, *Reithrodontomys*

Introduction

The origin, nature and evolution of B chromosomes remain largely unresolved. Several mechanisms of B chromosome evolution have been proposed. These include originating as the leftover centromere from centric fusion events (Patton 1977) or from fragments from trisomic pairing (Amos & Dover 1981). In both of these cases, the B chromosome originates as an intact fragment from the A complement. A radically different explanation (Brockhouse *et al.* 1989) for the origin of B chromosomes is that they are a eukaryotic nuclear counterpart to the prokaryotic plasmids. In the Brockhouse *et al.* (1989) proposal, these independent genetic elements within the cell nucleus are not derived from the chromosomes of the cell. An explanation that might be a hybrid between these two alternatives is that

some subset of sequences from the nuclear DNA from within the cell might be amplified into B chromosome-sized entities by mechanisms such as those that produce double minutes, homogeneous staining regions and megachromosomes (Gurstel & Burns 1970, Cowell 1982). Nuclear amplification has been shown to be responsible for production of numerous extrachromosomal double minutes in mammalian tumour cells (Cowell 1982, Hamkalo *et al.* 1985). As our understanding of the variation in genome size among closely related organisms documents the possibility of newly amplified regions, the possibility that B chromosomes arose as a side-effect of one of the mechanisms proposed to explain the C-value paradox (Thomas 1971, Cavalier-Smith 1985) should not be eliminated without empirical evidence from properly designed scientific tests.

B chromosomes are found in a variety of taxonomic groups ranging from higher plants to mammals (Jones & Rees 1982). B chromosomes are characterized by a non-Mendelian mode of inheritance, absence of pairing with the A chromosomes in meiosis, generally being smaller than chromosomes from the A complement (Jones & Rees 1982) and having some sequences unique from those present in the A complement (McQuade *et al.* 1994). The lack of similarity between A and B chromosomes suggests that significant differences exist between A and B chromosomes. Green (1990) has suggested that structural differences between B chromosomes and the A complement have arisen by the same mechanisms that allow the high degree of sequence divergence seen in the Y chromosome. This mechanism, known as Muller's Ratchet (Bull 1983), allows sequences in regions that are not constrained by selective pressure to rapidly evolve with each generation envisioned as a click of the ratchet. This hypothesized mechanism was supported by Peeters *et al.* (1985) who noted that newly formed B chromosomes in maize (*Zea mays*) were more heterochromatic in subsequent generations.

The majority of the work characterizing B chromosomes has been examined in higher plants (Jones & Rees 1982), and few studies on the molecular characterization of B chromosomes in mammals have been published. Wurster-Hill *et al.* (1986) examined B chromosomes in raccoon dogs (*Procyonoides viverrinus*) using

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G- and C-banding techniques and concluded that the B chromosomes were not entirely heterochromatic. McQuade *et al.* (1994) used microdissection and polymerase chain reaction (PCR) to hybridize B chromosomal DNA back to the A complement in the greater glider (*Petauroides volans*). They noted sequence homology between the B chromosomes and the centromeric regions of all of the chromosomes of the A complement except for the Y chromosome. Work on other mammalian taxa has been primarily descriptive of presence and morphology of B chromosomes (for review see Jones & Rees 1982).

B chromosomes were first described in the Western harvest mouse *Reithrodontomys megalotis* by Shellhammer (1967). *Reithrodontomys megalotis* is widely distributed from southwestern Canada to west of the isthmus of Tehuantepec in southern Mexico. B chromosomes have been reported from individuals collected from widely separated geographic areas, which suggests that B chromosomes arose in *R. megalotis* before the species became so geographically widespread or, alternatively, that B chromosomes have arisen several times in this species. In this study, we have examined B chromosomes in *R. megalotis*, the western harvest mouse, using fluorescence *in situ* hybridization (FISH) to determine the presence or absence of rDNA sites, LINE elements, telomeric repeat sequences and centromeric heterochromatin in this B chromosome system.

Materials and methods

Specimens examined

All specimens examined were collected from natural populations using Sherman live traps and were prepared as voucher specimens deposited in The Museum of Texas Tech University. TK numbers correspond to laboratory records and cell suspensions to voucher specimens. TK48817, TK48818, TK48821, TK48825, TK48564: Mexico, Durango; TK5118: Texas, Floyd County; TK54142: Texas, Presidio County; TK31755: New Mexico, DeBacca County; TK54340, TK54339, TK54385: Nebraska, Antelope County; TK46938, TK46939, TK46940: Nebraska, Lincoln County.

Cytological procedures

Cell suspensions were prepared from bone marrow using methods similar to those of Baker *et al.* (1982). Spreads were obtained by blaze drying one or two drops of cell suspension.

