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The specific organisation of satellite DNA sequences on the X-chromosome of Mus musculus: partial independence of chromosome evolution

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ABSTRACT

DNA was isolated from a chinese hamster/mouse hybrid cell line containing a single mouse chromosome, the X-chromosome, and digested with a variety of restriction endonucleases known to cut mouse satellite DNA. After agarose gel electrophoresis and transfer to nitrocellulose, hybridisation was carried out to a radioactive mouse satellite DNA probe. In this manner the organisation of satellite sequences at an individual chromosome was determined. We have found that the organisation of centromeric satellite DNA sequences on the mouse X-chromosome differs from that of other chromosomes in the complement. The nature of the differences suggests features of evolution of highly repeated sequences within a karyotype.

INTRODUCTION

Considerable attention has been paid to the analysis of bulk populations of highly repetitive sequences of higher organisms that are isolatable as distinct components on bouyant density gradients (satellite DNAs). However, until recently, little was known of the exact distribution of sequence variants of these satellite DNAs throughout the karyotype. Recent work in Drosophila (1) has indicated that the interspersed arrangement of four different blocks of satellites at each chromosome is unique to each of the four chromosomes. In the human genome (2,3) it has been shown that the complex satellite III consists of various populations of restriction fragments (some of them not cross-homologous) which are localized to one or a few chromosomes. The so-called 3.4kb 'male fragment' localized to the Y-chromosome in humans is a specific example of this (4). It has been suggested that such a unique organisation at individual chromosomes could play a role in homologous chromosome recognition (1,2).

The satellite of mouse (Mus musculus) is a homogeneous satellite (5) with a simpler restriction pattern in that on digestion with enzymes Ava II and Eco RII (or its isoschizomer Tsp I) all the satellite is reduced to a monomer of 240bp, and progressively smaller amounts of dimer, trimer etc.

(6,7). This is known as a type A pattern. However, digestion with other enzymes (Taq I, Alu I, Hinf and Eco RI) reveals that mouse satellite is composed of a number of segments distinguished by their susceptibility to these various enzymes. Taq I, Alu I, Hinf and Eco RI each cut only a portion of satellite DNA producing what is known as a type B pattern.

There are two extreme possibilities to explain this state of affairs: either the restriction sites which produce each type B pattern are localized to one or a few chromosomes which might implicate them in individual chromosomal processes, or, it is feasible that they are organised in smaller segments distributed throughout the chromosomes. It is not inconceivable that each restriction enzyme cuts a small and equal size segment from every chromosome. In either case, we must ask the question: is the pattern of restriction sites at any individual chromosome unique to that chromosome? We have attempted to answer these questions by examining the DNA from a chinese-hamster/mouse hybrid cell line which contains a single mouse X-chromosome (8).

MATERIALS AND METHODS

Cell Lines

The EV-1-5 cell line, derived from a cross between mouse BW5147-VI cells and chinese hamster E36-o cells, is a mouse/chinese hamster hybrid harboring a single mouse X-chromosome (8) as identified by a Giemsa/Hoechst sequential staining technique (9). 75% of the cells contain one X-chromosome and 10% of the cells contain two or more. The hybrid cell line was also examined for a variety of isozyme activities and was positive for mouse α-galactosidase activity. No other chromosome has been observed in this cell line. EV-1-5 cells were grown in Dulbecco's modified eagles medium plus 10% fetal bovine serum plus HAT. Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified eagles medium plus 10% fetal bovine serum. All cells were grown in monolayer culture and harvested by trypsinisation and pooled.

DNA Extraction

DNA from cell lines was extracted by a modification of the method of Gross-Bellard et. al. (10). The cell pellet was washed in 10 mM Tris-HCl pH 8, 10 mM EDTA, 10 mM NaCl and then lysed in the same buffer containing 0.5% SDS. Lysis was continued for 2 h with the addition of RNase at a concentration of 200 µg/ml. CsCl was added to the lysate to give a density of 1.70 $g.cm^{-3}$ and spun in a vertical rotor at 45,000 rpm for 18 h on a Sorvall OTD-65 ultracentrifuge. DNA fractions were collected, pooled and

dialysed against 10 mM Tris-HCl pH 8. DNA was ethanol precipitated and redissolved in 10 mM Tris-HCl pH 8. DNA from mouse livers was isolated essentially according to the method of Flamm et. al. (11).

