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A family of moderately repetitive sequences in mouse DNA

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ABSTRACT

When mouse DNA is digested to completion with restriction endonuclease Eco Rl, a distinct band of 1.3 kb segments comprising about 0.5-3% of the genome is observed upon agarose gel electrophoresis. This DNA is not tandemly repeated in the genome and is not derived from mouse satellite DNA. Restriction endonuclease analysis suggested that the 1.3 kb segments are heterogeneous. Specific sequences were selected from the 1.3 kb segments and amplified by cloning in plasmid pBR322. Southern transfer experiments indicated that three separately cloned mouse DNA inserts hybridized predominantly to the Eco Rl 1.3 kb band and to the conspicuous subsegments generated by secondary restriction endonuclease cleavage of the sucrose gradient purified 1.3 kb segments. Segments were also excised by Hha I (Hha I segments) from the chimeric plasmids containing mouse DNA inserts and subjected to restriction endonuclease and cross-hybridization analysis. It was found that the three Hha I segments were different, although two of them exhibited partial sequence homology. C_ot analysis indicated that each of the Hha I segments are repeated about 10^4 times in the mouse genome. These findings indicate that a family of related but non-identical, moderately repetitive DNA sequences, rather than a single homogeneous repeat, is present in the 1.3 kb Eco Rl band.

INTRODUCTION

The presence of repetitive DNA appears to be a general feature of the genomes of all eukaryotic cells. From studies of renaturation kinetics, three major classes of DNA sequences can be recognized. For example, when denatured mouse genomic fragments reassociate, 10% of the DNA renatures very rapidly at a low C_ot value. This represents primarily mouse satellite DNA. The largest fraction of the genome, having the highest C_ot values, consists of unique DNA. The remainder, the moderately repetitive DNA sequences, renature at intermediate C_ot values and are repeated between 10^3 and 10^5 times (1,2).

Mouse satellite is composed of a short nucleotide sequence (about 240 bp) arranged in tandem arrays repeated about 10^6 times in the genome (3). These sequences are located primarily in heterochromatic regions (4,5). There has not yet been any clear indication of a biological function for this DNA.

The moderately repetitive DNA sequences appear to be organized in the genome in at least two patterns. The first arrangement (called the Xenopus pattern (6)) consists of short repeated sequences (about 300 bp) which are interspersed within the unique sequences (about 1000 bp) and is found in the sea urchin (7), Xenopus laevis (8) and the genome of the rat (9). This pattern led to the speculation that moderately repeated sequences might function in the control of eukaryotic gene expression (10). The second pattern consists of moderately repetitive DNA (about 5.6 kb) interspersed within long stretches of non-repeated sequences (greater than 1.3 kb). This pattern occurs in Drosophila melanogaster (11), but the function of these moderately repetitive sequences is unknown (12).

A moderately repetitive DNA family which is not organized in tandem repeats or in either of the two patterns mentioned above has been observed in the DNA of D. melanogaster. This DNA is organized as short ($\leq$ 1 kb), moderately repetitive sequences in a complex arrangement occuring in large clusters (12). It has been suggested that some of these moderately repetitive DNA sequences in D. melanogaster are transposable elements (12,13).

When the mouse genome is digested to completion with the restriction endonuclease Eco Rl, a distinct 1.3 kb band (comprising about 0.5-3% of the total DNA) is observed after electrophoresis on agarose gels. We refer to the DNA present in this band as the 1.3 kb segments. We will present evidence suggesting that a family of moderately repetitive DNA sequences is present in the 1.3 kb segments.

MATERIALS AND METHODS

Materials: Modifications of standard procedures (14) were used to prepare mouse DNA from the Friend murine erythroleukemia cell line (Friend cell). The cell line (DS19) originally derived from clone 745A of Dr. C. Friend and culture conditions were as previously described (15). The chimeric plasmids and pBR322 were purified from detergent lysates by centrifugation in an ethidium bromide/CsCl density gradient (16). Ethidium bromide was extracted with an equal volume of n-butanol. Radiolabelled DNA was prepared using deoxycytidine 5'-[α-^{32}P] triphosphate (Amersham) by the nick-translation procedure (17). A Hind III digest of λ DNA was used as a molecular weight marker and all restriction endonucleases used in these experiments were purchased from New England Biolabs. For complete digestion 1 µg of DNA was incubated for 3 hours at 37°C with one unit

of endonuclease. Other materials were obtained from the following sources: Escherichia coli DNA polymerase I from Boehringer Mannheim; S1 nuclease from Sigma Chemical Company; salmon testes DNA from Worthington Biochemical Company.

