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Preferential Cleavage Sites for *Sau3A* Restriction Endonuclease in Human Ribosomal DNA

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Previous studies of cloned ribosomal DNA (rDNA) variants isolated from the cosmid library of human chromosome 13 have revealed some disproportion in representativity of different rDNA regions (N. S. Kupriyanova, K. K. Netchvolodov, P. M. Kirilenko, B. I. Kapanadze, N. K. Yankovsky, and A. P. Ryskov, *Mol. Biol.* 30, 51–60, 1996). Here we show nonrandom cleavage of human rDNA with *Sau3A* or its isoshizomer *Mbo*I under mild hydrolysis conditions. The hypersensitive cleavage sites were found to be located in the ribosomal intergenic spacer (rIGS), especially in the regions of about 5–5.5 and 11 kb upstream of the rRNA transcription start point. This finding is based on sequencing mapping of the rDNA insert ends in randomly selected cosmid clones of human chromosome 13 and on the data of digestion kinetics of cloned and noncloned human genomic rDNA with *Sau3A* and *Mbo*I. The results show that a methylation status and superhelicity state of the rIGS have no effect on cleavage site sensitivity. It is interesting that all primary cleavage sites are adjacent to or entering into *Alu* or Ψ cdc 27 retroposons of the rIGS suggesting a possible role of neighboring sequences in nuclease accessibility. The results explain nonequal representation of rDNA sequences in the human genomic DNA library used for this study.

The DNA secondary structure is not monotonous one and reflects peculiarities of the primary structure. DNA unwinding elements (DUEs), bent DNA, nuclear matrix or scaffold associated regions (MARs/SARs), polypurine–polypyrimidine tracts, hypervariable mini- and microsatellite sequences (ms and mcs) represent an usual accompaniment of hot spots of homologous recombination (2–4) and regions promoting transcription and replication processes (3, 5–9). The DNA sequences of these regions are partly shared and thus reveal common structural features. Some areas of eukaryotic genomes are extremely enriched in such unusual elements and can often display hypersensitivity to DNase I and S1 nuclease treatment. An example of such an area is a human rIGS. With some 400 copies of rDNA per human genome, it is to be expected that variation would arise among so many copies. The types of variability in human rIGS together with its complete primary structure are outlined in details in the paper of Gonzales and Sylvester (10). Previously, in the course of characterization of rDNA containing clones selected from the cosmid library of human chromosome 13 (LA13NCO1, Los-Alamos), we noted some variations in organization of microsatellite clusters $(gaca)_n$, $(cac)_n$, and $(tcc)_n$ in comparison with the published rDNA sequence and revealed disproportion in different rDNA regions representativity (1, 11).

Here we show nonrandom cleavage of human rDNA with *Sau3A* which can lead to non-equal representation of different rDNA sequences in the genomic DNA library prepared with the use of this enzyme. The primary cleavage sites were mapped in the rIGS, especially in the regions of about 5–5.5 and 11 kb upstream of the rRNA transcription start point.

EXPERIMENTAL PROCEDURES

Random rDNA containing clones were previously selected from the cosmid library of human chromosome 13 (LA13NCO1, Los-Alamos) using rDNA specific oligonucleotide probes (1, 11).

DNA preparation and analysis. DNA from the selected cosmid clones after a standard cell propagation (12) in the presence of canamycin (50 μg/ml) was isolated according to the CTAB-DNA method (13). DNA from blood samples was isolated according to Mathew (14). Restriction enzymes were purchased from commercial sources. Blot hybridization and hybridization in gel were done using oligonucleotide probes as described (1, 11).

Hybridization probes. 5′-ctctcttaaggtagc-3′ (r1), 5′-gttgatcctgccagt-3′ (r2), 5′-ttgcttgcttgcttgc-3′ (r3), 5′-tgactgaacgcctct-3′ (r4), and 5′-gaaaatgaaaatgaaagca-3′ (r5) were end-labeled in the reaction mixture with [γ-^{32}P]ATP and polynucleotide kinase (Amersham) and purified on prepacked column of Biogel P6 (1, 12).

