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**Novel classes of mouse repeated DNAs**

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**ABSTRACT**

Mouse DNA cleaved with Eco RII (Bst NI) displays two prominent restriction bands of 1.5 and 1.7 kb in agarose gels stained with ethidium bromide. These constitute novel subsets of repeated DNA in the mouse. Sequential Hoechst 33258-CsCl gradient fractionation of mouse DNA, yielding more GC rich main band DNA, and AT rich satellite DNA, revealed that both these fragments copurified with GC rich main band DNA. They were not detected in purified satellite preparations. Together these restriction bands constituted  $\geq 0.2\%$  of main band DNAs. Hybridization of  $^{32}\text{P}$  labelled satellite DNA to blots of Eco RII restricted mouse DNA showed positive hybridization only to smaller satellite restriction fragments, indicating satellite DNA had little or no homology with either the 1.5 or 1.7 kb fragments.

The 1.5 and 1.7 kb fragments were isolated from gels and labelled with  $^{32}\text{P}$  by nick translation. Using a series of restriction endonucleases each of these two fragments showed different cleavage patterns. Filter hybridization confirmed that these two fragments were distinct subsets as they did not cross hybridize with each other. They also did not hybridize to other more minor repeated non-satellite DNA bands noted in ethidium bromide stained gels. Neither of them could be assigned to ribosomal genes as they did not hybridize to  $^{32}\text{P}$  kinase labelled 18S and 28S RNA. Isolation of DNA from male and female mice showed comparable amounts of both the 1.5 and 1.7 kb fragments. Thus neither was Y chromosome specific. From restriction patterns, and preliminary chromosome hybridization studies, these fragments are thought to represent "interspersed" repeated sequences rather than very long tandem (satellite like) centromeric arrays.

The relationship between these repeated sequence subsets, their evolution and detailed organization, and their representation in different mouse species, remain to be determined.

**INTRODUCTION**

Mouse DNA in different tissues and cells contains a major AT rich satellite that accounts for ~9% of the genome as detected in CsCl isopycnic centrifugation (1, 2, 3). Renaturation studies have indicated that approximately 10% of the genome is highly repetitive (4). The observed banding pattern of satellite DNA in CsCl gradients indicates there are long stretches of AT rich repeated satellite sequences in the genome, and chromosome hybrid-

ization studies have shown discrete localization of mouse satellite DNA at the centromeres of the autosomal chromosomes (5), which is also consistent with long uninterrupted arrays of repeated sequences at these sites. Some sequencing studies have furthermore shown a nucleotide order based on an essentially simple 9 nucleotide tandem repeat (6).

Recent studies using restriction enzymes and recombinant DNA technology with a number of eukaryotic genes have indicated many non-transcribed or spacer regions between and around genes coding for specific proteins. The relationship of these "interspersed" sequences to known satellite DNAs is not resolved. For example, 1) are "interspersed spacer" sequences repeated, or represented at more than one site, 2) do some of these interspersed spacers constitute sequence families that are entirely distinct and separate from the major DNA satellites, and 3) are any satellite sequences present in main band DNA and in the chromosome arms? The mouse genome is an appropriate starting point to study some of these questions, since for a mammal it has a high proportion of a single defined separable satellite DNA. Furthermore renaturation studies have indicated that purified main band still contains appreciable amounts of repeated DNA sequences (7). The present report indicates there are indeed distinct sets of previously undescribed repeated DNA that are detectable in mouse main band DNA isolated by CsCl centrifugation.

### MATERIALS AND METHODS

Mouse DNAs were purified by CsCl centrifugation from purified nuclear lysates of liver using Hoechst 33258 at a concentration of 0.1 - 0.5  $\mu\text{g}/\mu\text{g}$  DNA in order to resolve the satellite DNA, and fractions were tested for purity by fluorescence densitometry of preparative tubes (8), or analytical centrifugation as described (3). Main band DNA was subject to more than three cycles of recentrifugation with shearing through a 18 gauge needle in order to completely release satellite.

