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Nucleotide sequence and genomic organization of a tandem satellite array from the rock vole *Microtus chrotorrhinus* (Rodentia)

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Abstract. A tandem satellite array (herein named MSAT-160) has been isolated and characterized from the rodent *Microtus chrotorrhinus*. Sequence data from 15 partial or complete monomers revealed a repeat unit length of 160 bp. This unit length was apparently derived from two shorter sub-motifs, one a tetramer (GAAA), the other a hexamer (CTTTCT), through polymerase slippage and mutation. Collectively, perfect or imperfect variants of these two motifs comprise nearly 60% of the component. Southern blot analyses of genomic DNA digested with 14 different restriction endonucleases indicated that most enzymes yielded either classical type A or type B restriction patterns, while *Rsa*I yielded a pattern that combined features of both the A and B types, and *Bam*HI appeared to lack sites altogether in MSAT-160. An examination of restriction patterns from 16 individuals with three enzymes failed to identify intraspecific variation, while a related study compared 11 species and documented interspecific distinctiveness (Modi, submitted). Fluorescence in situ hybridization indicated that the satellite DNA was located at the centromeres of several autosomes and at sex chromosome heterochromatin (GenBank accession No. M86843).

Introduction

Localized, highly repeated DNA sequences are known to comprise substantial proportions of higher eukaryotic genomes. Such components are traditionally called satellites because of their differences in buoyant densities from main band DNA in equilibrium cesium chloride gradients. These sequences are not transcribed and as a result are susceptible to rapid evolutionary change. A considerable amount of information exists regarding the DNA sequence, genomic organization, and chromosomal distribution of highly repeated sequences (Miklos 1985).

One observation that has emerged from the DNA sequence determinations of repetitive components is the extreme variation in size, composition, and putative origins of different satellite families. For example, the complexities of repeated DNA families in species of crabs (Crustacea) ranges from 2 to 2000 bp (Skinner et al. 1982). In the guinea pig, satellite I DNA has a repeating unit of CCCTAA (Southern 1970), and three components within the genome of the kangaroo rat *Dipodomys ordii* have core sequences of ACACAGCGGG, AAG, and GGGTTA (Hatch et al. 1976; Fry and Salser 1977). About one-half of the blue whale 422-bp heavy satellite is comprised of multiple copies of TTAGGG (Arnason and Widegren 1989), while a 337-bp array isolated from a South American rodent was found not to be composed of shorter repeats (Rossi et al. 1990). The domestic cow contains up to eight distinct satellites, one of which (the 1.711a satellite) is 1413 bp in length. The component consists of multiples of 23-bp subunits and a 611-bp stretch of alien DNA (Streeck 1981). On the other hand, the 1.715 bovine satellite was shown to be 1402 bp in length, has a complex sequence, and contains a variety of direct and inverted repeats (Gaillard et al. 1981). The laboratory mouse *Mus musculus* light satellite is a 234-bp repeat that contains eight diverged subrepeats. These eight subrepeats exhibit a weak periodicity of 30 bp and show homology ranging from 48 to 93% when compared with one another (Horz and Altenburger 1981). This component is thought to be derived from a 9–18 bp starter sequence (Southern 1975). The primate alphoid family consists of length variants of integer multiples of a fundamental 170-bp repeat. Adjacent monomers within a human alphoid multimeric array may differ by as much as 20–40% (Willard and Waye 1987). Finally, differences in substitution spectra have been reported in carvnivore satellites. A random pattern was observed in an array from the domestic dog, over 90% of all replacements involve G or C in a component from the domestic cat, and an excess of G or C

to A or T changes predominate in an element from the grey fox (Fanning 1989).

The current study presents results from the cloning, sequencing, and chromosomal localization of a newly isolated tandem satellite array derived from the rodent *Microtus chrotorrhinus*. This species belongs to the family Arvicolidae, which includes the voles, lemmings, and muskrats and contains approximately 125 species. *M. chrotorrhinus* was selected for the analysis of satellite DNA because previous cytogenetic observations had revealed the existence of extremely large-sized sex chromosomes, which are known to be comprised largely of constitutive heterochromatin. This study and several others (Modi 1990, 1992a, 1992b, in preparation) eleucidate the molecular structure of heterochromatin in *M. chrotorrhinus* and related species. Sequence information collected from a number of monomer units allows for the construction of a consensus sequence. Inspection of the consensus identifies two repeated sub-motifs suggesting that slipped-strand mispairing may have played a role in the origin of the satellite. Southern blotting was used to understand the genomic organization of the material, to document a lack of intraspecific variability, and to report that this particular element is unmethylated at CpT dinucleotides.

