

Comparative analysis of mouse chromosomal DNA digestion with restriction endonucleases in vitro and in silico

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Theoretical diagrams of mouse chromosomal DNA cleavage at 18 nucleotide sequences 4-6 bp in length, which are the recognition sites of restriction endonucleases, have been plotted based on earlier proposed method of mammalian genomes restriction analysis in silico [2]. A set of mouse LINE1 repeats from the data base has been analysed and diagrams of these repeats cleavage at the same nucleotide sequences have been constructed. In general, the diagrams of mouse chromosomal DNA digestion correspond to diagrams of LINE1 repeats cleavage. Mouse DNA hydrolysis with corresponding restriction endonucleases has been performed. A comparison of mouse DNA cleavage patterns and the computed diagrams has revealed a good correspondence between the experimental and theoretical data. Hydrolysis of mouse DNA preparation and subsequent gel-electrophoresis allows visualizing only LINE1 repeats and satellite DNA cleavage products. Additionally, experiments on mouse chromosomal DNA cleavage with novel methyl-dependent site-specific DNA endonucleases BslI, Glal and Glul have been conducted for the first time.

At the present time, a primary structure of the euchromatinic part of the mouse genome is determined for more than 96% [1] and this allows to analyze DNA structure *in silico*. In our earlier studies, we have proposed a method of mammalian DNA restriction analysis *in silico* [2]. The method includes a construction of the distribution diagrams of DNA fragments, which are produced in the course of chromosomal DNA cleavage at recognition sites of restriction endonucleases (REs). Distribution diagrams have been plotted for digestion of rat, mouse and human chromosomal DNAs at nucleotide sequences 5'-CCWGG-3', 5'-GATC-3', 5'-GGCC-3' and 5'-CCGG-3', which are recognition sites of restriction endonucleases Bst2UI, Kzo9I, HaeIII and MspI respectively. At the same time, experiments have been conducted on hydrolysis of the same chromosomal DNAs with these restriction endonucleases. A comparison of theoretical calculations and experimental results has been done. In our subsequent works we have carried out theoretical and experimental cleavage of rat [3] and human [4] genomic DNAs and found good correspondence of results obtained for *in vitro* and *in silico* studies in both cases.

The purpose of this work is 1) to plot the fragments distribution diagrams for mouse genomic DNA cleavage at 18 recognition sites (4, 5 and 6 bp in length), 2) to perform DNA hydrolysis with corresponding restriction endonucleases and 3) to compare computational data with experimental results.

MATERIALS AND METHODS

Mouse chromosomal DNA. In this study we used male A/He mice aged 5-6 months (Breeding Laboratory of Experimental Animals, Institute of Cytology and Genetics, Novosibirsk). Genomic DNA from the animal liver was isolated as described previously [3].

Hydrolysis of chromosomal DNA. The following restriction endonucleases manufactured by SibEnzyme Ltd. were used in the work (the recognition site of each respective restriction enzyme is given in the brackets):

AclI (5'-RAATTY-3'), AspA2I (5'-CCTAGG-3'), AspS9I (5'-GGNCC-3'), BglII (5'-AGATCT-3'), BlnI (5'-GmCNGmC-3'), Bme18I (5'-GGWCC-3'), Bse3DI (5'-GCAATG-3' and 5'-CATTGC-3'), Bsp19I (5'-CCATGG-3'), Bst2UI (5'-CCWGG-3'), BstFNI (5'-CGCG-3'), BstSCI (5'-CCNGG-3'), BstX2I (5'-RGATCY-3'), EcoRI (5'-GAATTC-3'), EcoRV (5'-GATATC-3'), Fsp4HI (5'-GCNGC-3'), Glal (5'-GmCGmC-3'), GluI (5'-GmCNGmC-3'), HpaII (5'-CCGG-3'), HspAI (5'-GCGC-3'), MspI (5'-CCGG-3'), PspN4I (5'-GGNNCC-3'), RsaI (5'-GTAC-3'), Sse9I (5'-AATT-3'), SspI (5'-AATATT-3') and TaqI (5'-TCGA-3').

Hydrolysis reactions were performed in 40 µl of the reaction mixture containing 6 µg of DNA, SE-buffers recommended by the manufacturer and 3 µl of restriction enzyme at optimal temperatures for 3 h.