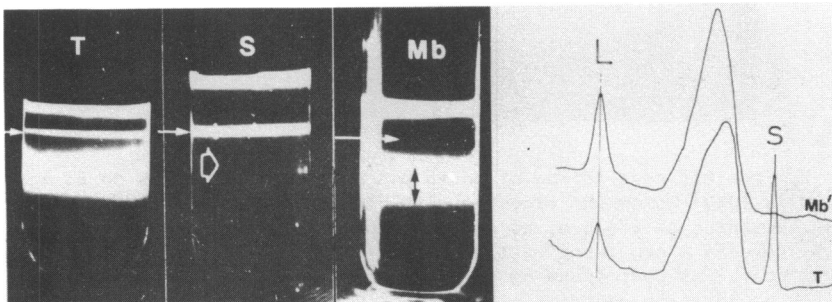
Radioactive labelling of purified satellite DNA and main band DNA was used to test various DNA fractions immobilized on filters (Southern blot hybridization). Restriction of DNA with Eco RI was carried out in 100 mM TrisCl pH 8.0 with 5 mM  $\text{MgCl}_2$  and Bst NI digestions were done at 60° under paraffin oil; other restrictions, electrophoresis, "nick translation" to label DNA with  $^{32}\text{P}$ , and Southern blot hybridization were done essentially as reported previously (9). 18S RNA containing some 28S RNA was isolated by sucrose centrifugation (10) and was labelled with  $^{32}\text{P}$  using polynucleotide

kinase (11) after incubation at 70°C for 10 minutes in 25 mM Tris Cl pH 9.5. Densitometry and computation of peak areas for quantitative analysis was done as described from negatives exposed in the linear range (3,9,12).

## RESULTS

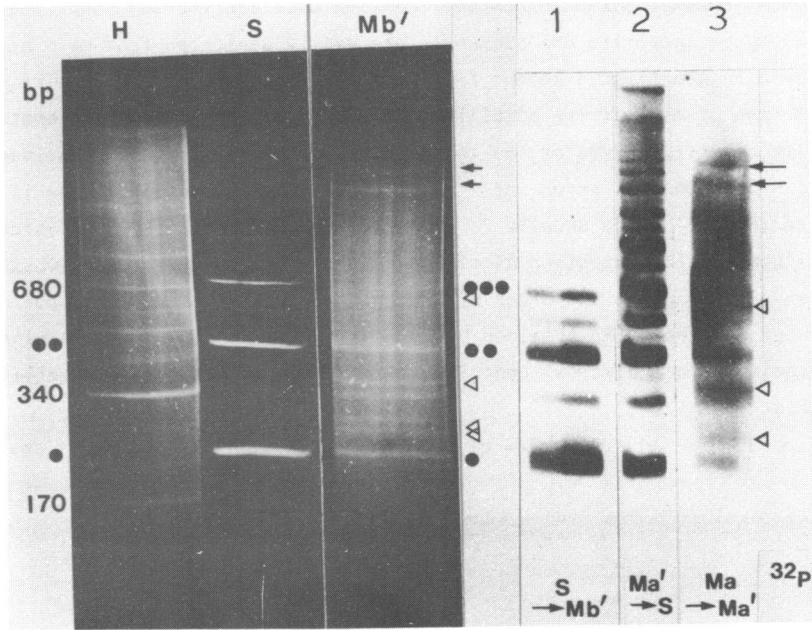
Using Hoechst 33258 in conjunction with CsCl equilibrium centrifugation, AT rich mouse satellite DNA sequences are easily separated from main band DNA; even in the first gradient fractionation the main band DNA can be obtained free of most of its satellite DNA (8), which prior to fractionation normally constitutes ~9% of the total DNA (3). In the present experiments the main band DNA was rerun until no satellite DNA was detectable by fluorescence (Fig. 1) which usually is somewhat more sensitive than analytical centrifugation for detecting AT rich sequences (10), and can unequivocally resolve a DNA band in the order of 0.3% of the total DNA. Further purification of main band DNA obtained by collecting the GC rich heavy portion of the gradient, on analytical centrifugation showed no detectable satellite DNA (Fig. 1, Mb').

