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Organization and chromosomal localization of a B1-like containing repeat of *Microtus subarvalis*

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Abstract. A repetitive DNA sequence, MS2, was isolated from *Eco*RI-digested genomic DNA of the vole, *Microtus subarvalis*. The fragment was cloned and sequenced. Sequence analysis of this 1194-bp fragment revealed a 156-bp region demonstrating a 55% homology with the mouse B1 repeat. The remaining MS2 sequence shows no significant homology with other known GenBank sequences. The results of in situ hybridization of MS2 on vole metaphase chromosomes indicate the fragment is confined to heterochromatin blocks of the sex chromosomes in all but one species (*M. arvalis*). Distribution of MS2 sequences provides evidence for heterogeneity of the giant heterochromatin blocks of the XY Chromosomes (Chrs) in voles, for the unique cluster-like localization of MS2 within these blocks.

Introduction

Studies of vole interspecific hybrids in the genus *Microtus* have led to the discovery of the phenomenon of nonrandom inactivation of parental X Chrs in particular combinations of crosses (Zakian et al. 1987, 1991). We have found that an X Chr, containing a block of heterochromatin, is preferentially inactivated when the other X Chr lacks any heterochromatic structures. This suggests X-linked blocks of heterochromatin play an important role in nonrandom inactivation (Zakian et al. 1991; Zakian and Nesterova 1992).

These results suggested a hypothetical mechanism accounting for the nonrandom inactivation of X Chrs in interspecific hybrids of vole. It was proposed that heterochromatin nonspecifically initiates the nucleation process in the inactivation center of its own X Chr (Nesterova and Zakian 1994a, 1994b). One basic assumption of this model is the presence of DNA repeats in the X-inactivation center (XIC) acting as the nucleation center of the X Chr under certain conditions. In addition, the model also assumes that specific repeated sequences are located along the whole length of the X Chr. The idea that specific X Chr repeats exist is also described in other hypothetical inactivation models for explaining the initiation and spreading of inactivation (see reviews by Gartler and Riggs 1983; Nesterova and Zakian 1994b). Recent cloning and sequencing of the human and mouse XIST/*Xist* genes have revealed unique repetitive sequences that may be involved in X-inactivation (Brockdorff et al. 1992; Brown et al. 1992). In addition, gene targeting experiments have demonstrated the requirement of the *Xist* gene for X Chr inactivation (Penny et al. 1996).

Mindful of the distribution of constitutive heterochromatin on the vole sex chromosomes and the fact that the bulk of this DNA is represented by repeated (cot.0.1) sequences (Graphodatsky et al. 1985), we initiated a search for X-specific repeats. During this search we identified and isolated the MS2 repeat that is heterochromatin-specific for sex chromosomes in common voles. We report herein a detailed analysis and chromosomal assignment of this repeat.

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The nucleotide sequence data reported in this paper (MS2) have been submitted to GenBank and have been assigned the accession number U36930.

Materials and methods

Cells and cell cultures. Four *Microtus* species were involved: *M. arvalis*, *M. subarvalis*, *M. transcaspicus*, and *M. kirgisorum*. Permanent cultures of the lung fibroblasts of four vole species were used. Cell lines were derived and maintained as described earlier (Nesterova et al. 1994).

Chromosome preparations. Colchicine, 0.4 mkg/ml, was injected into the cultures 1 h before fixation. Fixation was achieved in a 3:1 (vol:vol) mixture of methanol:acetic acid. The suspension was pipetted onto wet cold slides and then air-dried.

In situ hybridization. DNA was labeled with bio-11dUTP by nick-translation. Hybridization with denatured chromosome preparations was performed at 42°C in a mixture of 50% formamide, 10% dextran sulfate, and 2× SSC for 16 h. Preparations were washed twice for 2 min in 50% formamide, twice in 2× SSC, twice for 2 min in 2× SSC at 42°C, and incubated for 30 min in a blocking buffer (0.1 M Tris-HCl, pH 7.5; 0.1 M NaCl; 3 mM $MgCl_2$; 3% dry milk; 0.05% Tween-20) at room temperature. Detection was performed with the avidin-fluorescein conjugate with a three fold amplification of the signal (Lawrence et al. 1988).