Probes

LINE-1 DNAs (clones MX109 and MX28) were isolated from *Peromyscus maniculatus* (provided by H. Wichman). Ribosomal DNA was isolated from the 18s and 28s gene regions of the rDNA cistron from *Mus* (provided by N. Arnheim). The *Reithrodontomys* heterochromatic probe pMeg-1 is identical to that described in Hamilton *et al.* (1990) (provided by R. Honeycutt). The telomeric repeat probe consisted of (TTAGGG)₇ (provided by J. Meyne). Probes were labelled by either standard nick translation with biotinylated dUTP (Clontech Laboratories) (LINE elements, rDNA probes, pMeg-1) or 3' end labelling with biotinylated dCTP (substituted for digoxigenin-labelled dUTP, Boehringer Mannheim DIG oligo-

nucleotide 3' end labelling kit) (telomeric repeat probe). Probes were prepared for FISH by denaturing with 30% formamide in 2 × standard saline citrate (SSC) at 72°C for 5 min.

Fluorescence *in situ* hybridization procedures

Fluorescence *in situ* hybridization followed the procedures of Hamilton *et al.* (1990) and Baker and Wichman (1990) with slight modifications. Slides were treated with 200 μl of RNAase (100 ug/ml) at 37°C for 1 h. Slides were washed four times in 2 × SSC at room temperature, dehydrated in an ethanol series (70%–80%–95%) and allowed to air dry. Partial denaturing of the chromosomes was accomplished by placing the slides in 70% formamide in 2 × SSC at 72°C for 2 min and dehydrating in a cold ethanol series. Denatured probes were added to slides and hybridization was maintained at 37°C for 12–16 h in a humidity chamber. After hybridization, slides were washed five times in 2 × SSC at 42°C. Detection of signal was accomplished by treatment with fluorescein-conjugated avidin (Vector Labs), followed by two amplifications consisting of alternate treatments of biotinylated goat anti-avidin (Vector Laboratories) and fluorescein-conjugated avidin. Slides were counterstained with antifade containing propidium iodide (Sigma) and DAPI (4,6-diamidino-2-phenylindole, Sigma). For each probe, several individuals were examined and at least ten complete spreads per individual were analysed using an Olympus epifluorescent microscope.

Results

A chromosomes

A chromosomes in *Reithrodontomys* have the following characteristics revealed by FISH. Firstly, hybridization with (TTAGGG)₇ (the telomeric repeat) produces visible signal at the ends of every chromosome as well as blocks that encompass the centromeric regions of two pair (Meyne *et al.* 1990) (Figure 1A). Secondly, hybridization with the 18s and 28s ribosomal gene probes produces signal on eight pairs of chromosomes (Figure 1B). Thirdly, hybridization with LINE elements isolated from *Peromyscus* produces signal present on all of the A chromosomes with a banding pattern over the length of the chromosomes consisting of areas of lighter and darker hybridization. These probes are described in Casavant *et al.* (1996), and we used two LINE-1 probes (MX109, MX28) that differ by approximately 12% sequence divergence. Both probes gave the same pattern. There are two major blocks of heterochromatin associated with the X and the Y chromosomes in which there is little or no hybridization to LINE probes (Figure 1C). Finally, hybridization with the heterochromatic probe pMeg-1 produces signal on the centromeric region of all A chromosomes and in blocks as described by Hamilton *et al.* (1990) (Figure 1D). C-banding of the A complement produced a distribution of heterochromatin similar to that reported by Van Den Bussche *et al.* (1992), revealing C-band-positive centromeric regions as well as large blocks of C-band-positive material on the short arms of autosomes and large blocks on the X and the Y chromosomes.