Materials and methods

Genomic DNA from *Microtus chrotorrhinus* liver tissue was digested with a variety of restriction enzymes, run out in 1% agarose gels, and stained with ethidium bromide. It was observed that *Hae*III digestion produced an undegraded band that was present at the limit mobility of the gel, while *Alu*I totally digested the DNA and produced a smear less than 3 kb in size (Fig. 1). The high-molecular-weight, *Hae*III-resistant fragment was excised from the gel, electroeluted, and subsequently partially digested with *Alu*I. This double-digested DNA was then cloned into the *Sma*I site of the phagemid Bluescript KS$^+$ (Stratagene) and was used to transform either JM 101 or XL-1 blue host cells. Colony hybridization was performed with some of the original *Hae*III-resistant DNA as a probe. Either single-stranded [with M13 K07 helper virus (Promega)] or double-stranded (after Kraft et al. 1988) DNA was sequenced from either the 5′ or 3′ end by the dideoxy chain termination method (Sanger et al. 1977) with a Sequenase kit (USB). Sequence alignment, pair-wise distance comparisons, and dot matrix homology analyses were done by use of the UWGCG DNA sequence analysis software package (Devereux et al. 1984).

Southern blotting was carried out according to standard procedures (Maniatis et al. 1982). Restriction digestions were carried out containing 6 mM spermidine trihydrochloride and 100 μg/ml BSA in the buffer recommended by the supplier. Hybridization to Biotrace nylon membranes (Gelman Bioscience RP) was carried out with 5×10^5 cpm/ml of ^{32}P-labeled probe. The clone called 6D in Fig. 2, which contains three complete monomer units, was the labeled DNA. Filters were washed most stringently at 60°C in 0.1× SSC, 0.5% SDS for 20 min. A total of 16 specimens of *M. chrotorrhinus* were examined, and all were collected from natural populations in Cooke Co., Minn. or Wayne Co., W. Va., USA.

Fluoresence in situ hybridization with a biotinylated probe of clone 6D was carried out to metaphase chromosomes prepared from a primary fibroblast cell line. The detailed procedure utilized is given in Modi (1992b).

Results

The tandem satellite array isolated here is named MSAT-160, from *M*icrotus *sat*ellite-*160* bp in length.

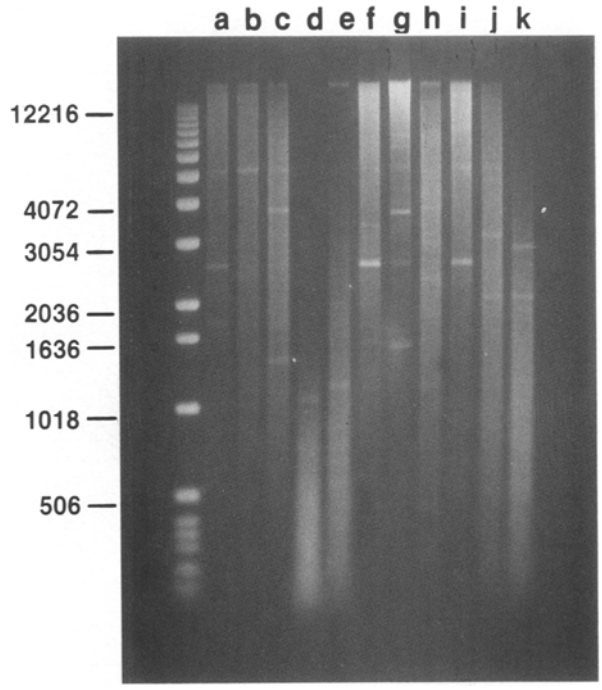

Fig. 1. Ethidium bromide-stained gel of restriction enzyme-digested genomic DNA from a male *M. chrotorrhinus*. Restriction enzymes are as follows: (a) *Eco*RI, (b) *Pst*I, (c) *Taq*I, (d) *Alu*I, (e) *Hae*III, (f) *Bcl*I, (g) *Hpa*I, (h) *Pvu*II, (i) *Sst*I, (j) *Msp*I, and (k) *Rsa*I. MSAT-160 is clearly seen as an undigested fragment at the limited mobility of the gel, particularly in lanes e and h. **One kb ladder** is the molecular weight marker.