Satellite Purification

Mouse satellite DNA sequences were prepared by repeated fractionation of mouse DNA on Hoechst 33258/CsCl gradients as described previously (12). In mouse a distinct fluorescing satellite band visualised by UV-light lies well separated from main-band material and was easily removed by a syringe inserted into the side of the gradient. Satellite DNA was judged pure when it produced a single symmetrical peak at density 1.691 $g.cm^{-3}$ after analytical centrifugation. In addition such a peak produces the expected series of restriction fragments with type A enzymes without any apparent background material. Similar criteria of purity have been used, Beauchamp et.al.(2). Purification of satellite was particularly facilitated by the use of 5 ml density gradients in a vertical rotor (see above) and we estimated that our preparations of satellite by these methods were 99% pure. One further indication of purity was revealed by the lack of hybridisation, after long exposures on autoradiographs, of the mouse satellite DNA sequences to the CHO DNA (see Results). Any contamination of mouse satellite DNA by main-band single-copy sequences would be expected on average to produce a background level of hybridisation with CHO single copy sequences. In the absence of this we are satisfied that all detected hybridisations were between homologous sequences of total and X-chromosome satellite.

Restriction Enzyme Digestions

Normally, 6 µg of DNA were digested with 5 units of enzyme for 2 h in a total volume of 25 µl. Digestion was complete under these circumstances. Incubation of Tsp I was in 20 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 1 mM DTT at 65°C; Taq I, 6 mM Tris-HCl pH 7.4, 6 mM $MgCl_2$, 1 mM DTT at 65°C; Alu I, 6 mM Tris-HCl pH 7.6, 6 mM $MgCl_2$, 50 mM NaCl, 6 mM 2-mercaptoethanol, 100 µg/ml BSA at 37°C; Hinf, 6 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 100 mM NaCl, 6 mM 2-mercaptoethanol at 37°C, and Eco RI, 100 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl at 37°C. Tsp I is an isoschizomer of Eco RII and has the advantage that Taq I/Tsp I double digestions can be carried out simultaneously at 65°C. A composite buffer was used in those circumstances. Reactions were stopped by the addition of 10λ of a solution containing 10% Ficoll, 0.06% BPB, 0.5% SDS, 0.5% Orange G and heating to 65°C for 10 min. Digests were fractionated on 1.6% or 1.8% agarose gels (15 x 15 x 0.3 cm) using a Tris-Borate buffer (Tris 89 mM, Boric acid 89 mM, Na_2EDTA 2.5 mM).

Current loading was at 20 mA for 30 min and then increased to 40 mA (10 V/cm) for 3 h. Gels were stained with ethidium bromide and visualised on a short-wave transilluminator. Size standards were provided by digestion of λ with Hind III and Eco RI (13) and ϕX174 digested with Taq I. Length measurements using these markers indicated that the ladder of fragments produced by digestion of mouse satellite DNA with various restriction enzymes is based on a monomer of approximately 240 bp. This is in good agreement with previously published work (6,7).

Nick Translation

Purified satellite was nick-translated essentially according to the procedure of Rigby et. al. (14). Satellite was labelled with [α-^{32}P]dATP (2,000 - 3,000 Ci/mmol; Amersham) to a specific activity of 0.5 - 2 x 10^7cpm/μg. Unincorporated nucleotides were separated from incorporated nucleotides on a Sephadex G-50 (fine) column.

Filter Hybridisation

Gels were denatured and neutralised according to Southern (15) and transferred overnight at room temperature to nitrocellulose paper (Schleicher and Schull, BA 85). Filters were baked in vacuo at 80°C for 2 h. Each filter strip representing one gel slot was hybridised with 0.5 ml of hybridisation mixture containing 5 x 10^5cpm of ^{32}P-labelled mouse satellite DNA after it had been heated to 100°C for 3 min to denature the DNA. Alternatively, whole filters representing 8-10 slots from one gel were hybridised in 5 - 15 ml of hybridisation mixture. Under these conditions labelled satellite was present at a lower concentration (approx. 250,000 cpm/ml). Hybridisation was carried out in 5x SSC, 50% formamide, 0.5% SDS at 37°C overnight with gentle agitation. Filters were washed extensively in 3 mM Tris (unneutralised), dried and autoradiographed at -70°C using preflashed Fuji X-ray film backed by a Fuji Mach II intensifying screen.