Eco RII (Bst NI) digestion of main band and satellite DNA fractions, on



**Figure 1.** CsCl centrifugation of mouse DNA with Hoechst 33258. The first panel shows total unfractionated DNA (T) and the white arrow points to the fluorescent satellite DNA band. A relatively pure satellite preparation was obtained by pooling the satellite band of several such gradients (seen in S), and this satellite was further purified by collecting the upper band (white arrow) and discarding the faint fluorescence in the main band position (open arrow). Purified main band preparations (as Mb), containing negligible fluorescence at satellite positions (white arrows) were further fractionated so that more AT rich upper segments of the main band were discarded (black arrows indicate region of main band collected). This more purified main band preparation is designated Mb' below. Analytical centrifugation of Mb' shows no detectable satellite DNA. M *Lysodeicticus* DNA (L) is density marker, and T is total DNA containing satellite DNA (S).

agarose gel electrophoresis yielded a series of ethidium bromide fluorescent restriction bands (Fig. 2). The satellite DNA displayed a series of major gel bands which were integer multiples of 237 b.p. Additionally more minor gel bands corresponding to 1/2 integer multimers were seen (e.g. 1.5 and 2.5 mer). These results are in accord with previous studies on Eco RII di-



**Figure 2.** Eco RII restriction of mouse DNA and electrophoresis on 2% Agarose gels. H is total human DNA digested with Hae III showing multimers of repeating DNA sequences of 170 b.p. as prominent bands (9,10). Mouse satellite DNA (lane S) reveals a prominent multimeric series of 237 b.p. as well as fainter bands at positions corresponding to 1/2 mers (e.g. 1.5 and 2.5 mer) This satellite preparation contains some partially digested products of the multimers. In the highly purified main band preparation (Mb') a series of other minor bands are seen. Arrows point to position of 1.5 kb and 1.7 kb fragments. Other minor fluorescent bands (open triangles) are also noted. All the main band preparations additionally contain Eco RII DNA fragments migrating at the same position as the satellite. One dot indicates the position of the 237 b.p. 1-mer, 2 dots the 2-mer and, 3 dots the 3-mer.

To the right (lanes 1,2,3) are hybridizations of <sup>32</sup>P 'nick translated' DNA probes to these same gel profiles (Southern blot), printed at the same magnification. <sup>32</sup>P labelled purified satellite DNA highlights only satellite sequences in the main band (lane 1). A <sup>32</sup>P labelled main band preparation (Ma') hybridizes to purified satellite DNA (partial satellite digest, lane 2). Hybridizations of <sup>32</sup>P labelled main band to itself (lane 3) displays bands that do not correspond to complete or partial digestion satellite multimers (arrows and open triangles). A smear of other background sequences is also seen.

gested mouse satellite DNA (13). Despite repeated isopycnic centrifugation and purification of main band DNA, restriction fragments corresponding to satellite DNA gel bands were visible in main band preparations. In the cleanest preparations these accounted for 0.1 - 0.2% of the total main band DNA fluorescence as determined by densitometry of negatives as described (3,12).

Several additional fluorescent bands were clearly visible in the Eco RII digest of purified GC rich main band DNA that were not present in purified satellite DNA preparations. Two restriction fragments, calculated to be  $\sim 1.5$  kb and  $\sim 1.7$  kb by their migration, were quite prominent (Fig. 2, arrows). By densitometry together these accounted for  $\geq 0.2\%$  of the total main band DNA fluorescence. Other more minor bands of lower molecular weight not corresponding to satellite DNA fragments were also noted above the background of heterogenous DNA (Fig. 2, open triangles). Together these more minor bands comprised  $\sim 0.1\%$  of the total DNA fluorescence of the main band DNA (Fig. 3).

Restriction with Ava II cleaves satellite DNA into a multimer pattern which very closely resembles the pattern obtained with Eco RII (237 b.p. integers and 1/2 integers). Direct sequencing of mouse satellite has confirmed the presence of an Ava II site overlapping the Eco RII restriction site (Manuelidis, in preparation). Digestion of purified mouse main band DNA with Ava II again showed gel bands corresponding to those of satellite digested with Ava II. However, the 1.5 and 1.7 kb fragments were not as prominent in main band DNA digested with Ava II. These results also indicated the 1.5 and 1.7 kb fragments were distinct in sequence from satellite DNA.