DNA primary structure was determined with the use of the fmol DNA sequencing system (Promega) according to the technical manual.

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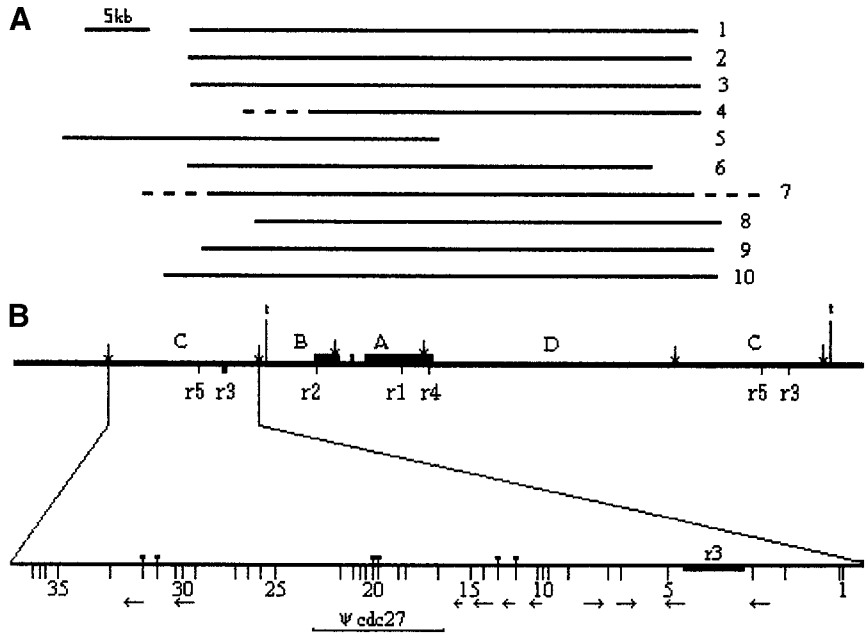

FIG. 1. (A) A general map of the human ribosomal DNA repeat unit. *Eco*RI cleavage sites producing fragments A, B, C, and D are indicated by vertical arrows. The positions of oligonucleotide probes r1, r2, r3, r4, and r5 used for the identification of *Eco*RI fragments are denoted by vertical bars under the main line; clusters of $(ttgc)_n$ hybridizing with r3 are denoted by one bold bar; t, transcription start point. The location of each cosmid insert on the rDNA map is shown above the major rDNA line by the numbered horizontal lines; the numbers correspond to the order of cosmid clones in Fig. 2; the rDNA insert ends that reveal no homology with the human rDNA are indicated by dotted lines. (B) An expanded scheme of the fragment C where all *Sau3A* sites are denoted by vertical lines under the main horizontal line and numbered in the direction upstream of the transcription start point, according to (10). The predominant *Sau3A* sites revealing hypersensitivity to the action of *Sau3A* are shown by hammer symbols above the line; the *Alu* elements are indicated by horizontal arrows, according to their direction. The location of Ψ cdc 27 is shown by a horizontal bracket.

Genomic DNA hydrolysis and analysis. Human genomic DNA (60 μg) in a total volume of 150 μl was completely digested with *Eco*RI (Amersham). Then 10 units of *Sau3A* endonuclease (BioLabs) were added and 20-μl aliquots were sampled periodically into reaction tubes, each containing 2 μl of 0.5 M EDTA, pH 8.3. The DNA restriction products were electrophoresed, transferred onto Hybond N^+ membrane filters (Amersham) and hybridized with the probe r3.

Cosmid DNA hydrolysis and analysis. DNA of cosmid clones containing rDNA inserts was mixed with λ phage DNA at a ratio of 1:4 and 60 μg of this mixture was digested with restriction endonucleases and analyzed as described above for genomic DNA.