Isolation of repetitive DNA fragments: Mouse DNA was digested to completion with Eco R1 and purified by sedimentation through a 10-40% sucrose gradient containing 100 mM Tris, pH 8.0/100 mM NaCl/1 mM EDTA/0.01% sodium dodecyl sulfate (SDS). These sucrose gradient purified 1.3 kb segments were inserted by poly(dA)·poly(dT) tailing and annealing into plasmid pBR322 which had been cleaved once by Pst I at the ampicillin resistance gene (amp^r) (18). E. coli strain χ1776 was transformed with the annealed chimeric plasmids as described (19). Transformed colonies were screened by the method of Grunstein and Hogness (20) using nick-translated probes.

Filter hybridization: DNA fragments obtained by restriction endonuclease cleavage were analyzed on 1% agarose gels (Seakem). This DNA was transferred to nitrocellulose filters (Millipore) by the Southern procedure. Pretreatment and hybridization of filters were performed as previously described (21). Our washing conditions were as follows: low stringency was 0.1 X SSC (SSC is 0.15 M NaCl + 0.015 M Na citrate) /0.1% SDS at 45°C and high stringency was 0.05 X SSC/0.1% SDS at 65°C. All hybridizations were carried out under conditions of high stringency unless otherwise noted. Nick-translated probes employed in hybridization had specific activities between 5 and 10 x 10^6 cpm/μg. Autoradiograms were obtained by exposing the x-ray film (Kodak, XR-5) to the filter at -70°C with two intensifying screens for 4 hours. Under these conditions, restriction endonuclease fragments containing single copy genes were not detected.

RESULTS AND DISCUSSION

Characterization and cloning of the 1.3 kb segments

The 1.3 kb segments and mouse satellite DNA are not related

When mouse genomic DNA is digested to completion with restriction endonuclease Eco R1, and electrophoresed in agarose gels, a distinct band approximately 1.3 kb in length is observed over the background of ethidium-stained DNA. This band had been observed in several laboratories in mouse DNA isolated from the BALB/c strain (22), and from the A9, 3T3, S107 and Friend cell lines. Microdensitometer tracings of ethidium bromide stained gels indicate that the amount of DNA in this band is between 0.5 and 3% of the genomic DNA. We considered the possibility that this DNA

contained some repeated sequences perhaps related to mouse satellite DNA which comprises about 8-10% of the genome. Mouse satellite DNA is organized in the genome in tandem arrays as evidenced by its presence in multimeric bands in agarose gels upon digestion of the DNA by restriction endonucleases (23,25). Limited digestion of Friend cell DNA with Eco R1, as shown in Figure 1 (b-g), did not result in multimeric bands based on a monomer unit of 1.3 kb, indicating that the 1.3 kb segments were not present in the genome as tandem repeats.

We then tried to determine how uniformly the 1.3 kb segments might be distributed throughout the genome. Our approach was to look for the 1.3 kb segments in fractions of mouse main-band DNA differing in their guanine plus cytosine (G+C) contents. Preparative density gradient centrifugation in CsCl was used to obtain several different fractions whose average G+C content was determined from their buoyant density (14). Each CsCl fraction was digested to completion with Eco R1 and electrophoresed in a 1% agarose gel and stained with ethidium bromide (Figure 2). The proportion of the 1.3 kb segments was significantly lower in fractions of higher G+C content than observed in those of lower G+C content or in the unfractionated Friend cell DNA. This result initially suggested that the 1.3 kb segments might be

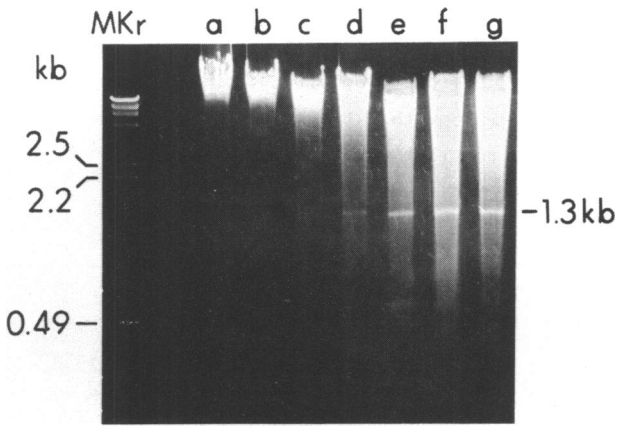

Figure 1. Agarose gel electrophoresis after limited digestion of mouse genomic DNA with Eco R1. Digestion was carried out (two separate experiments gave identical results) with decreasing ratios of DNA to endonuclease, and equal amounts (5 μg) of DNA were applied to each lane of the gel. The ratios of μg of DNA to units of endonuclease in the reaction mixture were (a) no enzyme (b) 1000 (c) 100 (d) 50 (e) 10 (f) 5 (g) 1. Each reaction mixture was incubated at 37°C for 3 hours. Hind III digested λ DNA was used as a molecular weight marker (Mkr).