From eight clones partial or complete sequence information was obtained for 15 distinct monomer units (Fig. 2). A total of 2075 bp was determined. The individual monomers ranged in length from 157 to 161 bp, while the consensus is 160 bp. The consensus is 64% A + T. An analysis of the consensus sequence reveals the existence of two complementary sub-motifs, each repeated several times in the consensus, often in a variant form (Fig. 2, Table 1). The consensus for the first motif is the polypyrimidine tract CTTTCT, and this sequence or variations of this occur at 11 positions in the 160-bp consensus. The consensus for the second sub-motif is the polypurine tract GAAA, and variants of this occur at 10 positions in the 160-bp consensus (Fig. 2, Table 1). These 21 individual motif members comprise 59% (95 of 160 bp) of the consensus sequence. A dot matrix comparison of the consensus with itself and its reverse complement graphically portrays the distribution of these complementary direct repeats (Fig. 3).

Examinaton of Fig. 2 indicates that most substitutional differences between individual monomers occur predominantly at two locations, between positions 38–75 and 125–148. Most differences are single bp substitutions, although a 1-bp insertion occurs at position 135 in clone 6A1, and deletions ranging from 1 to 3 bp occur at several sites (Fig. 2).

A pair-wise comparison of the 10 individual monomers for which complete sequence information exists (see Fig. 2) to each other indicates an average diver-