RESULTS

Type A Patterns:

Digestion of EV-1-5 DNA with Tsp I followed by fractionation on agarose gels, transfer to nitrocellulose, and hybridisation to a ^{32}P-labelled mouse satellite probe resulted in a pattern of bands identical to that seen from the control of mouse DNA against mouse satellite (Fig. 1). In both cases most of the homologus material was digested to a monomer of 240 bp and smaller amounts of dimer, trimer etc. This is the type A pattern. No hybridisation occurred to the CHO DNA similarly digested and we conclude

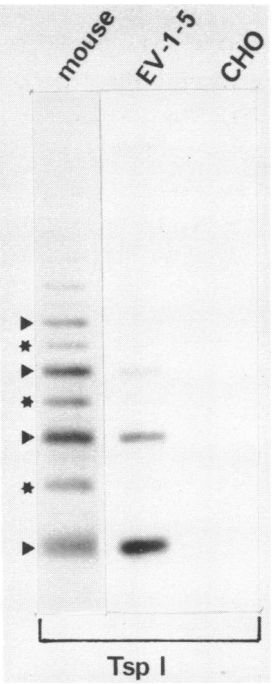

Figure 1. Autoradiographs of Tsp I digestion (Type A) of Mouse, EV-1-5 and CHO DNAs after fractionation on agarose, transfer to nitrocellulose and hybridisation to ^{32}P-labelled mouse satellite probe. EV-1-5 and CHO lanes were exposed for 3 days; mouse for 6 hrs. Arrows indicate an ascending series of multimers based on a monomer length of 240 bp (lowest arrow). Asterisks indicate intermediate size fragments: 1½-mer, 2½-mer etc.

that the pattern of hybridisation seen in EV-1-5 DNA is due to the presence of satellite DNA sequences from the mouse X-chromosome. It appears that the overall basic organisation of the X-chromosome satellite sequences does not differ from that of bulk satellite DNA and consists of a population of 240 bp repeats and their multimers. There is an underrepresentation of some intermediate size repeats (see Fig. 1) in the EV-1-5 DNA indicating that not all periodicities of total satellite are concomitantly amplified on the X-chromosome. Furthermore, there is a clear difference in intensity of the monomers with an apparent higher monomer abundance in the X-chromosome satellite. These differences in homogeneity are discussed later.

Type B Patterns:

A similar examination has been carried out using type B enzymes which cut only a portion of mouse satellite: Taq I, Alu I, Hinf and Eco RI.

(i) Taq I: Taq I cuts a considerable proportion of the X-chromosome satellite sequences to produce a pattern of bands similar to that seen in bulk satellite (Fig. 2). Little material was left near the origin. Due to the limitations of the Southern technique (15) no direct quantitation of the amount of cutting by Taq I can be made. Uncut material left near the origin is

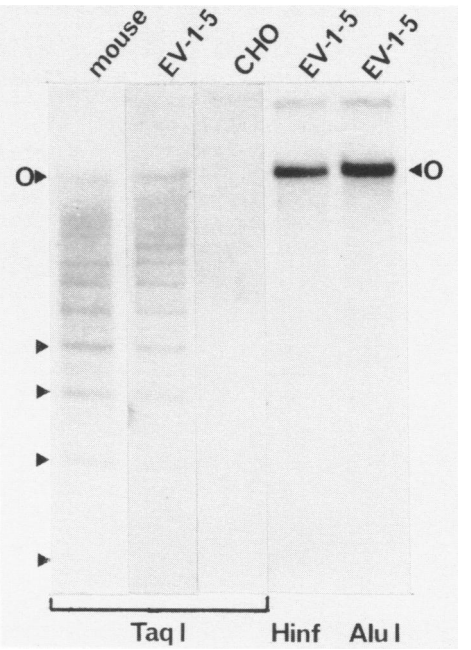

Figure 2. Autoradiographs of restriction enzyme digests (Type B) of mouse, EV-1-5 and CHO DNAs after fractionation on agarose, transfer to nitrocellulose and hybridisation to a ^{32}P-labelled mouse satellite probe. All tracks were transferred to and hybridised on the same filter. EV-1-5 and CHO lanes were exposed for 6 days; the mouse lane was exposed for 6 hrs. Arrows indicate an ascending series of multimers based on a monomer length of 240 bp (lowest arrow). O indicates undigested material left near the origin.

never completely transferred from the gel. However, by comparison of the homologous mouse and the EV-1-5 hybridisations it is evident that Taq I cuts a roughly similar proportion of the X-chromosome sequences to that in bulk satellite which has been estimated as approximately 35%, (unpublished results).