General methods and sources of enzymes. Standard procedures such as isolation of recombinant plasmids, preparations of competent cells, transformation and restriction analyses were performed as described (Sambrook et al. 1989). Maxam-Gilbert sequencing was performed following acetone precipitation (Baram and Grachev 1985). Restriction endonucleases, DNA ligase T4, Klenow fragment, DNA polymerase I ("Fermentas" Lithuania), and the kit for labeling DNA with digoxygenin (Boehringer Mannheim Biochemicals USA) were also used.

Isolation of DNA and cloning. Genomic DNA was isolated from fresh or liquid-nitrogen-frozen liver by a phenol-detergent method (Henry et al. 1990), digested with *Eco*RI, and separated by electrophoresis on a 1% agarose gel. The 1-kb band corresponding, approximately, to the MS2 repeat was cut from an ethidium bromide-stained gel and isolated by electroelution into a saturated solution of ammonium acetate. Resulting DNA fragments were ligated into the *Eco*RI site of pUC19; competent *E. coli* JM83 cells were then transformed (Mazin et al. 1990). Recombinant clones were grown in 3 ml LB. Plasmid DNA was isolated by the alkaline lysis method (Mazin et al. 1990). Clones with insertions the size of the initial repeat were selected for further investigations. DNA of these clones was

labeled with digoxigenin-dUTP and blot-hybridized with the digested genomic DNA previously transferred to a capron membrane by vacuum blotting.

Computer analysis. Computer analysis of sequences was performed with the software BLASTN and BLASTP (Altschul et al. 1990), program packages CONTEXT (Solovyev et al. 1992) and VOSTORG (Zharkikh et al. 1991). Search for homologous sequences to the cloned MS2 repeat involved the entire GenBank database of nucleotide sequences (Releases 76 and 79). Sequence alignment was obtained from applying the Needelman-Wunsch method (Needelman and Wunsch 1970). Computers used were IBM PC/486 and SUN SparkStation 10.

Results

Comparison of the electrophoretic spectra from *Eco*RI-digested genomic DNA from *M. arvalis*, *M. kirgisorum*, *M. transcaspicus*, and *M. subarvalis* revealed additional bands of repeated sequences in *M. subarvalis* not present in the DNA spectra of the other vole species (Kholodilov et al. 1993). This suggested to us that the presence of the excess DNA repeats in *M. subarvalis* was owing to the giant block of heterochromatin occupying nearly a half of the X Chr of *M. subarvalis* (Zakian et al. 1991). At least ten *M. subarvalis*-specific ethidium intense bands were observed. Cloning these repeats produced several clones with insertions similar to the approximate 1-kb size of MS2. One of these clones, pMS2, hybridized to the band of interest in *M. subarvalis Eco*RI-digested DNA (Fig. 1). In the other species studied, no hybridization in that region was observed (Fig. 1). However, two less intensive, higher molecular weight bands were revealed in the four species in question (1500-bp and 5000-bp regions). Perhaps significantly, the weakest signal was in *M. arvalis*. Several minor species-specific signals were detected in *M. subarvalis* and *M. transcaspicus* (Fig. 1).

The heterochromatin in *M. subarvalis*, 2n = 54, is represented by tiny centromeric blocks on autosomes; a giant distal block on the X Chr; and a totally heterochromatic, very large Y Chr. It should be noted that under some modes of differential staining, the Y Chr heterochromatin is represented by alternating C-bands of various densities (Fig. 2A).

Heterochromatin of *M. arvalis*, 2n = 46, is represented by small centromeric blocks on many autosome pairs, and the X Chr contains no heterochromatin. The Y Chr varies in size and morphology among individuals, from being the smallest in the set of biarmed chromosomes to the smallest acrocentric; the latter is mostly C-positive. In this species, one of the long autosomal pairs is polymorphic, being biarmed without C-heterochromatin. However, many populations of *M. subarvalis* are noted for an inversion

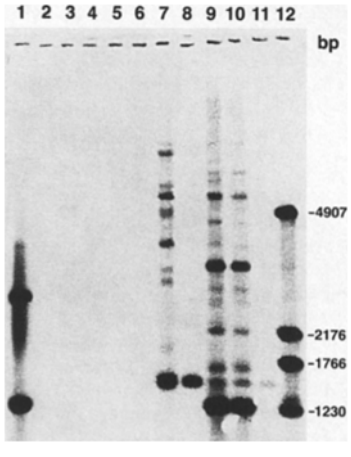

Fig. 1. Southern blot hybridization of *Eco*RI-digested DNA samples with digoxigenin-dUTP-labeled pMS2. (1) pMS2; (2) *Monodelphis domestica;* (3) *Nesokia indica;* (4) *Rattus norvegicus;* (5) *Microtus oeconomus;* (6) *M. agrestis;* (7) *M. kirgisorum*; (8) *M. transcaspicus*; (9) *M. subarvalis*, female; (10) *M. subarvalis*, male; (11) *M. arvalis*; (12) a mixture of equal amounts of *Eco*RI-*Bgl*I- and *Hinf*I-digested pBR328.