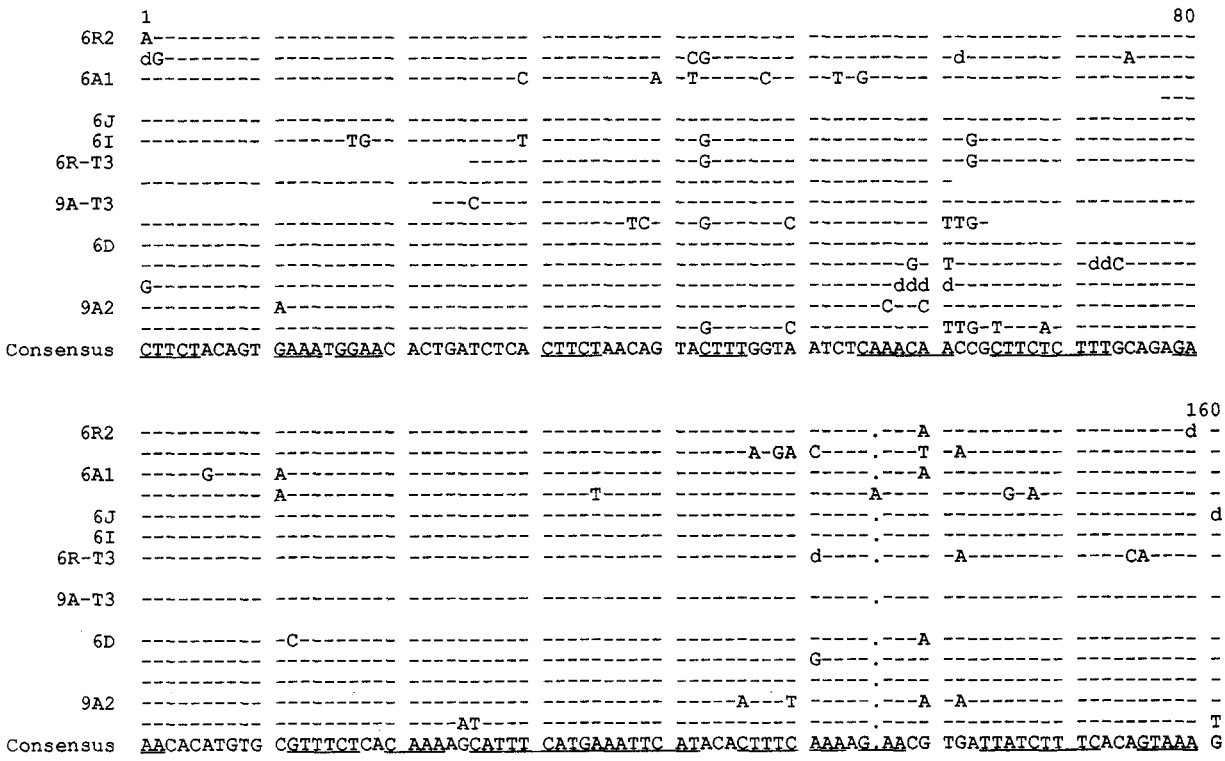

Fig. 2. DNA sequence of MSAT-160. Data were obtained from clones labeled 6R2, 6A1, 6J, and so forth. The consensus appears as the bottom row. **Blanks** indicate sequence not read, **dashes** indicate identity to the consensus, **d** represents deletion, and a **period** indicates the location of an addition. **Bases underlined** in the consensus are the individual submotif members discussed in the text and tabulated in Table 1.

gence of 6.9% per site. Several clones contain more than one monomer. The three monomers within clone 6D differ from one another by an average of 5.0% per site, and the two monomers within 9A2 by an average of 11.0% per site. A comparison of the individual monomers to the consensus illustrates that an approximately equal number of substitutions are occurring that result in new G or C bases as are those creating new A or T bases, that is, $X \rightarrow A$ or $T = 27$ and $X \rightarrow G$ or $C = 23$ where X is any base. Further, the transversion-to-transition ratio of 1.78 (32/18) is not significantly different from a random value of 2 ($p > 0.10$).

Genomic DNA from one individual was digested with 14 different restriction endonucleases and examined by Southern blot analysis with clone 6D as a probe. Eleven of these digests—*Bam*HI, *Dde*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hin*dIII, *Hin*fI, *Mbo*I, *Pst*I, and *Rsa*I—are shown in Fig. 4. *Sau*3AI is presented in Fig. 4, *Alu*I and *Taq*I are not illustrated. Two basic types of restriction patterns emerge. *Alu*I, *Hin*dIII, *Mbo*I, and *Sau*3AI all produce a type A or classical ladder pattern. Most of the satellite DNA is digested to fragment lengths that are integer multiples of 160 bp. The monomer is the fragment present in greatest abundance, with progressively decreasing copy numbers of each higher order n-mer.

The second basic pattern is produced by all remaining enzymes examined except *Bam*HI and *Rsa*I. This is a type B pattern, in which fragments occur that are integer multiples of the unit repeat length (160 bp) but where their size distribution is shifted upward relative to the type A pattern. The monomer and dimer are absent, and the intensities of the higher n-mers are approximately equal. With most enzymes a significant portion of the component remains undegraded and high in the gel. It can be seen that different enzymes degrade the DNA to different degrees. For example, although both enzymes produce type B patterns, *Hin*fI digestion is more extensive than is *Eco*RI digestion (Fig. 4). *Rsa*I digestion produced a pattern that combines features of both the A and B types and may here be designated a type AB pattern. *Bam*HI fails to digest the material to any notable extent. An examination of the GenBank database indicates that this component shows nothing but random homology with any other known sequence.

Inspection of the consensus sequence presented in Fig. 2 indicates that each of the five type A enzymes (*Alu*I, *Hin*dIII, *Mbo*I, *Rsa*I, and *Sau*3AI; as indicated above, *Rsa*I produced an AB pattern) have a recognition sequence at exactly one location in the 160 bp consensus. Seven of the eight type B enzymes require a 1-bp modification somewhere in the consensus in order for a type B site to be generated. For example, a change from T to C at position 89 would produce an *Hha*I site (GCGC), while a C to G at position 100 would yield a *Dde* I site (CTNAG). The number of positions at which a single bp substitution would produce a type B pattern for seven of the type B enzymes examined are *Dde*I (2 positions), *Eco*RV (1), *Hae*III (1), *Hha*I (1), *Hin*fI (5), *Pst*I (2), and *Taq*I (5). *Eco*RI requires two substitutions in any of at least three dif-

Table 1. Nucleotide sequences of the individual members and consensuses of the two sub-motifs found in the consensus sequence of MSAT-160.

CTT-CT	GAAA
CTT-CT	GGAA
CTTT--	CAAA
CTT-CT	CAA-
CTTT--	GAAA
GTTTCT	CAAA
A A	
CTTTCT	GAAA
A	
--TTCT	CAAA
CTTTC-	GAA-
	T
--TTAT	GAAA
CTTTC-	
CTTTCT	GAAA

ferent positions in order for a type B pattern to be produced. Further, substitutions at seven positions would yield a type B *Rsa*I site in addition to the type A site highlighted above.