(ii) Alu I, Hinf and Eco RI: In contrast to Taq I, Alu I and Hinf digests of EV-1-5 DNA hybridised on the same filter showed very little cutting of the X-chromosome satellite sequences and considerable material remained at the origin. Very faint bands were observed in the Alu I and Hinf digests, (more clearly observable on the original autoradiograph) of EV-1-5 DNA. In order to confirm the presence of these faint bands, further experiments, using Alu I, Hinf and Eco RI, were carried out by hybridising single nitrocellulose strips representing one gel slot in a small volume of hybridisation

mixture (see Materials and Methods), (Fig. 3). This increased the efficiency of the hybridisation reaction. Under these conditions restriction of EV-1-5 DNA with Alu I and Hinf was observed to produce a clear series of bands after probing with ^{32}P-labelled mouse satellite.

The patterns of bands produced by Alu I and Hinf were similar to that produced from bulk satellite DNA (Fig. 3). Again, no direct quantitation can be made because the degree of hybridisation is affected by differences in efficiency of transfer from the gel of high and low molecular weight material. However, it is known that Alu I and Hinf leave 90% of mouse satellite uncut near the origin (6), and similarly the bulk of the X-chromosome sequences is left uncut near the origin. Transfer of these uncut sequences of mouse total

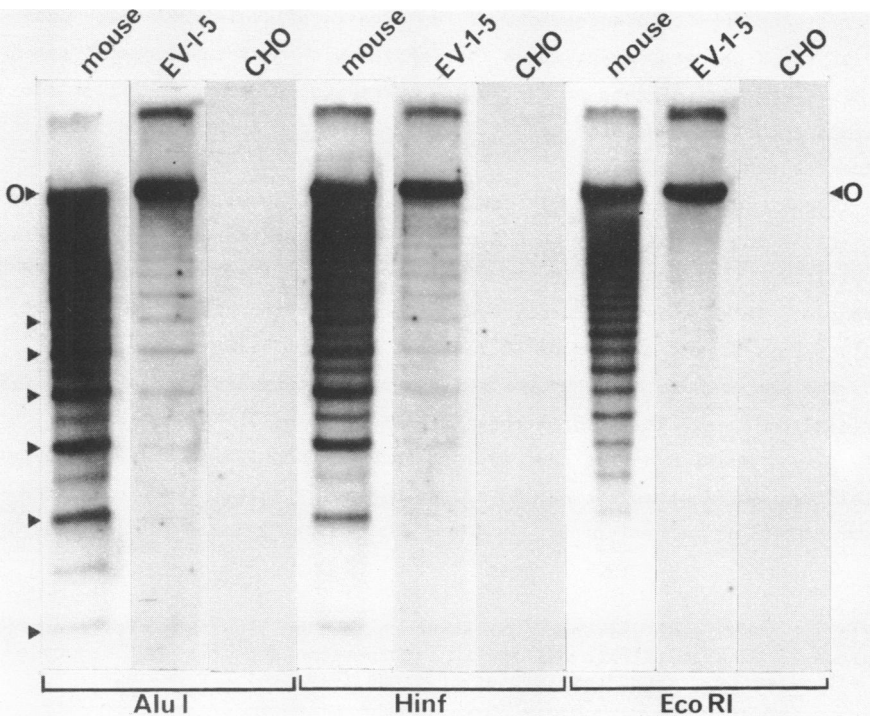

Figure 3. Autoradiographs of restriction enzyme digests (Type B) of mouse, EV-1-5 and CHO DNAs after fractionation on agarose, transfer to nitrocellulose and hybridisation to a ^{32}P-labelled mouse satellite. All tracks were transferred to the same filter and individual strips cut from this filter were hybridised under identical conditions. EV-1-5 and CHO lanes were exposed for 2 weeks, the mouse lanes were exposed for 18 hrs. Arrows indicate an ascending series of multimers based on a monomer length of 240 bp (lowest arrow). O indicates undigested material left near the origin.

satellite and EV-1-5 shown in Fig. 3 occurred from the same gel to the same filter and presumably with the same efficiency. Thus exposure to an equivalent intensity of the uncut bands of mouse DNA and EV-1-5 DNA that represent the bulk of satellite in each slot should permit a comparison of the relative amounts of material cut by Alu I and Hinf in bulk satellite and the X-chromosome. Using this procedure it is clear that the proportion of sequences cut by Alu I and Hinf in the X-chromosome is substantially less than that in bulk satellite. Furthermore, the proportion of Alu I sites on the X-chromosome can be seen to be greater than the proportion of Hinf sites. This was a consistent feature of repeated experiments carried out on whole filters rather than filter strips. However, in bulk satellite DNA these two enzymes cut a very similar proportion of sequences (6). By comparing microdensitometer traces from autoradiographs of Taq digests of EV-1-5 DNA which cuts 35% of the X-chromosome sequences (see above) with microdensitometer traces of Alu I and Hinf digests of EV-1-5 DNA hybridised on the same filter we calculate that Alu and Hinf cut 2-3% and 1-2% of the X-chromosome satellite sequences respectively.