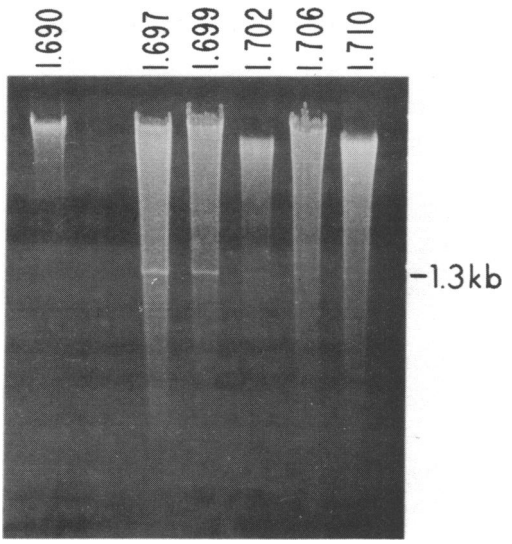

Figure 2. Agarose gel electrophoresis of Eco Rl digested fractions of mouse genomic DNA of different average G + C content. Purified mouse DNA was centrifuged to equilibrium preparatively in a CsCl gradient and fractions differing in mean buoyant densities (above each lane in g/ml) were collected. The results of analytical CsCl density gradient centrifugation indicated that the DNA of these fractions differed in their average G + C content. All but one of the DNA samples shown above had unimodal distributions in CsCl. The fraction containing main-band DNA of mean bouyant density 1.697 g/ml also contained satellite DNA (20%). None of the other main-band DNA fractions contained detectable satellite DNA. The DNA of density 1.690 g/ml is purified mouse satellite DNA. After fractionation on CsCl and Eco Rl digestion, equal amounts (3 µg) of each DNA sample were electrophoresed on a 1% agarose gel. Similar results were obtained using three different preparations of mouse DNA. Densitometer tracings of the negative were used to determine the following percentages for the integrated fluorescence in the 1.3 kb band in each lane: 1.697 = 1.6%; 1.699 = 2.6%; 1.702 = 1.2%; 1.706 = 0.5%; 1.710 = 0.8%; unfractionated mouse DNA (see Fig. 1g) = 1.6%.

derived from mouse satellite DNA which has a lower buoyant density and G+C content than the main-band DNA. To explore a possible relationship to the 1.3 kb segments, mouse satellite DNA was isolated from main-band DNA by using the fluorescent dye Hoechst 33258 which binds preferentially to AT-rich sequences; this procedure achieves further separation in preparative CsCl density gradients (24). Analytical centrifugation indicated that the purified mouse satellite DNA obtained in this manner was contaminated by less than 5% of main-band DNA. After Eco Rl digestion, mouse satellite

DNA has been observed to be in large fragments with a small percent in an ascending series of multimers based on a monomer length of 240 bp (25). When purified mouse satellite DNA was digested to completion with Eco Rl, no significant band of DNA was observed at 1.3 kb (Figure 2).

Preparation and cloning of the 1.3 kb segments

In order to isolate the 1.3 kb segments for further studies, mouse genomic DNA was digested to completion by Eco Rl and fractionated according to size by sedimentation through a 10-40% sucrose gradient. All of the fractions were assayed on an agarose gel. As shown in Figure 3 the 1.3 kb segments were present in fractions 16-18 and were most prevalent in fraction 17. These were the only fractions containing detectable amounts of the 1.3 kb segments. Fraction 16 contained additional mouse genomic fragments of higher molecular weight whereas fraction 18 contained additional lower molecular weight DNA fragments.