RESULTS AND DISCUSSION

A full length of the repeating rDNA unit in the human genome is about 43 kb, whereas DNA insert sizes in the cosmid library of the human chromosome 13 LA13NCO1 (Los-Alamos) lie between 30 and 40 kb (15). *Eco*RI restricted human rDNA usually gives rise to four characteristic fragments: A, B, C, and D (16), where A contains the 3′-end of 18S rDNA, an internal transcribed spacer and the greater part of 28S rDNA; B contains ~200 bp of rIGS, an external transcribed spacer and the greater part of 18S rDNA; D involves the 3′-end of 28S rDNA and ~19 kb of rIGS, and C includes only ~12 kb of rIGS. With the use of oligonucleotide probes specific for each of these fragments we checked their nativity in the products of *Eco*RI hydrolysis of the rDNA containing clones. Location of the oligonucleotides used as the probes for the fragments A(r1), B(r2), C(r3), and D(r4) is shown in Fig. 1. For the fragments A, B, and D these probes were complementary to the most conservative parts of 18S and 28S rDNA. For the fragment C, which is devoid of the transcribing part of the rRNA gene, we have constructed an effective oligonucleotide probe $(ttgc)_4$ (r3) that is highly specific for the human rIGS (17). Hybridization with the probes r1 and r2 demonstrates the nativity of the fragments A and B in 100 and 80% of the clones, respectively (Figs. 2A and 2B). Hybridization with the probe r4 reveals that 4 of the 10 fragments D are shortened (Fig. 2D). Hybridization with the probe r3 shows a disruption of the fragment C integrity in 9 of the 10 DNA samples tested (Fig. 2C) suggesting a reproducible loss of a part of the fragment C in the course of the library preparation.

To define more precisely the points where the fragment C was preferentially cleaved we used an additional oligonucleotide probe (r5) synthesized according to the published rDNA primary structure (Accession No. U13369) (10). Hybridization with the probe r5 demonstrates the loss of signals in two more samples (lanes 7 and 9 in Fig. 2E). It means that the ends of the inserts in these two cosmid clones lie between the r3