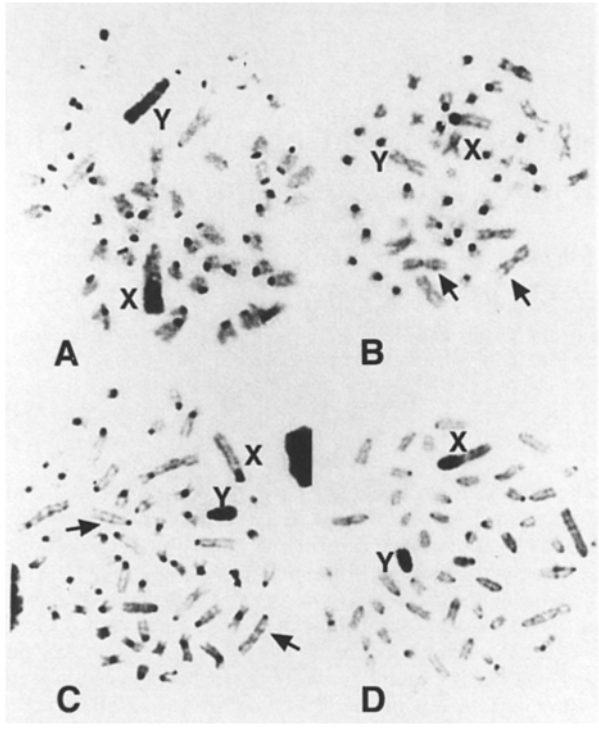

Fig. 2. C-banded chromosomes of the four *Microtus* species. (A) *M. subarvalis*, (B) *M. arvalis*, (C) *M. kirgisorum*, (D) *M. transcaspicus*. XY Chrs and sites of MS2 repeats on autosomes are indicated with arrows.

of one of the homologs of this pair when heterozygous, and an additional heterochromatic arm emerges on it (Fig. 2B).

The heterochromatin in *M. kirgisorum*, 2n = 54, is comprised of notable centromeric blocks on almost all pairs of autosomes; an additional, large arm on the X Chr; and an entirely heterochromatic Y Chr (Fig. 2C). Heterochromatin in *M. transcaspicus*, 2n = 52, is represented by small centromeric blocks on autosomes; an entirely heterochromatic, acrocentric Y Chr; and a large centromeric block on the acrocentric Y Chr (Fig. 2D). Under less harsh modes of treatment, each of the four species exhibits several autosomes with small blocks of interstitial heterochromatin staining not as densely as the centromeric blocks and the blocks on sex chromosomes.

MS2 in situ hybridization on *Microtus* chromosomes is shown in Fig. 3. This repeat is located along the entire length of the giant C-block on the *M. subarvalis* X Chr. On the Y Chr, MS2 sequences are concentrated at the distal third of the chromosome, an interstitial band, and a thin centromeric band. About 50% of the Y Chr is free of MS2 sequences in any concentration. No MS2 was detected on *M. subarvalis* autosomes. In *M. arvalis*, MS2 produced a weak signal, comparable to the intensity of unique sequences, and is located on the largest pair of autosomes in the interstitial heterochromatic region. MS2 was not detected in *M. arvalis* sex chromosomes, in the centromeric regions of autosomes, or in the additional arm of the inverted autosome.

In *M. kirgisorum*, the MS2 repeat is concentrated nearly the entire length of the Y Chr. Only about one-sixth of its pericentromeric region is unoccupied. On the X Chr, the MS2 repeat occupies the pericentromeric half of the additional heterochromatic arm. MS2 produces an intensive hybridization signal with the interstitial heterochromatin block on the large part of acrocentric autosomes. In *M. kirgisorum*, the centromeric blocks of autosomes lack MS2.