Seven unrelated wild-caught animals were examined by Southern blotting following *Alu*I and *Pst*I digestion. An additional nine individuals were examined with *Hin*dIII (not shown). No individual variation other than minor differences in certain fragment intensities in the *Hin*dIII digestions was observed with any of the three enzymes.

*Mbo*I and *Sau*3AI are isoschizomers, both cleaving at the recognition sequence (GATC; positions 24–27 in Fig. 2), but differing in their abilities to cleave methylated DNA. *Mbo*I does cleave when the C residue is 5-methylcytosine, whereas *Sau*3AI does not cleave when this cytosine is methylated. Methylation was subsequently measured at the CpT dinucleotide at positions 27–28 (Fig. 2). Figure 5 shows the results of digesting three different DNA samples: cells from a lung fibroblast cell line, liver tissue, and kidney tissue, all from a single individual with both *Mbo*I and *Sau*3AI. No differences in restriction patterns between the two enzymes are seen in any of the three tissues, indicating that MSAT-160 is not methylated either in vivo or in vitro. However, the DNA sample from the lung cell line shows a different restriction pattern from that of the two seemingly identical tissue samples. Fewer fragments in the 8-mer to 20-mer size range are present in the lung sample than in the kidney or liver preparations.

Microtus chrotorrhinus has a diploid chromosome number of 60. All autosomal pairs (except the smallest pair) are acrocentric. The X is a large submetacentric, and the Y a large acrocentric. Fluorescence in situ hybridization to metaphase chromosomes of a male *M. chrotorrhinus* is shown in Fig. 6. Hybridization was noted at many of the autosomal centromeres and in certain areas on the long arm of the X and the Y. All sites of hybridization are known to be C-band positive.

Discussion

A viable explanation for the origin of the present array is through slipped-strand mispairing (Levinson and Guttman 1987). Under this process, the two submotifs described above would increase in copy number by intrahelical mispairing followed by excision repair. This may have occurred for several rounds yielding a number of motifs equal to or greater than the number currently present in the 160-bp consensus. Subsequent mutations and/or recruitment of small stretches of unique sequences from elsewhere in the genome could