Electrophoresis. Electrophoresis in 8% polyacrylamide gel was used to separate DNA fragments from 40 to 500 bp (6 µg of hydrolyzed DNA was applied on each lane of gel). Electrophoresis in 1.5% of low melting point agarose ("Sigma", USA) was used to separate DNA fragments in the range of 200-2000 bp. Electrophoresis in 1% agarose "Type I-A, Low EEO" ("Sigma", USA) was used to separate DNA fragments in the range of 500-20000 bp. 3 µg of hydrolyzed DNA was applied on each lane of agarose gel. Pulsed field electrophoresis in 1% agarose was used to separate DNA fragments in the range of 10-50 Kbp and 1 µg of hydrolyzed DNA was applied on each lane of agarose gel. Voltage of the direct impulses was 6 v/cm, impulses duration was 1,5 sec. Voltage of the reverse impulses was 6 v/cm. Duration of reverse impulses was changed in the sequence 0.7; 0.6; 0.5; 0.4; 0.3; 0.2; 0.1 sec. Tris-acetate buffer was used for electrophoresis in all the cases. After electrophoresis DNA bands were stained with ethidium bromide and photographed in the UV light.

The following DNA preparations were used as DNA fragment length markers: 50 kb DNA ladder, 1 kb DNA ladder and pUC19/MspI DNA ladder (SibEnzyme Ltd., Russia).

Nucleotide sequence of the mouse genome. Mouse genomic DNA sequence was obtained from <ftp://ftp.ensembl.org/pub/> (version as of June 2, 2006).

Nucleotide sequence of the major fragment of mouse monomeric γ-satellite DNA was acquired from the article [6].

The sequences of LINE1 repeats were obtained from the genomic mouse DNA database using Table Browser service on the site (<http://genome.ucsc.edu/cgi-bin/hgTables>) [8]. The studied LINE1 repeats included 854172 sequences with a total length of ~510 million bp.

Software. Theoretical diagrams of the relationship of total fragment mass to their length (in bp) were constructed by the method described in [2].

RESULTS AND DISCUSSION

Analysis of DNA fragments distribution diagrams

In our previous work, we have constructed diagrams of mouse genomic DNA fragments distribution after cleavage at the recognition sites of the following restriction enzymes: HaeIII (recognition site GGCC), MspI (CCGG), Kzo9I (GATC) and Bst2UI (CCWGG). A good correlation has been observed between the calculated data and the experimental results on DNA hydrolysis with these enzymes [2].

In the present work we have constructed similar theoretical diagrams of distribution of mouse chromosomal DNA fragments after DNA digestion at a wider range of recognition sites, including 6-nucleotide sequences. The distribution diagrams have been constructed in arctangent scale, which imitates electrophoresis separation of the DNA fragments in agarose gel. Only the diagrams with peak values of ≥ 5.5 million bp have been selected. As was demonstrated in our previous work, this is the threshold value necessary for visualization of corresponding bands in the analysis of DNA cleavage products [2]. At the same time the background spot, which is formed by cleavage of the main non-repetitive DNA, can conceal the peaks on the electrophoresis lanes in some digestion patterns [2].

We have analyzed DNA cleavage at recognition sites of 18 REs. Figure 1 shows the calculated diagrams of mouse genomic DNA fragments distribution, which have been plotted for these recognition sequences, and provides a pattern of DNA hydrolysis with corresponding RE.



Fig. 1. Comparison of DNA digestion pattern and calculated distribution diagrams.

Electrophoresis of DNA digestion products with restriction enzymes AspA2I (recognition site 5'-CCTAGG-3'), BstV2I (5'-GAAGAC-3'), BglII (5'-AGATCT-3'), Bse3DI (5'-GCAATG-3'), Bsp19I (5'-CCATGG-3'), EcoRI (5'-GAATTC-3'), EcoRV (5'-GATATC-3'), NdeI (5'-CATATG-3'), PstI (5'-CTGCAG-3') in 1% agarose, for PspN4I (5'-GGNNCC-3'), SspI (5'-AATATT-3'), AspS9I (5'-GGNCC-3'), Bme18I (5'-GGWCC-3'), BstSCI (5'-CCNGG-3'), BstDEI (5'-CTNAG-3'), BstX2I (5'-RGATCY-3'), Fsp4HI (5'-GCNGC-3') has been performed in 1,5% agarose and with RsaI (5'-GTAC-3') - in 8 % PAAG. The lengths of peak's DNA fragments are indicated (in bp).