In *M. transcaspicus*, MS2 is located on the large part of the heterochromatic block of the X Chr and on the distal third of the

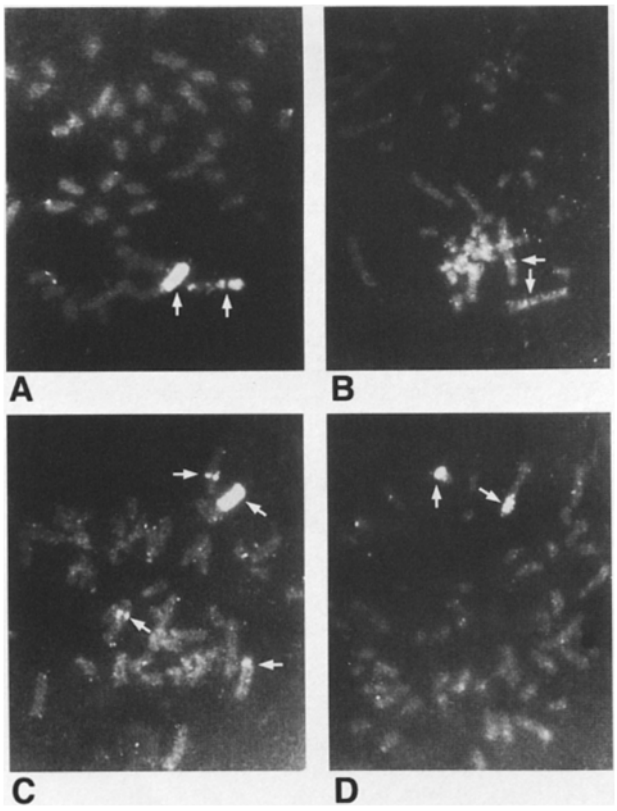

Fig. 3. Localization of the MS2 repeat on *Microtus* chromosomes by fluorescent in situ hybridization (FISH). (A) *M. subarvalis*, (B) *M. arvalis*, (C) *M. kirgisorum*, (D) *M. transcaspicus*. Hybridization sites on the autosomes and XY Chrs are indicated with arrows.

Y Chr block. Two-thirds of the Y Chr, the centromeric region of the X Chr, and autosomes are free of MS2.

Thus, for three of the four *Microtus* species studied, the MS2 repeat is found in the heterochromatin of the XY Chrs, but is concentrated in separate bands of different widths. This result provides evidence for heterogeneity of the giant heterochromatic blocks of the XY Chrs in the voles, and for the unique cluster-like localization of MS2 within these blocks. In addition, MS2 sites were localized as unique sites in the interstitial regions of autosomes in *M. arvalis* and *M. kirgisorum*.

Maxam-Gilbert sequencing of both strands of MS2 demonstrated an extended open reading frame in the complementary chain (Fig. 4). A search of the amino acid database PIR (Issue 37) for homology with the aid of the software BLASTP (Altschul et al. 1990) revealed no significant similarity between the sequence and a protein of the database. Therefore, a functional role for the open reading frame could not be implied.

A search of the GenBank database (Issue 76) with the aid of the software SCAN (Solovyev et al. 1992) revealed the region between MS2 nucleotides 344 and 500 is homologous to the mouse B1-like repeat sequence. Alignment of this region and the mouse B1-like element consensus sequence (Krayev et al. 1980) demonstrated a 55% homology (Fig. 5). The vole B1-like sequence contains two potential promoter regions of RNA-polymerase III (A- and B-blocks) and a 3′-terminal A/C-rich region; both are typical of the B1 element (Krayev et al. 1980; Weiner et al. 1986). The two promoter regions lack deletions or insertions, and the A-block exhibits high homology with RNA polymerase III. The MS2 sequence is characterized by a high density of A + T nucleotides (58%) and poly-A/poly-T tracks. Features like these are typical of insertion sites of ALU and B1 elements (Krayev et al. 1980).

Analysis of the structural organization of the MS2 repeat revealed no expressed regularities (tandem or inverted repeats) typical of the sequences from the heterochromatin regions of chromosomes. The most significant imperfect repeat structures are shown in Fig. 4. As seen, both direct and inverted repeats fall within poly-A tracks which are, perhaps, because the MS2 sequence has a high abundance of these tracks.