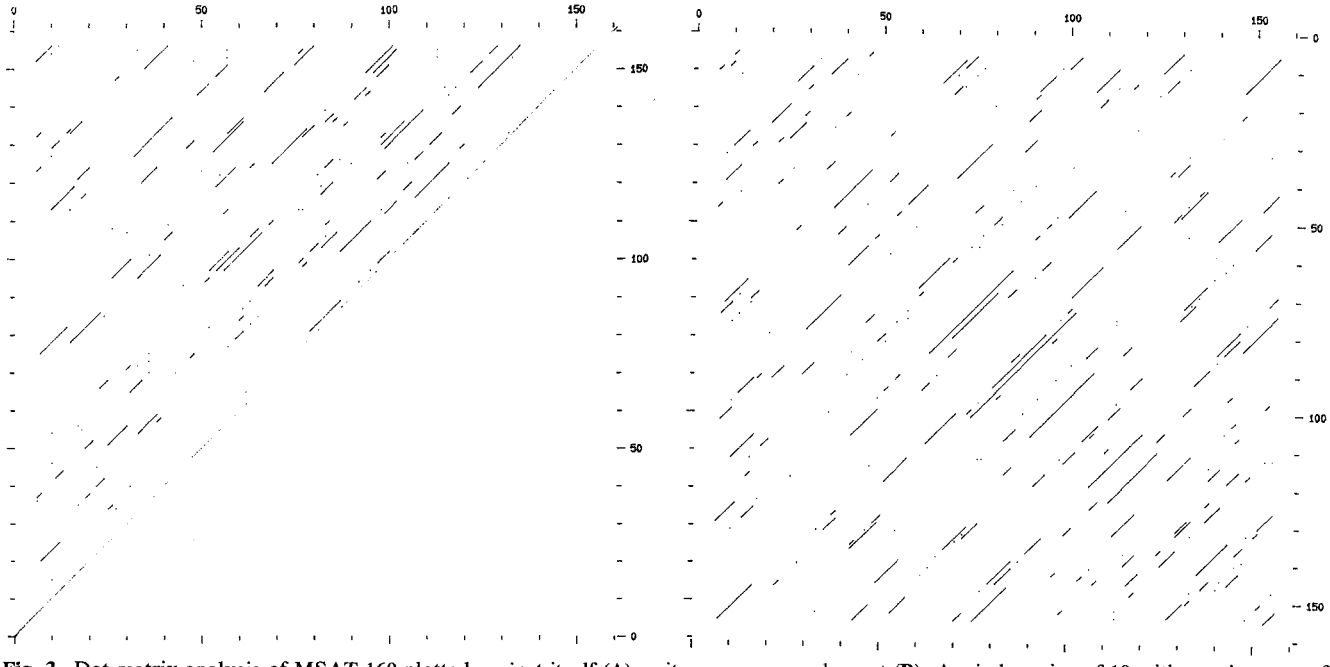

Fig. 3. Dot matrix analysis of MSAT-160 plotted against itself (**A**) or its reverse complement (**B**). A window size of 10 with a stringency of 6 was employed. Note the large number of complementary direct repeats that are identified in Table 1.

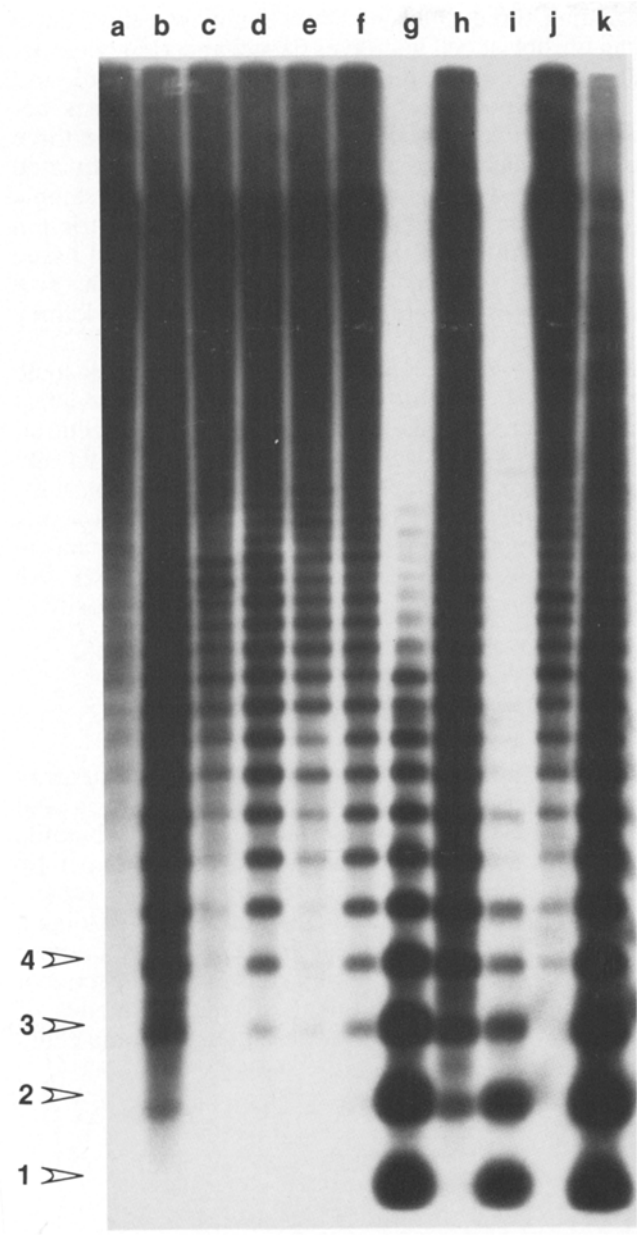

Fig. 4. Southern blot of restriction enzyme-digested DNA from *M. chrotorrhinus* after probing with MSAT-160. Enzymes used are: (a) *Bam*HI, (b) *Dde*I, (c) *Eco*RI, (d) *Eco*RV, (e) *Hae*III, (f) *Hha*I, (g) *Hind*III, (h) *Hinf*I, (i) *Mbo*I (with a longer exposure, bands in the 8-mer to 20-mer size range are apparent, as seen in Fig. 5), (j) *Pst*I, and (k) *Rsa*I. In this and Fig. 5, **arrow heads** at lower left indicate integer multiples of the unit repeat length (160 bp).