The DNA from fraction 17 of the sucrose gradient enriched in the 1.3 kb segments (referred to below as sucrose gradient purified 1.3 kb segments) was digested with various other restriction endonucleases. Bgl II produced two distinct segments: 0.49 kb and 0.82 kb (Figure 4). The sum of the lengths of these two segments was close to the expected value of 1.3 kb. Other restriction endonucleases such as Hae III (Figure 4) or Hind III cleaved sucrose gradient purified 1.3 kb segments into subsegments of several sizes, the sum of which exceeded 1.3 kb. This suggested the existence of heterogeneity of the 1.3 kb segments. In addition, the sucrose gradient purified 1.3 kb segments were nick-translated and used as a probe for hybridization to a Southern transfer of mouse genomic DNA that had been digested to completion by Eco Rl. The nick-translated probe hybridized not only to the 1.3 kb segments but also to Eco Rl generated DNA fragments of

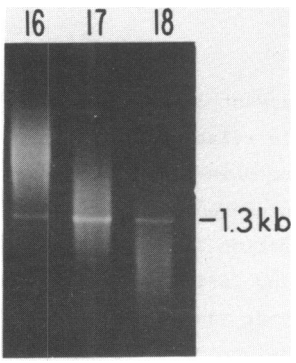

Figure 3. Agarose gel electrophoresis of Eco Rl fragments of mouse genomic DNA after fractionation by sucrose gradient centrifugation. DNA was digested to completion by Eco Rl and centrifuged through a 10-40% sucrose gradient containing 100 mM Tris (pH 8), 100 mM NaCl, 1 mM EDTA and 0.01% SDS. Thirty fractions were collected and assayed on a 1% agarose gel. In two independent experiments, only fractions 16, 17 and 18 contained significant amounts of the 1.3 kb segments.

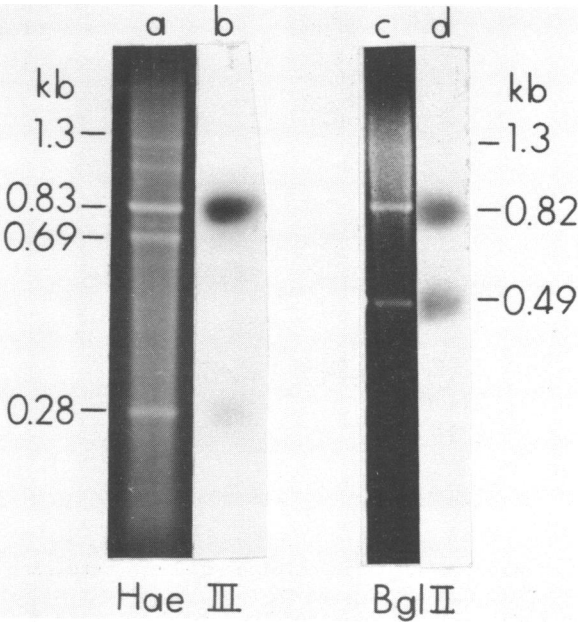

Figure 4. The relationship between the mouse DNA insert of pSM104 and the subsegments produced by restriction endonucleases from the purified 1.3 kb segments. (a, c) Restriction fragments generated from sucrose gradient purified 1.3 kb segments by Hae III and Bgl II, respectively, were electrophoresed on agarose gels and stained with ethidium bromide. (b, d) Autoradiograms resulting after nick-translated pSM104 was used as a probe for hybridization to DNA transferred from these gels to nitrocellulose filters.

many other sizes (Figure 5). This might be due to the contamination of the 1.3 kb segment probe by other DNA species of the same size. For both reasons, it was decided to make use of recombinant DNA to prepare a unique probe. The DNA sequences present in fraction 17 of the sucrose gradient were cloned using plasmid pBR322 (for details, see Methods).

The DNAs from fractions 16 and 18 of the sucrose gradient, both of which contained the 1.3 kb segments, were nick-translated individually to make radioactive probes. This procedure involving 2 probes was employed to avoid selecting repeated sequences other than those represented in the 1.3 kb segments.

The Grunstein-Hogness colony hybridization method (20) was used to obtain recombinant colonies containing 1.3 kb segments inserted into chimeric