Eco RI, digestion of EV-1-5 and mouse DNA is shown in Fig. 3. Barely detectable bands appeared with DNA of EV-1-5. We estimate that under these conditions 0.5% digestion of the satellite sequences on the X-chromosome would be detected. This is approximately equivalent to 125 repeats of 240bp which would result from a minimum of 25 restriction sites being present.

The proportions of X-chromosome sequences cut by Alu I, Hinf and Eco RI differ from those cut in total satellite DNA (Table 1). Alu I and Hinf cut only a very small fraction that is less than the proportion they cut in total satellite, while sites for Eco RI are barely detectable. The proportions of X-chromosome sequences cut by Alu I and Hinf differ yet they cut identical

Table I. Summary of percentage of sequences cut by a variety of restriction enzymes in total satellite DNA and in X-chromosome satellite DNA sequences as estimated from microdensitometer tracings (see text for details).

	Percentage of satellite DNA sequences cut:			
	Taq I	Alu I	Hinf	Eco RI
Total satellite DNA	35	10	10	5
X-chromosome satellite DNA	35	2-3	1-2	--
	Eco RI sites are present at too low a frequency on the X-chromosome to be determined accurately.			

proportions of bulk satellite.

Mapping of Taq I sites with respect to Tsp I sites on the X-chromosome:

Since Taq I cuts a considerable proportion of the X-chromosome sequences it is possible to map Taq I sites with respect to Tsp I sites on the X-chromosome and compare their location with that found in bulk satellite DNA. As shown in Fig.4 double digestion with Taq I and Tsp I on total mouse DNA produced a new band at 160 bp approximately after hybridisation with ^{32}P-labelled mouse satellite. This band results from Taq I sites occurring within the monomer at a position 160 bp from one Tsp site and 80 bp from the other (see diagram in Fig.4). The 80 bp fragment would not be retained during the hybridisation reaction. In addition a 400 bp fragment is produced in total mouse DNA by cutting within the dimer produced by Tsp I. A similar fragment produced after Tsp I/Taq I double digestion of EV-1-5 DNA is of very low abundance and too faint to reproduce clearly. It can be observed in the original autoradiograph. Similar results to total mouse DNA were obtained after double digestion of

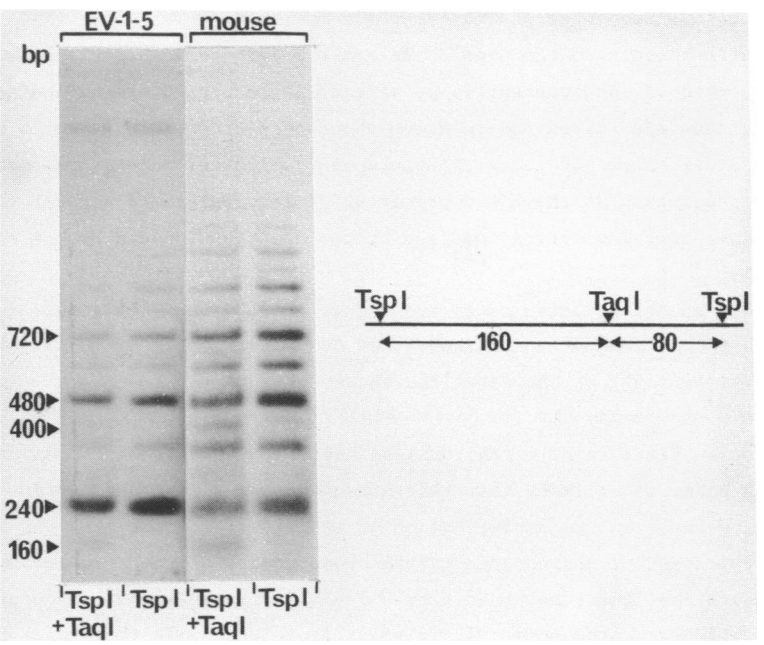

Figure 4. Autoradiographs of Tsp I and Tsp I/Taq I restriction digests of mouse and EV-1-5 DNAs after fractionation on agarose, transfer to nitrocellulose and hybridisation to a ^{32}P-labelled mouse satellite. All tracks were transferred to and hybridised on the same filter. EV-1-5 lanes were exposed for 2 weeks; the mouse lanes were exposed for 18 hrs. The accompanying diagram indicates the relation of Tsp I and Taq I sites. Figures are in base pairs.