then account for the present composition of the consensus sequence. Once reaching a unit length of 160 bp, the copy number of the component was then increased through unequal crossing-over (Smith 1976) and/or some type of saltatory amplification mechanism (Britten and Kohne 1968; Southern 1970). It is estimated that there are 10^4–10^5 copies per genome in *M. chrotorrhinus* (Modi, unpublished). A somewhat similar mechanism has been proposed to account for the origin of the mouse light satellite DNA from a 9–18 bp precourser (Southern 1975), for the calf satellite I from

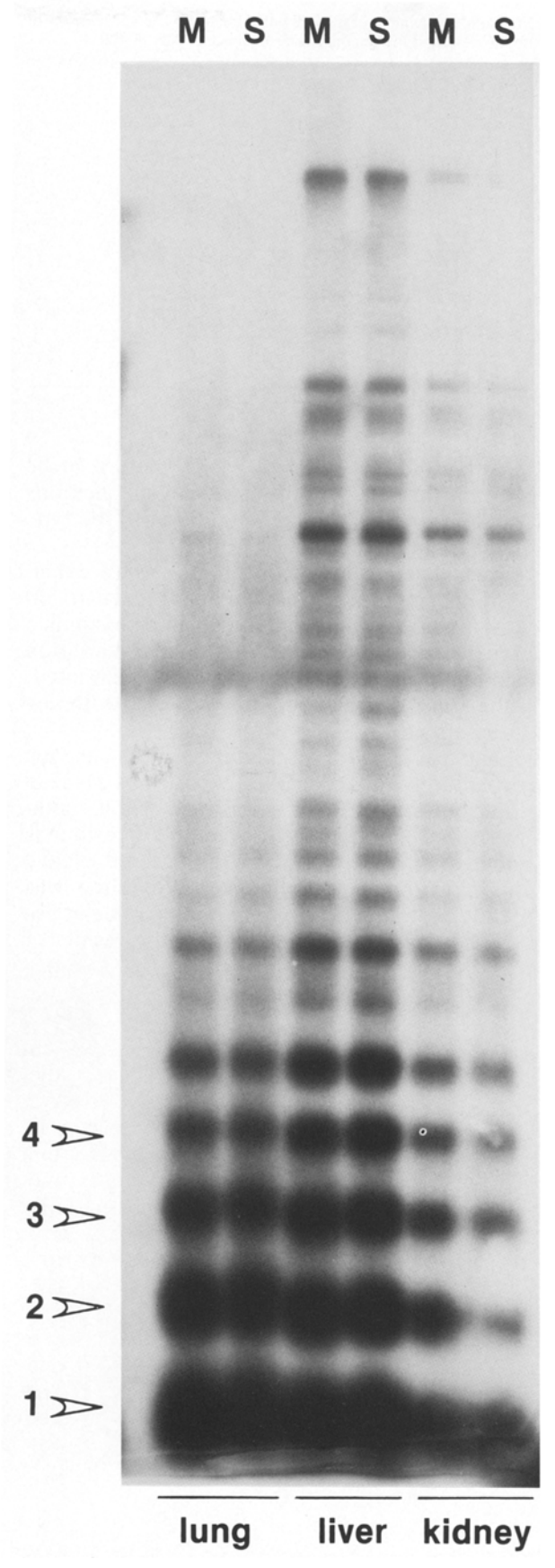

Fig. 5. Southern blot of *Mbo*I (M)- and *Sau*3AI (S)-digested DNA from a single male individual of *M. chrotorrhinus* from three different sources: a lung fibroblast cell line, liver tissue, and kidney tissue.