Analysis with the aid of the software BLASTN (Altschul et al. 1990) found the most similar sequence in GenBank (Issue 79) was HAMSHCA (α-cardiac myosin heavy chains gene exons 1-39 from *Mesocricetus auratus*). In MS2, the region homologous to HAMSHCA corresponded to the potential B1 element (positions

```
1     ACTTAGAAGA  GATATAAAAA  TTAACAGTTA  CTGAGGAGCT  GTTACTGAAA  CAATTTAATC
61    AACAGATATT  AAATTGATAA  GCAATCGAAT  TTAGAGATGA  ACTCCATAAA  TCATTAGCAG
                                                     i---------  ----------
121   TCTCAAGAGG  ACACTCTAAA  CTGGTGCAAA  AGAGAAGAAA  TGTTGTCAGT  AGGTTGTATT
      ----------  ----------  ->
181   TCTGAAATCA  TATTTGAATA  ATGCTTAAAA  GTTATAAAAA  TATAATAGAT  CCTCAATTAC
241   CCATGAAATG  CAAGGGGAAA  AAAGAGAGGG  AGGGAATAAT  TACCTAAATT  CAGTACACAT
      <---------  ----------  ----------  ----------i
301   TTGTGGAAAC  ATCATTTAAG  AAAAAAAACT  CAATAAAATA  GAGattggcc  tggtggtcat
361   ggtcggggct  ccagcaccag  cactttagag  gcagaagcag  gcctgtctgt  gtgctttcaa
421   agccagactg  atcaatgtag  attgagttta  ggcactctat  accgtaagat  ctgcagagaa
481   CAGGACAAAG  TTCCAAGTAT  GGAATATGCA  TATCGGTCAC  TGCCCAATAA  TACCATCTGT
                                                               d----------
541   TGTATTATAT  ATTTGCTAGG  AAGTGTCCCA  ACCTGCATTA  TTATATTACA  ATCAGGAAAA
      ---------->
601   AGCAGCATCG  TGTCTAGATC  TGAGTGAGGA  TGAAGGCAGA  TTGACTAGGA  TTTCCATGCC
      d---------  ---------->
661   CACACAGGAA  AAAGCAGACA  CTTCGTCTTG  TAATTGTCAT  CCTACATAGC  ACCACGGATC
721   AGTCTTAACT  CATTTGCTTG  GGATATGTAA  GGGAACATAG  GTGCTAGACT  CTCCCACTCA
781   GGACAACGTG  GAGATAACAC  AGTCCCTCCC  AGGCAGCCCA  GGTAGAACAT  TTCCATCTCA
841   AGTGTTTCCA  ACTCTCTTCT  TGCTTCCTAC  AGAAAATTCC  CCAAATGCAC  CTTTCGCTGG
901   GACAGAATGT  GCTTCTGCAG  CCCTCTCTGC  CTTTGCTGAG  GAATGCCACA  CCCTCTGCAG
961   GAAGCGTGTG  TAATTTTCTA  CGGGGTCTCC  ACTCCGCTCA  GAATGAGCTC  AGCATGGCTC
                              P  T  E     V  G  S  L     I  L  E     A  H  S
1021  CTTTCATCAG  ATTCCAGCAC  AGCTGCCAGC  AGCTCAGGGA  CGGTGACTGT  CTTTAAAAAC
      R  E  D  S     E  L  V     A  A  L     L  E  P  V     T  V  T     K  L  F
1081  GAGCTGGCAC  GACCATGCTG  ACTGAGCCAT  CAGACCAGAA  CCAGATTGCT  ACTTCCACAT
      S  S  A  R     G  H  Q     S  L  W
1141  TTTTCTATCC  AAGTGTTCTC  CACTGGGATC  TCAGAGAAAG  CACCACCAGA  ATTC
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Fig. 4. MS2 sequence from *M. subarvalis*. The region homologous to the B1 element is written in small letters. The translated amino acid sequence of the open reading frame is provided for the complementary chain. The 13-bp direct repeat is indicated by arrows with the letter "d." The 38-bp inverted repeat is indicated by arrows with the letter "i."

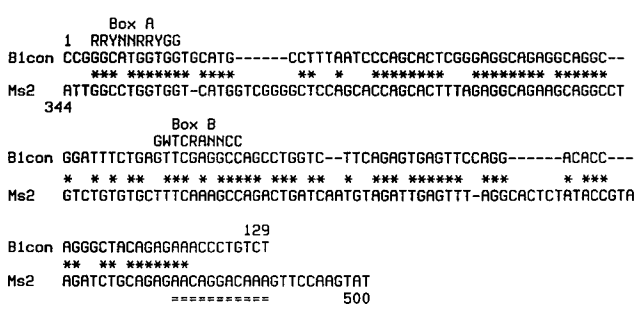

Fig. 5. A comparison of the mouse B1 element consensus sequence (Krayev et al. 1980) and the vole region from MS2 nucleotide positions 344 to 500. Asterisks denote nucleotide matches. Consensus sequences of boxes A and B of the RNA polymerase III promoter (Weiner et al. 1986; Perez-Stable and Shen 1986) are given above the corresponding sequence fragments: Y, pyrimidine (T or C); R, purine (A or G); W, bases with weak interaction (A or T); N, any nucleotide; = =, A/C-rich region (Krayev et al. 1980; Weiner et al. 1986).

347-500). In the HAMSHCA sequence, the homology falls within intron 34 (positions 26113-26276). The percentage of matching nucleotides is about 65% (Fig. 6). No significant homology for the remaining MS2 sequence with other known sequences was found.