“sat” – products of satellite DNA cleavage. “M” – SE 1 kb DNA fragment length marker was used for agarose gel-electrophoresis (fragment lengths: 10000, 8000, 6000, 5000, 4000, 2x3000, 2500, 2000,

1500, 2x1000, 750, 500 and 250 bp); pUC18/MspI DNA ladder was used for PAAG (fragment lengths: 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 2x34 bp).

The highest peaks (more than 10 million bp) have been observed after *in silico* DNA cleavage at recognition sites for the following restriction enzymes: Bse3DI (1059 and 2590 bp fragments), Bst2UI and BstSCI (1826 bp fragment), Fsp4HI (695 and 1613 bp fragments), PctI (2859 bp fragment) and EcoRI (1373 bp fragment). Visualization of the latter fragment was described in the literature earlier [7]. The DNA bands, which correspond to these fragments, are clearly visible on electrophoregrams (Fig.1). The DNA fragments with weaker intensity, as seen on Figure 1, are also represented as bands on the gel photographs for DNA hydrolysis with enzymes: AspA2I (1273, 3343-3344 and 4613 bp), BglII (876 bp), Bsp19I (3253 bp), EcoRV (1883 bp), Fsp4HI (583 and 1035) and PspN4I (444, 508, 716 and 1721 bp).

Bme18I DNA hydrolysis produces a visible 1619 bp fragment, as well as 809 and 818 bp fragments. In addition, we can observe a band, which is most likely formed as a result of a merge of 708 bp fragment with ~705 bp fragment of γ -satellite DNA [6].

Some electrophoregrams have significantly large areas of high intensity background spot, which obscures DNA fragments that correspond to the calculated peaks. In particular, after DNA hydrolysis with enzymes Bst2UI and BstSCI, no bands are visible, except those corresponding to fragments 1511 and 1826 bp in length. DNA cleavage with BstX2I enzyme produces visible 203, 397, 508, 514 and 713 bp DNA fragments, however, the predicted 1856 bp long fragment is not visible on the gel, as it is probably obscured by the background "spot". A similar phenomenon is observed in the case of RsaI. No bands, which correspond to peak fragments greater than 503 bp in length, are seen on electrophoregram.

In our earlier work [3], we have demonstrated that the clustering of nearby DNA fragments allows in some cases to visualize peak fragments that are less than 5.5 million bp. The electrophoregram of DNA hydrolysis with enzyme SspI (Fig.1) demonstrates that there is a visible band corresponding to 1037 bp DNA fragment. However, on the corresponding diagram this fragment has a height of only 3.7 million bp, which is less than the aforementioned threshold value necessary for peak observation. We believe that this peak visualization on the electrophoregram may be attributed to a presence of several DNA fragments of near same length, which can be seen on detailed distribution diagram in Fig. 2. Although each of the smaller fragments has significantly lower intensity, their cumulative presence on the gel picture leads to single band visualization.

Fig. 2. Distribution diagrams of mouse genomic DNA fragments obtained at SspI recognition site (5'-AATATT-3') cleavage. Axis X – DNA fragment length (bp); axis Y – height of DNA fragment peak (bp).

As can be seen in Fig. 1, in the case of some REs (AspS9I, Bme18I, Bst2UI, BstSCI and SspI) there are clearly visible bands on electrophoregrams, which do not correspond to any peak fragments in the diagrams. These bands are likely the products of hydrolysis of mouse γ -satellite DNA [6], primary sequence of which is not provided in the genomic DNA [1]. The brightness of these bands is significantly higher than those, which result from genomic DNA cleavage. This is due to a high proportion of satellite DNA, which is about 10%, in the preparation of total mouse DNA [6]. A percentage of satellite DNA in total rats and human DNA is significantly less [3], [4].

Fig. 3. Patterns of mouse DNA cleavage with restriction endonucleases. Electrophoresis in 1.5% agarose, TAE buffer. “M” – DNA fragment length marker SE 1 kb DNA ladder.