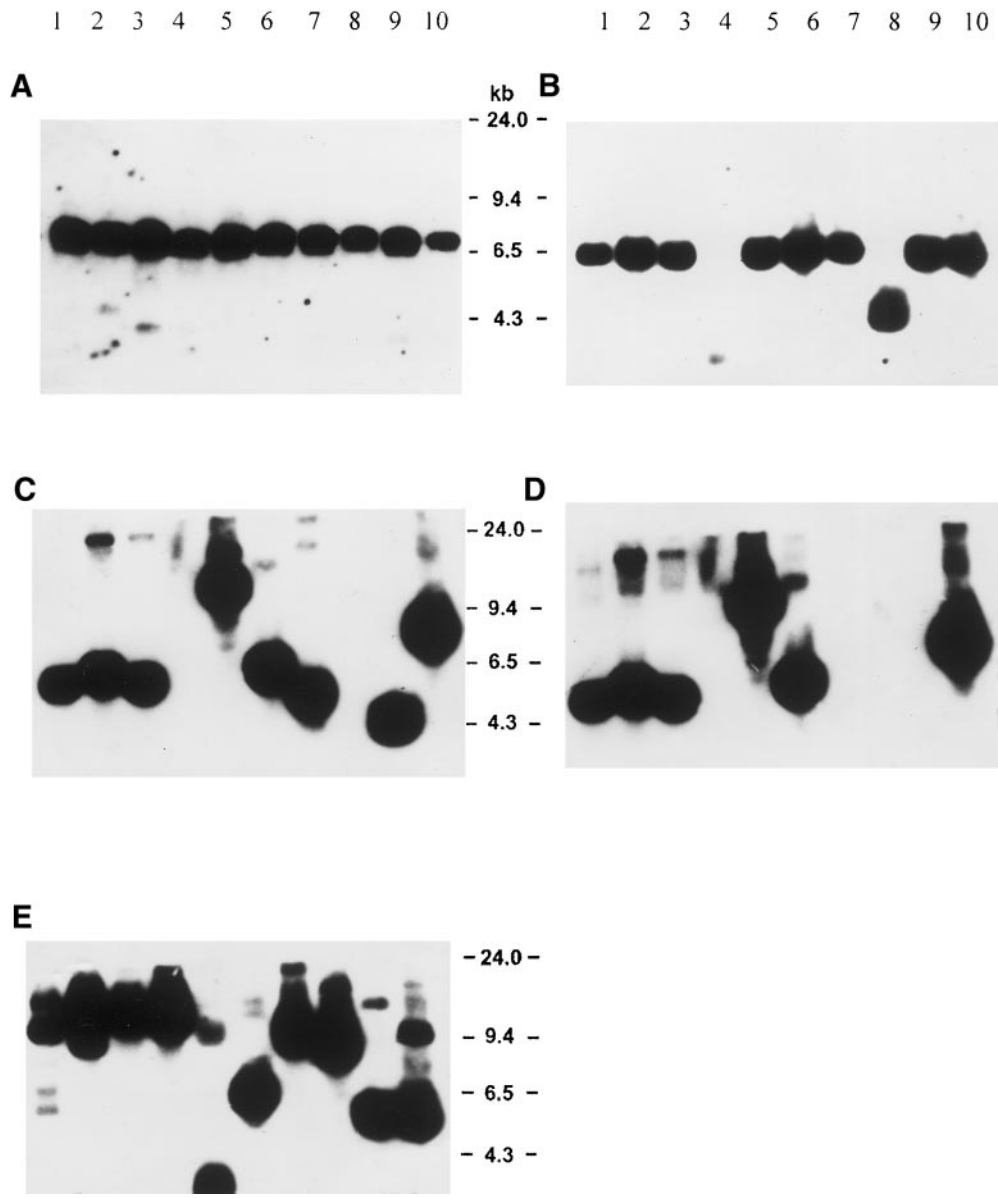

FIG. 2. Successive hybridization of the agarose gel carrying *Eco*RI fragments of 10 cosmid clones with the probes r1 (A), r2 (B), r3 (C), r4 (D), and r5 (E). *Hin*dIII-digested λ phage DNA was used as a molecular weight marker.

and r5 markers (Fig. 1). To establish properly which of the rIGS *Sau3A* sites were preferentially cleaved and ligated with the vector DNA, we have sequenced and mapped all the insert ends in the selected cosmid clones. The results of this experiment are schematically shown by numbered horizontal lines in the Fig. 1A. It is seen that the fragment C harbors 14 of 20 insert ends. Three ends are located in the fragment D (samples 5 and 6) and three ends reveal no homology with the rDNA sequences (lanes 4 and 7). Although a nature of these clones remains unknown, one can suppose that nonribosomal sequences belong to unclustered members of ribosomal DNA family. It is important to note that no one of the inserts contain the both identical ends thus suggesting the individuality of the clones. The majority of the ends in rDNA inserts are mapped in the fragment C in the regions of about 5–5.5 and 11 kb upstream of the rRNA transcription start point that corresponds to the *Sau3A* sites numbered 12–13 and 32–33 according to (10) (Fig. 1B). These data can mean that the *Sau3A* sites of just these regions of the fragment C are preferentially cleaved and ligated with the vector DNA. The results of the insert ends mapping for clones 9 and 10 (both located in the fragment C) do not agree with the data of the probe r4 blot hybridization that reveal substantially truncated D fragments in these clones. The most probable explanation of this phenomenon can be an existence of addi-