In order to confirm the independent identity of the 1.5 and 1.7 kb fragments, as well as that of the more minor fluorescent gel bands, a series

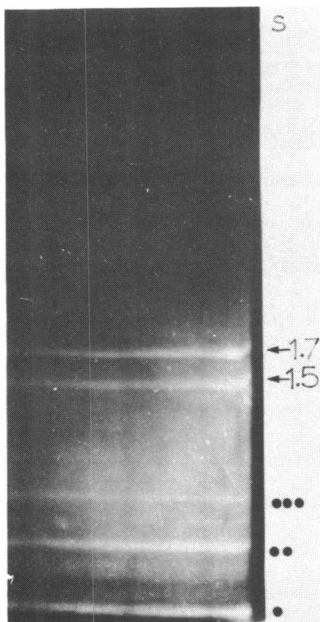


**Figure 3.** Densitometry of ethidium bromide fluorescence of mouse DNA preparations as seen in Fig. 2. Open triangles indicate minor smaller fragments, arrows indicate the 1.5 kb and 1.7 kb fragments, and dots indicate the peaks corresponding to satellite. s is slot. Such tracings were used to calculate the amount of non-satellite fragments in the various main band preparations. Sat is tracing of partial Eco RII digest of purified satellite DNA for reference in same gel.

of filter hybridizations utilizing different  $^{32}\text{P}$  labelled nick translated probes were undertaken.  $^{32}\text{P}$  labelled satellite DNA hybridized to fragments in the main band that corresponded to satellite DNA. It did not hybridize to either the 1.5 or 1.7 kb fragments (Fig. 2). The presence of satellite sequences in the main band fraction was also confirmed in studies using  $^{32}\text{P}$  labelled main band DNA. This probe clearly hybridized to purified satellite DNA profiles (Fig. 2). Hybridization of main band DNA to itself also demonstrated that the main band DNA contained repeated sequences other than satellite DNA. For example the 1.5 and 1.7 kb fragments (Fig. 2, arrows) as well as the other minor fluorescent bands noted above (open triangles) were now detected above a background of other DNAs. Thus these restriction fragments were not conventional mouse satellite DNA, and were likely to represent repeated copies of distinct DNA subsets within mouse main band DNA.

In order to begin to resolve and define some of these repeated DNA subsets, the 1.5 and 1.7 kb fragments were isolated by preparative gel electrophoresis for further study (Fig. 4). Such isolates were used to determine if each of these two fragments were homologous in sequence, and if they were related to the other more minor non-satellite gel bands.

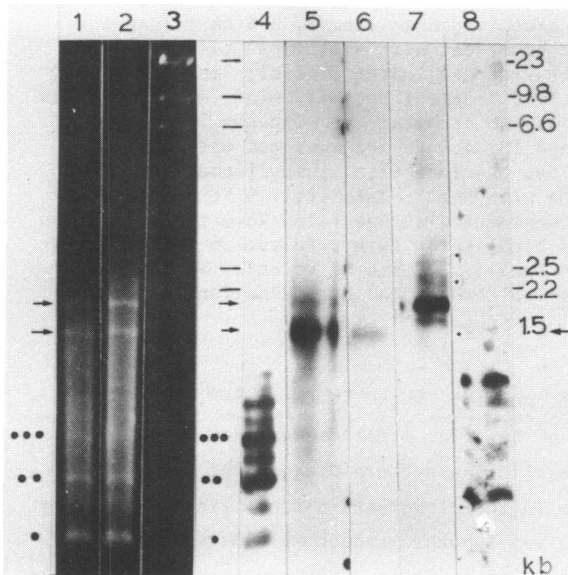
Nick translated probes of the 1.5 and 1.7 kb fragments were hybridized