The hydrolysis of mouse DNA with enzymes AspS9I, Bme18I, BstSCI and Bst2UI results in formation of the band, electrophoretic mobility of which corresponds to 234 bp DNA fragment of the so-called main fragment of the mouse γ -satellite DNA [6] (Fig. 3). Recognition sites of these enzymes are located in the nucleotide sequence of the monomeric fragment of satellite DNA in positions 1, 1, 4 and 4, respectively, and hydrolysis of the tandem satellite DNA repeats at these positions leads to the formation of 234 bp fragment [6]. A fragment of the same length is also formed after cleavage of the mouse γ -satellite DNA with enzymes BstF5I (5'-GGATG-3', position 166), FatI (5'-CATG-3', position 150), Sse9I (5'-AATT - 3', position 98), AclI (5'-RAATTY-3', position 97). Additionally, as it was described in the literature [6], the bigger fragments are formed (468 bp, 702 bp), comprising of two or more monomeric fragments. Electrophoregram on Fig. 3 shows such dimers, trimers and even tetramers of the major satellite DNA fragment in almost all lanes. Evidently, the presence of multimeric satellite DNA fragments is a consequence of high variability of satellite DNA structure and changes of

nucleotide sequence in the recognition sites of REs. As a result, enzymatic hydrolysis does not occur in each DNA repeat at the specified positions and consequently dimers, trimers, etc. are formed. The number of multimeric fragments varies, most likely depending on the size of the enzyme recognition sequence. In particular, AclI recognition site is an expanded site of Sse9I RE. As a result, after DNA hydrolysis with AclI, the observed number of trimers and tetramers is greater and number of monomers is fewer, than in course of DNA cleavage with enzyme Sse9I. On the electrophorogram of DNA cleavage with enzyme SspI, which recognizes 6 bp nucleotide sequence, fragments that correspond to dimer (468 bp), trimer (702 bp), tetramer (936 bp) and even pentamer (1170 bp) are observed, however, the band corresponding to the major monomeric fragment of γ -satellite DNA is practically not visible.

Thus, DNA fragments, which correspond to the bright bands on the electrophoregrams, are the products of mouse γ -satellite DNA cleavage.

The cleavage of euchromatinic mouse DNA with restriction endonucleases produces fragments with weaker intensity, but with much more diversification in size due to the presence of interspersed repeated DNA. Short and long interspersed repeats constitute ~39% of the mouse genome [10]. Most of mouse repeats are long interspersed repetitive elements belonging to the LINE class, mainly to LINE1 (L1) family [1], which has been observed in most mammals [9]. LINE1 repeats, which have been studied in this work, include more than 854 thousand sequences with a total length of over 500 million bp. This represents approximately 1/5 of the complete mouse genome [1]. Full-length LINE1 repeats are 6-7 kb in length; however the majority of L1 repeats in genome are shortened versions [11] and only 5713 of all L1 sequences are more than 6 kb in length. The relatively big length of LINE1 repeats causes a significant variability in the size of DNA digestion products.

Short interspersed repeated DNAs (SINEs) occupy a much smaller part of mouse genome, than do L1 repeats [1]. Since in our experiments we haven't observed the bands, which correspond to the products of SINE hydrolysis, SINE digestion is not discussed in this work.

We have compared the above described distribution diagrams of genomic DNA fragments (see Figure 1) and diagrams of L1 repeats digestion, which have been constructed (see "Materials and Methods"). Fig. 4 shows both types of diagrams for the recognition sites of enzymes BglII and AspS9I. As can be seen from this figure, all peak fragments on the genomic DNA cleavage diagrams are also present on the diagrams of L1 repeats digestion.