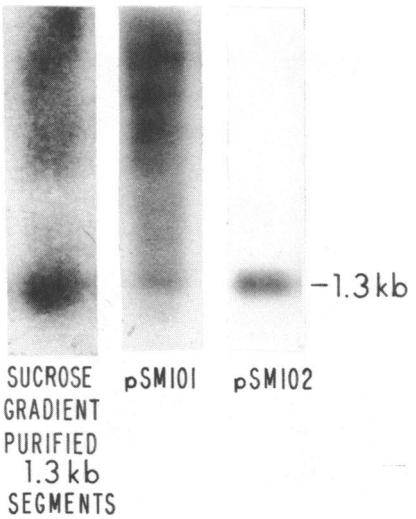

Figure 5. Autoradiogram of a nitrocellulose filter containing Eco R1 digested mouse genomic DNA hybridized to three different nick-translated probes. Mouse genomic DNA was digested to completion with the restriction endonuclease Eco R1 and identical aliquots were loaded on each lane of an agarose gel. After electrophoresis the DNA was transferred to a nitrocellulose filter by the procedure of Southern. The filter was cut into three portions so that the DNA transferred from each lane could be hybridized (three independent experiments) to a different nick-translated probe. Similar results were obtained using two different preparations of each plasmid DNA. The sucrose gradient purified 1.3 kb segments were prepared as described in Figure 3. Plasmid DNA was purified as described in Methods. The hybridization pattern using pSM103 DNA as a probe was similar to that obtained using pSM101 DNA as a probe. The pSM102, pSM104, and pSM106 DNA probes gave identical hybridization results.

plasmids. Only five transformed colonies out of eighty seven hybridized with both probes. Their efficiency of hybridization was much greater than any of the other colonies suggesting that they contained repeated sequence DNA. Next we determined that all five selected transformants, each contained a chimeric plasmid (designated pSM101, pSM102, pSM103, pSM104 and pSM106) with an insert of the size expected for the Eco R1 1.3 kb segments. This was demonstrated by isolating the plasmid DNA and subjecting it to digestion with Eco R1. Since the S-DNA inserts should not have any Eco R1 internal cleavage sites and pBR322 DNA had one Eco R1 site (Figure 6), the length of each chimeric plasmid after cleavage by Eco R1 should be at least 1.3 kb longer than the plasmid itself. This expected result was indeed obtained (Table 1).

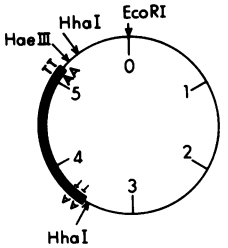

Figure 6. Schematic drawing of chimeric plasmid containing pBR322. Only the restriction sites of the pBR322 DNA (thin line) located near the inserted mouse DNA (thick line) are shown. Numbers located within the circle are distances in kb. The shortest distance between the Hha I and Hae III sites shown in pBR322 is 0.17 kb.

Analysis of mouse DNA inserts in pSM102, pSM104 and pSM106

Identification in chimeric plasmids of DNA from the 1.3 kb segments

Southern transfer experiments were used to determine the relationship between the DNA in the 1.3 kb band produced by Eco Rl and the mouse DNA inserts of the chimeric plasmids purified from the five selected transformed colonies. The restriction fragments of mouse genomic DNA generated by Eco Rl cleavage were electrophoresed and transferred to a nitrocellulose filter and the hybridization patterns obtained using different nick-translated chimeric plasmids as probes were compared. Under hybridization conditions used here, only repetitive DNA sequences should be detected on the autoradiograms. As can be seen from the autoradiograms in Figure 5,

Table 1. Size (kb) of mouse DNA inserts and associated pBR322 DNA

Plasmid	Size (kb) of Total Insert[a]	Size (kb) of Restriction Segments Generated by			
		Hha I[b]	Bgl II[c]	Hae III[c]	Hind III[c]
pSM102	1.45	1.00	0.84	0.46 0.15 0.17	1.00
pSM104	1.40	1.30	0.80 0.50	0.83 0.17	1.05 0.25
pSM106	1.40	1.20	0.62 0.61	0.51 0.26 0.17	0.79 0.42

a. Sizes were determined as described in the text after Eco Rl cleavage of the chimeric plasmids.
b. The purified chimeric plasmids were cleaved by Hha I and fractionated through a sucrose gradient to separate the inserted mouse genomic DNA segments (Hha I segments).
c. The Hha I segments were then redigested with the different restriction endonucleases listed above and the sizes of the resulting DNA fragments are shown. The smallest subsegment (0.17 kb) generated from the Hha I segment by digestion with Hae III corresponds to the smallest distance between the Hha I and Hae III sites on pBR322 shown in Figure 6.

all five chimeric plasmids possess sequences which hybridze to the 1.3 kb segments generated from mouse DNA by Eco Rl. Using the chimeric plasmids as probes, two types of hybridization patterns were observed. When nick-translated DNA of pSM102, pSM104 or pSM106 was used as a probe, the hybridization was predominantly to the DNA of the 1.3 kb band (Figure 5). On the other hand, nick-translated DNA of pSM101 or pSM103 hybridized to many genomic Eco Rl fragments in addition to those in the 1.3 kb band (Figure 5). This pattern was similar to that observed using the sucrose gradient purified 1.3 kb segments as a probe (Figure 5). Thus, pSM101 and pSM103 may contain DNA species derived from the 1.3 kb segments that also show homology to many other genomic DNA fragments produced by Eco Rl. Additional characterization will be required in order to determine the exact relationship between the mouse DNA inserts in pSM101, pSM103 and the DNA in the 1.3 kb band produced by Eco Rl. The present study concentrates on the mouse DNA inserts of pSM102, pSM104 and pSM106.