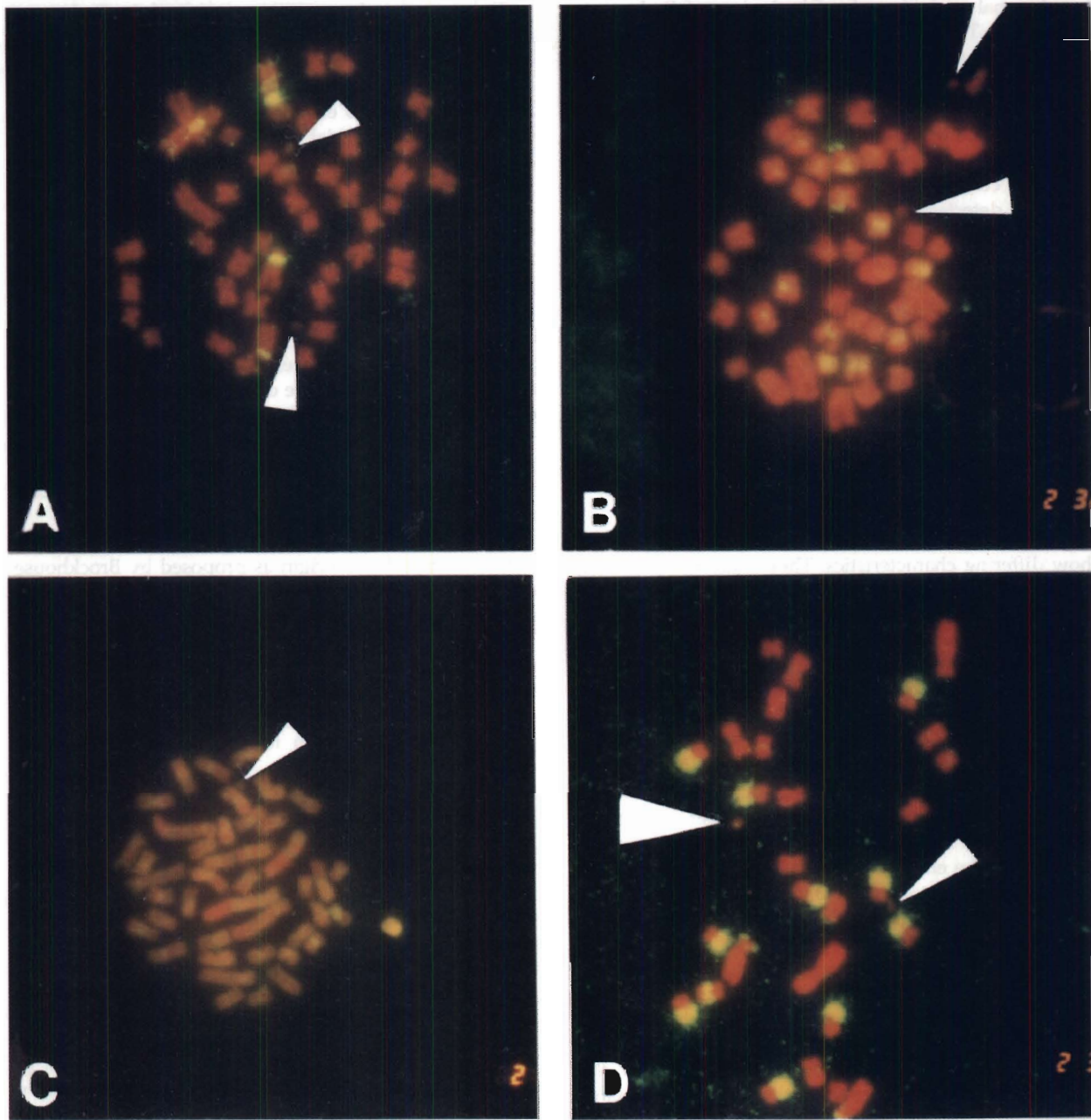


Figure 1. Fluorescence *in situ* hybridization to metaphase spreads of *Reithrodontomys megalotis* with B chromosomes using several labelled DNA probes. **A** Hybridization with the telomeric repeat sequence (TTAGGG)_n. Both sizes of B chromosomes are indicated (arrows). **B** Hybridization with the rDNA probes. Note lack of hybridization to both sizes of B chromosomes (arrows). **C** Hybridization with LINE element probe. Large B chromosome indicated (arrow). **D** Hybridization with centromeric heterochromatin (pMeg-1) to the large B chromosome (large arrow). Lack of hybridization to small B element is indicated (small arrow).

B chromosomes

Although the number of B chromosomes in *R. megalotis* has been reported to vary from zero to as high as seven (Shellhammer 1967), in our sample the range was zero to four. As reported in Shellhammer's original study of B chromosomes in the harvest mouse (Shellhammer

1967), we have also noted two different sizes of B elements. In our sample, when B chromosomes appear in the karyotype, the larger B element is always present. The smaller B element may or may not be present in individuals with more than one B chromosome. Hybridization with the (TTAGGG)₇ (telomeric repeat) pro-

duced signal on both ends of both sizes of B elements (Figure 1A). The 18s and 28s ribosomal gene probes did not hybridize to either sized B element (Figure 1B). The clones representative of LINE elements hybridize evenly and are present on both sizes of B chromosomes (Figure 1C). As with the A chromosomes, the results for both LINE-1 probes were indistinguishable. Hybridization with the centromeric heterochromatic probe pMeg-1 produced detectable signal on the larger B element but no hybridization was detectable on the smaller element (Figure 1D). C-bands on B chromosomes appear to be restricted to small amounts at the centromeric region on both sizes of B elements, although detection of C-band-positive regions on the smaller element is not obvious in all spreads. In C-band preparations in both sizes of B chromosomes, there are regions that stain with the same intensity as seen in the euchromatic regions of the A complement.