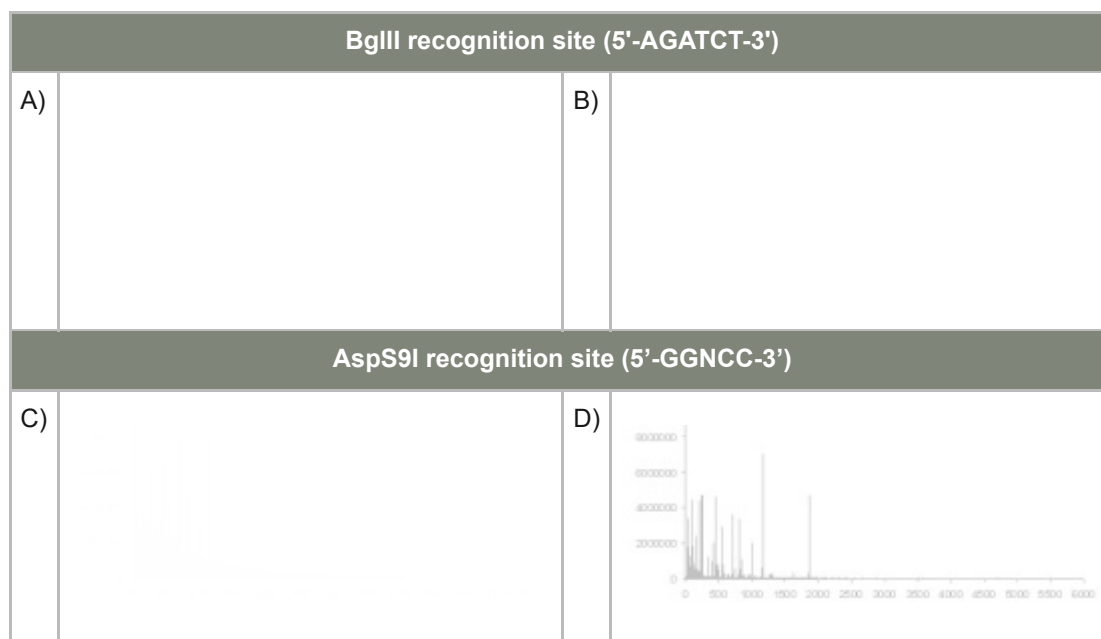


Fig. 4. Comparison of calculated distribution diagrams for genomic DNA (A, C) and LINE1 repeats (B, D).

Axis X – DNA fragment length (bp); axis Y – height of peaks, obtained at site specific cleavage of total mouse (A, C) and LINE1 (B, D) DNA (bp).

In most cases, DNA fragment peak heights, which have been calculated for genomic DNA, do not differ considerably from those observed on the diagrams for cleavage of L1 repeats (taking into account the background values, which are the result of the rest genomic DNA cleavage). For example, according to the data in Figure 4, a height of peak of 876 bp fragment, which is formed in the course of DNA digestion at the recognition site of enzyme BglII, is almost identical on both diagrams.

However, in some cases, the calculated peak heights for L1 repeats are significantly lower than that for the same fragments in diagrams of genomic DNA digestion. For example, in a diagram of DNA cleavage at site GGNCC (recognized by enzyme AspS9I) 708 bp fragment has a height of 8.0 million bp for genomic DNA and only 3.6 million bp for L1 repeats (Fig. 4). This difference may be due to incompleteness of mouse L1 repeats data base.

The influence of methylation on chromosomal DNA hydrolysis with site-specific endonucleases

Mammalian DNA hydrolysis with some restriction endonucleases is blocked due to methylation of CG dinucleotide. Approximately 70-80% of all CG pairs in DNA of somatic cells are methylated [12] and DNA hydrolysis with restriction endonucleases, which contain CG sequence in their recognition sites, can be restricted. In addition, as was demonstrated for mouse and other mammalian genomes, proportion of CG-dinucleotides in these DNAs is about 5 times lower than that of CC, GG and GC dinucleotides [13,14]. This lower portion of CG-dinucleotides also decreases a number of RE recognition sites with CG dinucleotides [3].

Fig. 5 demonstrates results of mouse chromosomal DNA digestion with restriction endonucleases that contain CG dinucleotide in their recognition sites. The figure shows electrophoregrams of DNA hydrolysis products separation in 1% agarose gel using constant and variable (pulse electrophoresis) voltages, Fig.5A and 5B, respectively.

As shown on Fig. 5A, HpaII does not significantly cleave DNA, while MspI, which has the same recognition site CCGG, but not sensitive to the CG methylation), cleaves mouse DNA much more

effectively. Similarly, CG dinucleotide methylation in genomic DNA may explain weak hydrolysis with enzymes BstFNI (CGCG) and HspAI (GCGC).