**Figure 4.** Preparative 1.2% agarose gel of Bst NI restricted Mb DNA used for isolation of the 1.5 and 1.7 kb fragments (arrows). Satellite monomer, dimer, and trimer are indicated by dots, s is slot. The 1.5 and 1.7 kb bands, and the satellite monomer were cut from the gel and reembedded in agarose. A small slit was cut beneath each band and filled with hydroxyapatite. After complete electrophoresis of the DNA into hydroxyapatite, the slurry was eluted with 1 M  $\text{KPO}_4$  pH 6.8, and the eluates were exhaustively dialyzed against 1 M NaCl and 10 mM Tris Cl pH 7.4. Isobutanol was used to reduce the volume and remove the residual ethidium bromide, and the DNA was precipitated with ethanol, resuspended in 10 mM Tris Cl pH 7.4 and used for "nick translation." Picture is 0.3x actual gel dimension.

to total mouse DNA. In these studies, each of these components hybridized to itself and did not hybridize to satellite DNA (Fig. 5). Long autoradiographic exposures did reveal a few other very minor additional restriction DNA bands, however these bands were not at positions corresponding to satellite DNA. The faint additional bands also did not correspond to the other non-satellite fluorescent gel bands noted previously. In studies where the stringency of the hybridization was increased by washing the hybridized filters in 0.2x SSC, 0.1% SDS at 55°C, these very minor additional hybridized bands were no longer detectable even in long autoradiographic exposures (Fig. 5, lane 6).

Analysis of each of the isolated 1.5 and 1.7 kb bands with a series of restriction enzymes further confirmed that each had an independent identity. For example, each band yielded quite different patterns in parallel digestions with the same enzymes (Fig. 6). The restriction patterns obtained were compatible with patterns highlighted by hybridization to total mouse DNA, indicating that a significant proportion of these sequences were contained in the 1.5 and 1.7 kb isolates. For example Hae III digestion of the nick translated 1.7 kb DNA isolate yielded a major large fragment in the order of 1.2 kb; hybridization of this <sup>32</sup>P labelled 1.7 kb fragment to a Hae III digest of total mouse DNA also highlighted a comparable pattern of restriction frag-



**Figure 5.** Total male DNA (lane 1) and female DNA (lane 2) restricted with Bst NI show comparable amounts of the 1.5 and 1.7 kb fragments.  $\lambda$  DNA digested with Hind III on same gel (lane 3), ethidium bromide fluorescence. Lane 4 is Southern blot of <sup>32</sup>P labelled monomer to whole mouse DNA. Lane 5 is <sup>32</sup>P labelled 1.5 kb DNA to parallel total DNA blot. Lane 6 is <sup>32</sup>P 1.5 kb fragment hybridized, and washed under conditions of increased stringency. Lane 7 is <sup>32</sup>P labelled 1.7 kb fragment to total DNA, parallel lane. Lane 8 is <sup>32</sup>P labelled ribosomal RNA to total DNA. Note the 1.5 and 1.7 kb fragments do not hybridize to each other or satellite DNA, and the <sup>32</sup>P ribosomal RNA labels smaller molecular weight fragments at positions different than the satellite, and the 1.5 and 1.7 kb fragments.