Two additional types of evidence indicate that the mouse DNA inserts in pSM102, pSM104 and pSM106 originate from the Eco Rl 1.3 kb band. The first type of evidence is from analysis using restriction endonucleases Hae III, Hind III or Bgl II. After complete digestion of the sucrose gradient purified 1.3 kb segments, the resulting fragments were electrophoresed on agarose gels, transferred to nitrocellulose filters by the Southern procedure and hybridized to each of the nick-translated probes prepared from pSM102, pSM104 and pSM106 DNA. The autoradiographic patterns obtained were very similar to the patterns observed after staining the agarose gel with ethidium bromide. The autoradiograms did not reveal extensive homology between these plasmid DNA probes and any DNA fragments other than those in the most prominent bands visible in the gels described below. An example is given in Figure 4 which shows the sucrose gradient purified 1.3 kb segments after digestion by Bgl II followed by electrophoresis and staining with ethidium bromide. Two conspicuous DNA bands are observed (Figure 4c) and when pSM104 DNA was used as a probe, hybridization was almost exclusively to the DNA located in these two bands (Figure 4d). These results indicate that pSM104 contained mouse DNA inserts that originated from the 1.3 kb segments. The absence of homology to the DNA of one (0.69 kb) of the Hae III bands was observed (Figure 4b). This was in contrast to the results obtained when the sucrose gradient purified 1.3 kb segments were nick-translated and used as a probe. In this instance, hybridization was to all of the prominent bands. These results provide

further evidence that the DNA in the 1.3 kb band is heterogeneous.

A second type of evidence providing further indication that the mouse DNA inserts were derived from the 1.3 kb segments is provided by the association of these sequences with DNA of low G+C content. DNA fractions of different buoyant density were cleaved with Eco Rl and electrophoresed as shown in Figure 2. After transfer by the Southern technique, hybridization (using nick-translated pSM104 DNA as a probe) was predominantly to the 1.3 kb DNA present in the DNA fractions of lower G+C content.

The availability of chimeric plasmids containing unique mouse DNA inserts made it possible to repeat some of the studies described above using purified homogeneous components of the 1.3 kb segments. Further evidence that mouse satellite DNA and the 1.3 kb segments are not homologous was provided by the absence of cross-hybridization under conditions of low stringency between a mouse satellite DNA probe and mouse DNA inserts derived from pSM102, pSM104 or pSM106. Additional data indicating that the 1.3 kb segments are not tandemly repeated in the genome was provided by the following experiment. Mouse DNA that had been partially digested with Eco Rl was electrophoresed as described in Figure 1 and transferred to nitrocellulose filters. After hybridization with nick-translated DNA of pSM102, pSM104 or pSM106 as a probe, no multimeric bands were observed.

Restriction endonuclease analysis of three chimeric plasmids

Since the DNA in the 1.3 kb band was found to be heterogeneous, the next question considered was the relatedness among the mouse DNA inserts in pSM102, pSM104 and pSM106. Restriction endonuclease analysis was employed to identify specific cleavage sites for each of the three chimeric plasmids. First it was found that Hha I could excise a large mouse genomic DNA segment carrying relatively few pBR322 sequences and also cleave the rest of the plasmid vector into small fragments (26). This facilitated the separation of the mouse genomic DNA segments from plasmid DNA by sucrose gradient centrifugation. The mouse genomic segments from pSM102, pSM104 or pSM106 will be referred to in the text as Hha I segments 2, 4 or 6. Digestion of the pSM102, pSM104 and pSM106 DNA with Hha I produced segments which were a little less than 1.3 kb in size (Table 1) indicating that at least one of the Hha I sites was within the segment of mouse genomic DNA originally inserted into the plasmid. The Hha I site at the opposite end of the mouse DNA inserts appears to be located within the plasmid DNA.

This suggestion is supported by the appearance of a 0.17 kb segment after digestion of these Hha I segments with Hae III. The 0.17 kb segment is of the size expected after cleavage between the known Hha I and Hae III sites on the pBR322 (Figure 6).