Pulse field gel electrophoresis allows to separate DNA fragments of up to 50,000 bp long [5]. With this method, the products of mouse chromosomal DNA hydrolysis with HpaII and HspAI are much better visualized, as can be seen on Figure 5B. The majority of DNA fragments obtained by the cleavage with the aforementioned REs are 10 to 50 kb long.

Fig. 5. Patterns of mouse DNA cleavage with restriction endonucleases, which contain CG dinucleotide in recognition site: BstFNI (5'-CGCG-3'), HspAI (5'-GCGC-3'), HpaII (5'-CCGG-3'), MspI (5'-CCGG-3'), TaqI and methyl-specific endonucleases BlnI, GluI and GluII).

A – a standard electrophoresis of DNA fragments; B - pulsed field electrophoresis of DNA fragments. I - non-treated DNA; M and M1 – SE 1 kb DNA fragment length marker.

Chromosomal DNA cleavage with BstFNI, even in the case of pulse field electrophoresis, doesn't produce a visible cleavage pattern. Apparently, this is caused by the presence of two CG dinucleotides in the recognition site of BstFNI. This results in a much lower occurrence of BstFNI site, unlike that of enzymes HpaII and HspAI, since their recognition sites contain only one CG dinucleotides. In addition, the presence of two CG dinucleotides also means that the fraction of BstFNI recognition sites, which are modified *in vivo*, is likely higher than that of HpaII and HspAI recognition sites.

In recent years, a new tool has been developed for analysis of DNA methylation status - site-specific methyl-dependent endonucleases that only cleave DNA when their recognition sites contain 5-methylcytosine [15], [16], [17], [18]. GluII is an example of these endonucleases; it effectively cleaves a number of different 4 bp DNA sequences, which contain 2-4 5-methylcytosines [16], [19]. BlnI [17] and GluI [18], other enzymes that belong to this group, effectively cleave DNA sequence GCNGC that contains 2-4 5-methylcytosines and only 4 5-methylcytosines, respectively. The results of mouse DNA cleavage with these three enzymes are given in Fig. 5.

Fig. 5A shows that an effective chromosomal DNA hydrolysis is observed only for GluII and that a pattern of hydrolysis is nearly the same as in the cases of MspI and TaqI enzymes.

BlnI and GluI hydrolysis is not visible on Fig.5A because of low frequency of these enzymes methylated recognition sites occurrence within chromosomal DNA. However, with pulsed field electrophoresis (Fig. 5 B) we can observe that BlnI, unlike GluI, only slightly hydrolyses chromosomal DNA producing fragments of high length.

BslI can cleave methylated sequences CGCGGC or GCCGCG, which are two variants of this enzyme's recognition site expanded by one nucleotide (the core recognition site is underlined), since in these cases there are two methylated cytosines in the recognition site. GluI cleaves sequence GCNGC with four methylated cytosines only. Thus, DNA hydrolysis of these methylated sequences with GluI is impossible because of lack of four 5-methylcytosines in the recognition sites.

CONCLUSIONS

in silico restriction analysis of mouse DNA has been performed for a wide range of nucleotide sequences, and experimental patterns of DNA cleavage with the corresponding restriction endonucleases have also been obtained. We have demonstrated that experimentally observed DNA bands are the products of LINE1 repeats and mouse γ -satellite DNA cleavage. More than fifty peak fragments (or clusters of fragments) have been found theoretically for the cleavage of mouse genomic DNA at 18 nucleotide sequences, which are the recognition sites of restriction endonucleases. It has been found that theoretically calculated diagrams of mouse genomic DNA cleavage in general correspond to diagrams of LINE1 repeats cleavage, however peak height difference for several fragments has been observed between aforementioned types of diagrams. The comparison of calculated DNA fragments distribution diagrams and DNA hydrolysis patterns for the corresponding restriction enzymes shows a good match of theoretical and experimental data.

Electrophoretical analysis of products of mouse chromosomal DNA cleavage with methyl-dependent endonucleases has shown that DNA cleavage is significant for enzyme GluI; considerably weaker with endonuclease BslI and is virtually absent with enzyme GluI.

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