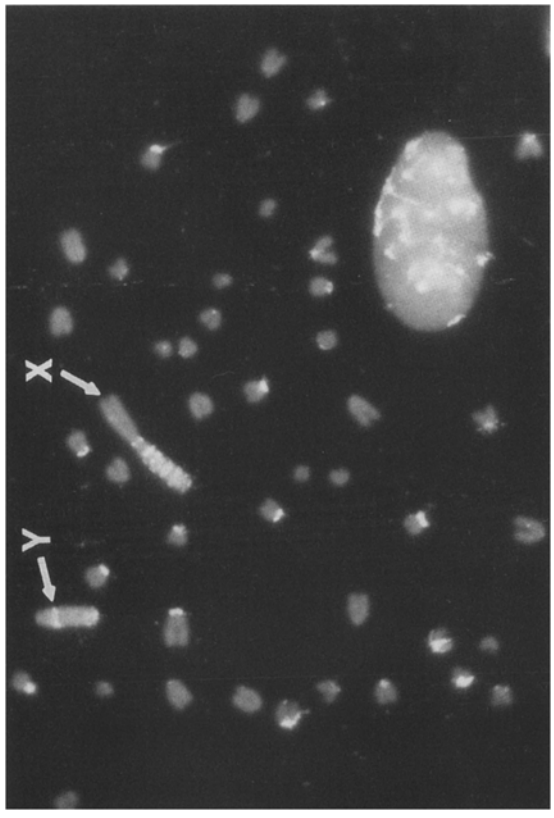

Fig. 6. Metaphase chromosomes from a male *M. chrotorrhinus* following fluorescence in situ hybridization. The signal was detected with avidin-fluorescein-isothiocyanate (FITC) (brightly fluorescing areas) while the chromosomes were counterstained with propidium iodide.

a 23 bp basic unit length (Roizes 1976), and for human satellite III that is thought to be derived from 5 bp and 26 bp subunits (Deininger et al. 1981).

Several different processes appear to be going on simultaneously within MSAT-160, as evidenced by the sequence variants and restriction patterns that have been observed. The type A restriction pattern is thought to come about when most family members contain the site of interest. Random mutation in one base of a recognition sequence would subsequently prevent the enzyme from cutting at that site, thus producing a dimer. Mutations in two consecutive restriction sites would produce trimers, and so forth. The presence of dimers, trimers, and other higher n-mers, and the observed sequence differences seen between monomers in Fig. 2 indicate that stochastic mutations are occurring throughout the arrays. Further, the near equivalence of changes producing A or T versus those producing G or C, and the nearly expected transversion-to-transition ratio suggest that most substitutions are taking place randomly. However, this process of random mutation seems to be contradicted by the presence of type B restriction patterns and the lack of intraspecific variability in restriction patterns. Type B patterns represent small, non-overlapping tandem arrays that define different subsegments of the satellite DNA (Southern 1975; Horz and Zachau 1977). These subsegments may originate by mutation followed by unequal crossing-over. If the origin of a type B pattern depends upon the probability with which a recognition sequence for the particular enzyme will be generated through random mutation, then certain sites should result more frequently than others; for example, it was shown above that *Hinf*I sites should occur five times more frequently than *Eco*RV or *Hae*III sites and even more frequently than *Eco*RI, which requires two substitutions for its site to be created. Examination of Fig. 1 indicates, as expected, greater digestion in the *Hinf*I lane (lane h) than in the *Eco*RI lane (lane c). This observation suggests that at least certain novel variants are homogenized roughly in proportion to their frequency of origination. However, the sequence data indicate that many variants have either escaped homogenization (if they are reasonably old) or are of recent enough origin that homogenization has not yet acted upon them. Sequence data from one or more additional species possessing this satellite would enable an estimate of the approximate age of certain sequence variants.

One final point warrants discussion. The submotifs documented in Figs. 2–3 are complementary and potentially capable of pairing, forming various intrastrand dyad structures. The compact chromatin typical of heterochromatin may contain such dyad structures stabilized by specific proteins. It has been suggested that methylated DNA would have an increased affinity for H1 histones and subsequently form a compact chromatin structure in calf DNA (Davie and Delcuve 1988; Pages and Roizes 1988). Eukaryotic DNA methylation (5-methylcytosine, 5mC) occurs most frequently at CpG dinucleotides (Grippo et al. 1968; Pech et al. 1979). The enzymes *Hpa*II and *Msp*I both recognize the sequence CCGG but differ in their methylation sensitivity. An examination of Fig. 2 illustrates that this recognition sequence is not present in the consensus, and, when DNA digested with these enzymes is probed with MSAT-160, a smear is obtained (not shown). This result prevented the ascertainment of CpG methylation status with conventional Southern blotting. However, 5mC is found in small amounts at CpC and CpT dinucleotides (Salomon and Kay 1970; Woodcock et al. 1987). Although the presently studied DNA was shown not to be methylated at CpT dinucleotides, many satellites in other species similarly exhibit potential dyad sequences and, although unmethylated, may nonetheless be involved in heterochromatin condensation (Fowler et al. 1988; Bigot et al. 1990). Indeed, the chromosomal locations where this satellite resides in *M. chrotorrhinus* are areas known to be constitutively heterochromatic.

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