The results of the restriction endonuclease analysis of the mouse DNA inserts and the associated pBR322 DNA from three chimeric plasmids are summarized in Table 1. Hae III, Hind III and Bgl II generated from each of the three Hha I segments different sets of subsegments of various sizes. In addition, a cross-hybridization experiment was carried out to explore sequence homologies among the Hha I segments of three chimeric plasmids. Under conditions of high stringency the three Hha I segments did not cross-hybridize. These findings indicated that Hha I segments 2, 4 and 6 did not contain extensive sequence homologies.

Homologies among the three Hha I segments

Hha I segments 2, 4 and 6 (derived from pSM102, pSM104 and pSM106, respectively) hybridized predominantly to the Eco Rl 1.3 kb band, but did not hybridize to each other under conditions of high stringency. The Southern transfer technique was applied to determine whether hybridization under less stringent conditions would allow the detection of partial homologies within the Hha I segments.

First, we had to consider that under conditions of low stringency, some pBR322 DNA sequences at the ends of the Hha I segments could provide sequence homology that would make it appear as though there were some relatedness among the mouse DNA inserts. In order to eliminate this possibility, each chimeric plasmid was double digested with Hha I and Hae III to generate one major segment (referred to below as the major Hae III segment) which was larger than 450 bp in size (see Table 1), as well as some minor segments (Figure 7a). Under these conditions, pBR322 DNA sequences were detected in the minor segments (Figure 7b). There was, however, some cross-hybridization between the major Hae III segment 6 and the nick-translated pBR322 probe (Figure 7b). This was probably due to the presence of linked plasmid sequences since additional studies revealed no cross-homologies between pBR322 DNA and mouse DNA. The major Hae III segments 2 and 4 did not appear to have any detectable homologies to pBR322.

Thus, to avoid possible complications caused by the presence of pBR322 DNA sequences, only the major Hae III segments 2 and 4 were examined in cross-hybridization experiments to detect partial homologies. The filter shown in Figure 7b was cut so that only the major Hae III segments 2 and

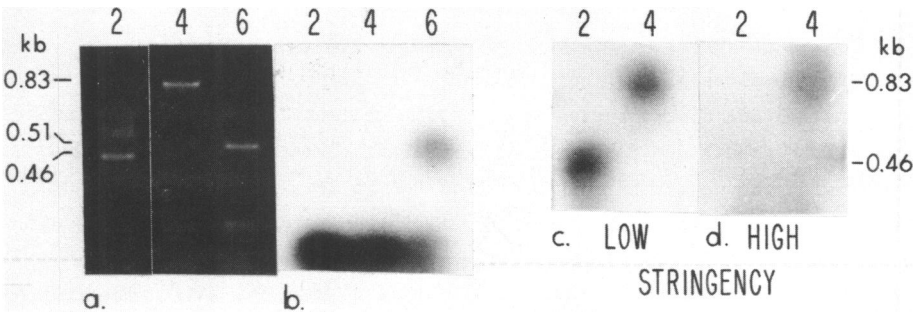

Figure 7. Identification of partial homologies between Hha I segments 2, 4 and 6 (derived from pSM102, pSM104 and pSM106) by cross hybridization under conditions of low and high stringency. (a) pSM102, pSM104 and pSM106 were double digested by restriction endonucleases Hha I and Hae III, electrophoresed on a 1% agarose gel and then stained with ethidium bromide. (b) DNAs from (a) were transferred to a nitrocellulose filter by the Southern transfer procedure and hybridized using nick-translated pBR322 as a probe. Hybridization was carried out in 50% formamide and 5 X SSC at 37°C for 12-16 hours. The autoradiogram was prepared after washing the filter under conditions of low stringency (0.1 X SSC at 45°C). The autoradiogram (not shown) prepared after washing the filter under conditions of high stringency (0.05 X SSC at 65°C) was identical. (c) The upper left portion (containing DNA from the major Hae III segments 2 and 4 but not the pBR322 DNA) was cut out of the filter whose autoradiogram is shown in (b). Nick-translated DNA from Hha I segment 4 was hybridized to this filter under the conditions described in (b) and the filter was washed under conditions of low stringency and autoradiographed. (d) Autoradiogram of the filter described in (c) after rewashing under conditions of high stringency. The absence of hybridization to lane 2 was not due to extensive loss of DNA from this filter since a strong band was subsequently observed when Hha I segment 2 was used as a radioactive probe.