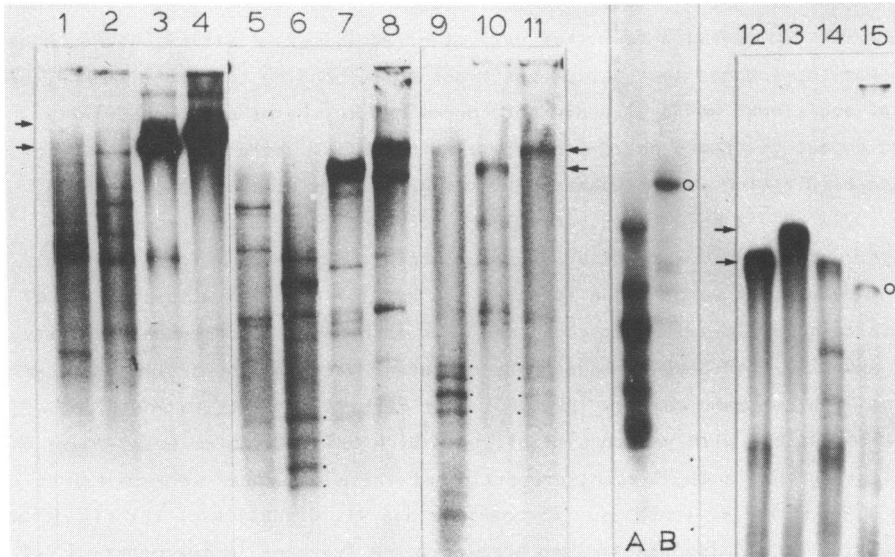


Figure 6. Restriction digests of  $^{32}\text{P}$  (lanes 1-11) or  $^{125}\text{I}$  labelled (lanes 12-15) 1.5 and 1.7 kb fragments. A and B are Southern hybridizations of the 1.7 kb fragment to total DNA digested with Alu and Hae III. The restriction fragment pattern is different for each fragment; lane 1, 1.5 kb fragment digested with Alu, lane 2, 1.7 kb fragment cut with Alu; lane 3, 1.5 kb fragment, uncut control; lane 4, 1.7 kb fragment, uncut control; lane 5, 1.5 kb fragment cut with Hinf; lane 6, 1.7 kb fragment cut with Hinf; lane 7, 1.5 kb fragment cut with Ava II, lane 8, 1.7 kb fragment cut with Ava II; lane 9, 1.7 kb fragment cut with Mbo I; lane 10, 1.5 kb fragment cut with Sau 3A; lane 11, 1.7 kb fragment cut with Sau 3A; lane 12, 1.5 kb fragment cut with Hha I; lane 13, 1.7 kb fragment cut with Hha I; lane 14, 1.5 kb fragment cut with Hae III; lane 15, 1.7 kb fragment cut with Hae III. Note the major band in lane 15 corresponds to the band highlighted by hybridization of this fragment to whole mouse nuclear DNA in Hae III digests (circles). The  $^{125}\text{I}$  labelled probes were used for the in-situ chromosomal hybridizations cited in the text.

ments, with a single major band of the same length in total DNA, as seen in the purified 1.7 kb restriction profile (Fig. 6, lanes B, 15).

It is of interest that the restriction pattern of both the 1.5 and 1.7 kb fragments were somewhat complex in that fragments did not yield completely simple ladders or multimers of a given length. Ladder multimers have been observed in a number of tandemly arrayed satellite DNAs, even those with fairly complex sequences (9,11,13,). The complex restriction patterns suggest that neither of these isolates contain only extremely simple sequence tandem



arrays. Although a multimer ladder is seen in some of the digest (for example Fig. 6, lanes 6,9), not all the major fragments can be accommodated by a multimer relationship. Such patterns may indicate there are at least two different sequence subsets in each of these isolates. More direct sequencing data will be needed to resolve these internal fragment units and their relationship to each other. Such studies will also be useful for determination of fold-back or significant dyadic features; these features are of interest because of the relatively high GC content of these fragments.

The question of whether these fragments were related to ribosomal genes was also experimentally investigated, although the quantity of these fragments in total DNA was higher than expected for ribosomal DNA (200 copies of 13 kb ribosomal DNA (14) in a mouse genome of  $10^{10}$  bp would yield only 0.026% of the total DNA, which is approximately an order of magnitude less than the amount found for these fragments). Ribosomal RNA (18S containing some 28S RNA) labelled with  $^{32}\text{P}$  was hybridized against total mouse DNA. Hybridized bands were observed (Fig. 5), but none of these hybridized bands corresponded to either the 1.5 or 1.7 kb fragments. This indicated the 1.5 and 1.7 kb fragments did not contain 28S or 18S ribosomal coding sequences. Consideration of the quantities of the 1.5 and 1.7 kb fragments also suggested that if these sequences constituted spacer sequences within the 13 kb ribosomal genes, they would have to be represented at other non-ribosomal sites within the genome.