purified satellite DNA, (data not shown). The double digestion of EV-1-5 with Taq I and Tsp I probed with ^{32}P-labelled mouse satellite gave essentially an identical result to that seen from total mouse DNA, (Fig. 4). A new band arises at 160 bp approximately. We conclude that the position of the Taq I site on the X-chromosome with respect to the Tsp I sites is similar to that of bulk satellite DNA.

DISCUSSION

Examination of the satellite DNA sequences of the mouse X-chromosome has revealed a basic organisation similar to that of the bulk satellite DNA . However, its sequence organisation with respect to the type A and type B patterns as originally defined by Horz and Zachau (6) shows it to be different in detail.

With respect to the type A pattern there are no apparent major differences in the periodicities of type A sites. However Tsp I (Fig.1) has revealed a greater abundance of the monomer and a lesser abundance of intermediate size repeats in the X-chromosome DNA. These differences, that indicate greater homogeneity of sequence, might reflect a more recent and localised amplification event generating the satellite sequences on the X-chromosome.

With respect to the type B pattern the data demonstrate that a single chromosome does not necessarily possess a single type B segment unique to itself. Secondly, given the relative abundances of different type B segments on the X-chromosome it is not feasible that all other chromosomes would have similar amounts. On these two premises, it is possible to suggest that chromosome individuality is defined by the distribution and amount of such segments.

The questions arise as to the nature of the events that have led to the observed distributions of sequences on individual chromosomes and the functions (if any) of the satellite sequences.

The similarity in the positioning of the Tsp I and Taq restriction sites in both the total satellite DNA and the X-chromosome (see Fig. 4) makes it unlikely that this arrangement would have occurred independently by mutation and amplification on individual chromosomes. On the other hand, the apparent increase in overall homogeneity of the X-chromosome type A suggests that there has been a relatively later amplification event on this particular chromosome. It is possible to reconcile these data by suggesting that there is a low but significant level of interchange of sequences between chromosomes at different evolutionary time points. Simplistically, we suggest that a Tsp/Taq unit(s) might have transferred to the X-chromosome from elsewhere in the genome (so maintaining the basic similarity in the distances between sites) followed by its amplification. Such

subsequent amplification would explain any differences in homogeneity and also the differences in abundance of particular type B segments. It is possible that amplification occurs to varying extents at slightly different locations producing the differences in type B abundance or, alternatively, it is possible that the differences reflect the evolutionary time points at which particular segments were introduced to the X-chromosome.

A state of flux of type B patterns at any individual chromosome might exist with one type B prevalent at any time, others being introduced by interchromosomal exchange. A relatively low rate of interchromosomal exchange could act in preserving the relatively unique type B organisation at an individual chromosome centromere.

Beauchamp et.al. (2) also considered that the unique arrangement of restriction sites of human satellite III at individual chromosomes might be due to limited interchromosomal exchange. However, unlike human satellite III, mouse satellite is a relatively more homogeneous satellite consisting of a basic uniform repeat structure spread throughout the karyotype (19). Mouse chromosomes are telocentric and it is possible to envisage considerable interchromosomal exchange at the centromeres (without disturbing the karyotype) that maintains the overall uniformity and spread of satellite sequences. Such a situation might not apply to the human karyotype where the chromosome morphology is metacentric.

With respect to function it is tempting to suggest that the unique organisation of type B segments at the X-chromosome plays a role in some individual chromosome recognition process. However, recent data in Drosophila (16,17) and data from this laboratory in rodents (18) indicate that it is unlikely that the majority of centromeric sequences are involved in homologous chromosome pairing. It is possible that the distinctions of chromosomes with respect to these sequences affects other aspects of chromosome behaviour that need to differentiate between long stretches of sequences.

Any discussion of the evolution or function of such sequences would need to take into account the additional interesting finding that the same type A and some of the type B segments of the mouse satellite are present in the genome of the related species Mus spretus, with the same relative abundances but with an overall reduction in amount (18)

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