4 were present. When nick-translated Hha I segment 4 was used as a probe (in two separate experiments) under washing conditions of low stringency, some homology to the major Hae III segment 2 was observed (Figure 7c). These sequences were only partially homologous, since hybridization was eliminated by washing under conditions of high stringency (Figure 7d). Thus, although Hha I segments 2, 4 and 6 do not contain identical DNA sequences, there appears to be partial homology between the regions of Hha I segments 2 and 4 described above.

Kinetics of reassociation of the three Hha I segments

Analysis of rates of reassociation (C_0t analysis) was used to estimate the number of copies in the mouse genome of some of the cloned DNA derived from the 1.3 kb Eco R1 band. Mouse DNA was sheared to an average length of 700-800 bp, denatured and allowed to anneal in the presence of each of

the three nick-translated Hha I segments. Figure 8 shows the percent renaturation (determined from the susceptibility of the radioactive DNA probe to S1 nuclease) as a function of the logarithm of the product of the total initial DNA concentration and the time of incubation at 60°C. From these curves, the time ($t_{\frac{1}{2}}$) required for 50% renaturation of the probe was determined. The number of copies of this DNA in the genome was determined from $C_o t_{\frac{1}{2}}$ and the linear relationship between the rate of reassociation and DNA complexity (27). To determine, for our conditions, the exact proportionality constant which depends on factors such as the salt concentration and size of fragments, purified mouse satellite DNA was used as a standard (Figure 8). Using this proportionality constant and the size of each Hha I segment for the complexity, the initial concentration of each Hha I segment present in the non-radioactive driver DNA in the reaction mixture was determined. From the ratio between the total DNA concentration in the

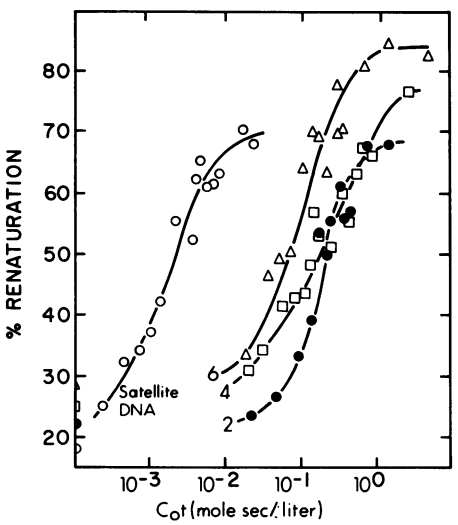

Figure 8. $C_o t$ analysis of the three Hha I segments and mouse satellite DNA. Each Hha I DNA segment (2, 4 or 6) was nick-translated and added to unlabelled sheared (700-800 bp) mouse genomic DNA. After heat denaturation, the DNAs were allowed to reassociate at 60°C (0.2 M NaCl, 10 mM PIPES, pH 6.8). The percent renaturation was determined by susceptibility to S1 nuclease. Similar results were obtained in two separate experiments. In a control experiment in which the unlabelled sheared mouse genomic DNA was omitted, no increase in the percent of renaturation of the probe (Hha I segment 4) was observed. C_o refers to the total concentration of unlabeled DNA.

Table 2. Reiteration frequencies of the three Hha I segments

DNA	$C_o t_{\frac{1}{2}}$*	% of genome	Number of copies per haploid genome
Hha I Segment 2	1.7×10^{-1}	0.39%	9.0×10^{3}
Hha I Segment 4	1.8×10^{-1}	0.48%	8.5×10^{3}
Hha I Segment 6	1.5×10^{-1}	0.53%	1.0×10^{4}

*The incubation was carried out at 60°C with $[Na^+]$ = 0.2M. This is the average of two determinations, one of which is shown in Figure 8.

reaction mixture and the initial concentrations calculated for each Hha I segment studied, the number of copies in the genome was determined (Table 2).

The data in Table 2 indicate that the DNA of each Hha I segment that has been cloned from the sucrose gradient purified 1.3 kb segments is represented in the genome in a large number of copies (about 10,000 per haploid genome). This is comparable to the proportion in the total genome of the DNA in the 1.3 kb Eco R1 band estimated from the intensity of this band observed in agarose gels by ethidium bromide staining. Hha I segments 2, 4 and 6 possess reiteration frequencies indicating that they are derived from a family of moderately repetitive DNA sequences in the mouse genome. As demonstrated above, these Hha I segments are homologous to the DNA in the Eco R1 band. These findings indicate that the 1.3 kb segments may represent a family of related but non-identical, moderately repetitive DNA sequences rather than a single homogeneous repeat.

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