The possibility that one or both of these bands could comprise a Y chromosome specific reiterated sequence was also considered. The mouse Y chromosome is devoid of satellite DNA (5) and thus might conceivably contain other repeated sequence subsets. In human DNA for example, a 3.4 kb band is visible in restriction digests of male but not female DNA (15). This human DNA restriction band is roughly comparable in fluorescent intensity to either the 1.5 or 1.7 kb Eco RII fragments described here. To investigate the possibility that one or both of these fragments were Y chromosome specific, total male and female mouse DNA were separately isolated and subjected to Eco RII digestion. Both showed comparable amounts of both the 1.5 and 1.7 kb fragments (Fig. 5) and thus no Y specific assignment to either fragment was demonstrable. In keeping with this finding, in situ chromosomal hybridization of each of these fragments has shown widespread representation on chromosome arms (Manuelidis, in preparation). Although further in situ chromosome hybridization studies with more purified segments of these fragments are in progress, the data thus far would indicate that each of these sequences are likely to constitute novel sets of repeated mouse DNAs that

are interspersed in the mouse genome.

### DISCUSSION

Previous studies on mouse main band DNA, using Cot renaturation kinetic analysis and electron microscopy have suggested there are repeated sequences within main band DNA (7). The present results clearly delineate a series of minor restriction DNA fragments in mouse main band DNA that are different from mouse AT rich satellite sequences. On the basis of their amount, and Southern blot hybridization, at least several of these bands are likely to be repeated DNAs. Two novel restriction bands of this series have been isolated, and by hybridization and restriction analysis, each of these appear to constitute a distinct repeated DNA subset. Neither fragment is related to the presence of the Y chromosome, and these fragments do not contain ribosomal RNA coding sequences. Preliminary analysis by in situ hybridization indicates each of these sequences is widespread and probably interspersed. The complexity of the restriction fragment pattern is also not compatible with huge tandem arrays such as centromeric arrays of AT rich mouse satellite DNA. The mode of integration of these fragments into larger chromosome structures and their presence at defined non-centromeric sites, such as secondary constrictions, or telomeric regions, needs further investigation.

The possible relation between these two fragment groups and satellite DNA will be clarified by direct DNA sequencing, and such studies should be able to address the definition and origin of repeated mouse DNA subsets. Distinct subsets of repeated DNA have been found for example in the human genome (10), and the classification and relation of various repeated DNAs considered (11). The features that separate centromeric satellite-like DNA from subsets of reiterated interspersed sequences, such as those presumably involved in replication, transcription and transposition, appear most conspicuously related to long range organization and length (11). The clarification of such repeated DNA subsets is essential, as the present lack of knowledge about the function of various repeated DNAs may prematurely relegate some of them to essentially meaningless categories (16).

In the present studies a small amount of AT rich satellite DNA was found in the main band, and although this could conceivably result from incomplete extraction, as for example from shearing of satellite at points where it joins more GC rich DNA, it is possible that a very minor portion of satellite DNA resides in the main band as interspersed copies. Such satellite may represent "primal" copies of origin that exist prior to their am-

plification in long tandem arrays, as proposed for a largely centromeric human repeated DNA subset (11). In this context the recent finding of satellite DNA sequences in Mus Spretus, which contains no demonstrable satellite DNA by CsCl centrifugation, is notable (17). Such interspersed copies may have been chosen for amplification in Mus musculus but not in Mus spretus. It would thus be of interest to know if other "interspersed" repeated sequences, such as those observed in the present study, are amplified to a commensurate degree in Mus spretus, and for example can account for centromeric domains in that species.

Conservation of these sequences in more distantly related species, both in sequence and long range organization may have specific evolutionary ramifications. It will also be useful to know if such sequences are highly conserved in amount and distribution in aneuploid cells within a given species, as are some centromeric repeated DNAs (3, 12, 18).

The mouse genome presents an approachable model system for study of the mechanism of differential amplification, and long range chromosome organization of different repeated DNA subsets. Such studies are enhanced by the available well defined mouse species and cell lines.

#### ACKNOWLEDGEMENTS

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NOTE: Eco RI digests of mouse DNA yield a prominent band of 1.3 kb as recently reported by S. Chen and C. Schildkraut (Fed. Proc. (1980) 39, 1784). The 1.7 kb fragment isolated here hybridizes to this Eco RI band on Southern blots (data not shown).

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