Discussion

In *R. megalotis*, the two different sizes of B chromosomes show differing characteristics. They share the following features: (a) telomeres present on the ends of both arms; (b) hybridization to LINE probes; (c) absence of hybridization to the ribosomal gene probes; (d) C-band-positive centromeric regions; and (e) euchromatic arms. They differ by the following: (a) the larger B element hybridizes to the centromeric heterochromatin (pMeg-1) probe whereas the smaller B element does not; (b) the amount of C-band-positive material is reduced in the smaller B chromosome relative to that present on the larger B chromosome; and (c) the smaller element is reduced in size by about a third. The feature of telomere presence on the ends of both arms is shared with the B elements in racoon dogs (Wurster-Hill *et al.* 1988). The presence of interspersed mobile elements on B chromosomes has not been reported previously.

Patton (1977) proposed that B chromosomes originate as leftover centromeric regions produced as a byproduct of centric fusions. B chromosomes generated by this mechanism would be expected to have telomeres on at least one end, and potentially on both ends, of the chromosome. As all *Reithrodontomys* chromosomes have substantial blocks of heterochromatin flanking the centromeres, a heterochromatic region would be expected on a B chromosome resulting from a leftover centromere. The larger B element of *Reithrodontomys* appears to fit these two criteria, whereas the smaller B element fails the criterion of heterochromatin flanking the centromeric region.

Centric fusions are the most common euchromatic rearrangements in mammalian chromosomal evolution. One explanation for the high tolerance for centric fusions in karyotypic evolution is the conservation of euchromatic regions in Robertsonian events. In other words, a leftover centromere would be accompanied only by heterochromatic sequences that could be lost without any deleterious effects to the genome. Euchro-

matin appears to be present on some of the B chromosomes of pocket mice, *Perognathus baileyi*, (Patton 1977) and all of the B chromosomes of raccoon dogs, *Nyctereutes procyonoides* (Wurster-Hill *et al.* 1986). In *R. megalotis*, both sizes of B elements have a substantial region of euchromatic material as revealed by C-banding. The euchromatic nature of these C-band-negative regions in *R. megalotis* is further substantiated by the hybridization to LINE probes, which in the A complement do not hybridize to blocks of heterochromatin and hybridize extensively to the euchromatic segments (Figure 1C). Perhaps these euchromatic portions are the reason that B chromosomes are maintained in populations of *R. megalotis* throughout its geographic range. However, such a need for the presence of these euchromatic fragments in the karyotype does not explain the absence of B elements in some individuals. The forces that have maintained these B elements across a wide geographic range most likely involve a complex set of selective forces, as was envisioned for mice of the genus *Apodemus* (Blagojevic & Vujosevic, 1995).

If B chromosomes are eukaryotic nuclear plasmids with an extracellular origin as proposed by Brockhouse *et al.* (1989), genomic sequences of *R. megalotis* would not be expected to be present on the B chromosomes. Therefore, the combination of hybridization to LINE probes, telomeric repeat sequences and the centromeric heterochromatin specific to the subgenus *Reithrodontomys* does not make the Brockhouse proposal the most parsimonious explanation for the origin of these elements.

Concerning the hypothesis that B chromosomes might be generated by amplification of a segment of the nuclear genome, it is impossible to determine whether the smaller B chromosome was generated from an intact fragment from the A complement or whether it is an amplified region from the A complement. The absence of the centromeric heterochromatic sequence found on all the A chromosomes of *Reithrodontomys* makes it unlikely that this B chromosome is the result of an intact fragment from the centromeric region.

The centromeric heterochromatic sequence pMeg-1 is unique to the subgenus *Reithrodontomys* (Hamilton *et al.* 1990). The presence of this sequence on the larger B chromosome suggests an origin occurring after the divergence of the subgenus *Reithrodontomys* from the subgenus *Aporodon*. The documented presence of B chromosomes in *Reithrodontomys megalotis* throughout much of the geographic range of the species (Mexico, Texas, Nebraska, California) suggests that these elements arose before the species became so widely distributed or, alternatively, suggests multiple origins for B chromosomes in this species.

Note added in proof

Since the submission of this manuscript, we have analyzed individuals of *R. megalotis* from an additional population. Of the five individuals in this sample, one

individual contained one small B chromosome and no large B chromosomes.

Acknowledgements

Meredith Hamilton generously provided technical assistance and advice. The authors wish to thank H. Wichman, R. Honeycutt, J. Meyne, N. Arnheim and D. Kass for providing probes. Holly Wichman critically reviewed the manuscript. Assistance in collecting specimens was provided by members of the field methods course (1995 and 1996) at Texas Tech University and by Hugh Genoways, Mike Hobson, Lottie Peppers and Frank Yancey. Support for this research was provided (in part) by a grant from the Howard Hughes Medical Institute through the Biological Sciences Undergraduate Education Program at Texas Tech University, DOE and USACERL Grant RW025 to R.J.B. and a cooperative research program with Texas Parks and Wildlife.

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