Discussion

The cloned MS2 fragment is a 1194-bp repeated sequence, containing a B1-like element between positions 344 and 480. In situ hybridization results and additional bands following blot-hybridization indicate the fragment is confined to heterochromatin blocks. Distribution of MS2 sequences provides evidence that the giant heterochromatin blocks of the XY Chrs of *Microtus* voles are heterogeneous and the MS2 sequences are clustered within the blocks.

Data suggest heterogeneity within the majority of the heterochromatic regions of mammalian genomes, including the XY Chrs in a variety of North American and European species of the genus *Microtus* (Modi 1992; 1993a, 1993b, 1993c). The classified and characterized repeat groups are different in structure, yet dispersed along the entire length of large, heterochromatic blocks. Thus, the MS2 repeat is different in principle from any other known repeat of vole genomes. Contrasted to *Microtus* MSAT repeats, MS2 is not present in the centromeric heterochromatin on the autosomes.

Notably, blot hybridization revealed a common fragment about 1500 bp in length in the genomes of four species of *Microtus*. These results are in agreement with the data of MS2 in situ hybridization on metaphase chromosomes in common voles, suggesting the copy number of this fragment was greatest in *M. kirgisorum*, with fewer copies in *M. transcaspicus*, *M. subarvalis*, and *M. arvalis*, respectively. These data may indicate the MS2 repeat arose from an ancestral sequence and is present in these species as a result of considerable amplification.

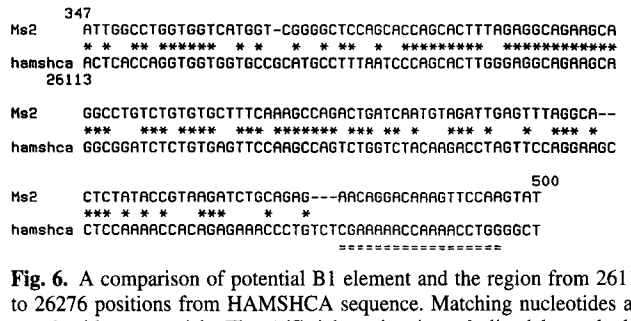

Fig. 6. A comparison of potential B1 element and the region from 26113 to 26276 positions from HAMSHCA sequence. Matching nucleotides are noted with an asterisk. The A/C-rich region is underlined by a double dashed line (Krayev et al. 1980; Weiner et al. 1986).

Computer analysis with the Genbank database showed this is a new representative of the B1 element family, significantly different from the other members. It is of importance for study of the evolution of the B1 and ALU families of repeated elements.

An interesting feature of MS2 is the absence of three properties typical of heterochromatin DNA (Modi 1993b). First, there are no clear-cut direct or inverted repeats. Secondly, no match is found between MS2 and the other heterochromatin nucleotide sequences. Finally, MS2 is shown to carry a B1-like element relating to the SINE class (short dispersed repeated elements). Such elements are not a characteristic feature of heterochromatin DNA of other organisms (Brutlag 1980).

We failed to find any connection between the MS2 repeat and X-inactivation. It is obvious MS2 cannot be a candidate sequence for X-specific repeat involved in spreading X-inactivation along the chromosome owing to its distributional specificity on the sex chromosomes. However, unique features of MS2 sequences make it interesting for evolutionary studies in voles. Other X-specific repeats have been cloned and are being characterized from the vole in an attempt to find unique repeats similar to those found in the XIST gene in both human and mouse (Brockdorff et al. 1992; Brown et al. 1992). X-Chr specific repeats may be linked to an understanding of the repeats bound within the XIST gene and its function as the master regulator of X-inactivation (Penny et al. 1996).

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