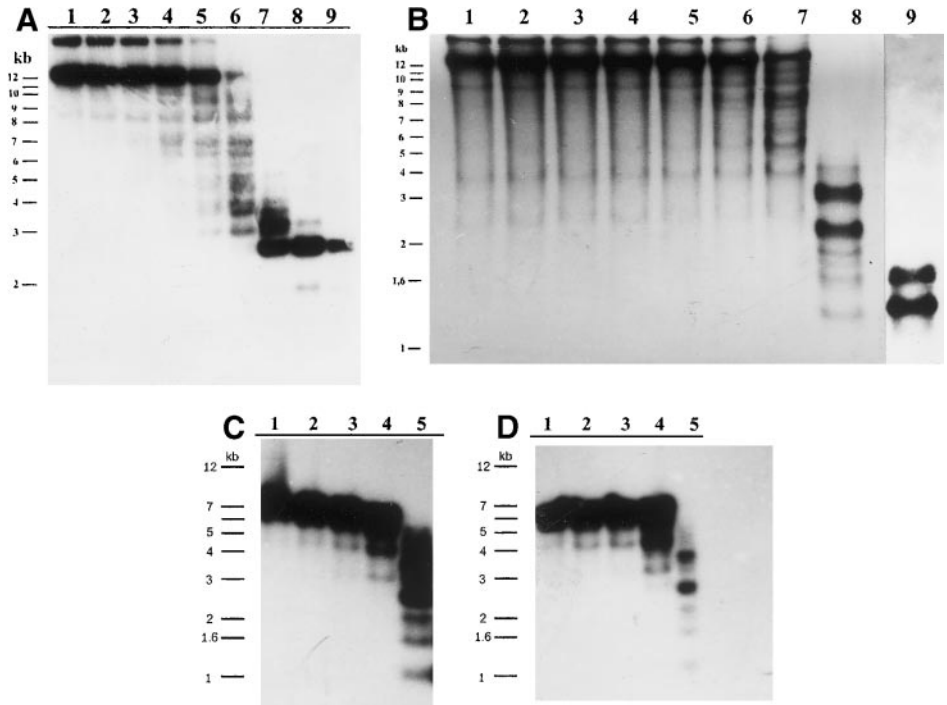

FIG. 3. Kinetics of rDNA fragment C hydrolysis with *Sau3A* or *Mbo*I. (A) Total DNA was digested with *Sau3A* and analyzed as described under Experimental Procedures. 1–7: 0, 1, 3, 5, 30 and 60 min; 8, 9: 6 and 16 hrs of digestion, respectively. 1-kb ladder was used as molecular weight marker. (B) Total DNA was digested with *Mbo*I and analyzed as in (A). 1–8: 0, 0.5, 1, 3, 5, 10, 30, 60 min; 9:16 h of digestion, respectively. (C) $(rDNA)^+$ cosmid, containing truncated 6.5-kb fragment C, adjoining to the promoter was digested with *Sau3A* as described under Experimental Procedures. All designations as in A. (D) As in C, but *Sau3A* treatment preceded to *Eco*RI exhaustive hydrolysis.

tional *Eco*RI sites in the fragment D owing to point mutations, all the more so that examples of restriction fragments lengths polymorphisms in rDNA have been reported earlier (1, 18).

The question arises if similar *Sau3A* hypersensitive regions can be detected in native noncloned rDNA. To answer this question, an experimental procedure was developed including *Eco*RI exhaustive treatment of genomic DNA followed by *Sau3A* endonuclease action at a low enzyme concentration for different periods of time and subsequent blot hybridization with the probe r3. After *Eco*RI treatment alone, one can observe, besides a full sized fragment C (~12 kb), its polymorphic variant (~18 kb) devoid of the *Eco*RI site between the fragments B and C (18) and an about 9-kb fragment containing the interface between the distal part of the rDNA cluster and nonribosomal DNA (Fig. 3). Identity of these 9-kb fragments for all genomic rDNA arrays in humans was shown earlier (19, 20). In the course of a mild *Sau3A* endonuclease treatment three DNA bands of about 10.5, 7.5, and 7 kb arise primarily (Fig. 3A, lanes 2 and 3) later followed by bands of 5–4.5 kb (Fig. 3A, lanes 4 and 5) that corresponds to the *Sau3A* cleavages 11, 8, 7.5, 5.5, and 5 kb upstream of the transcription start point, respectively. In parallel, weakening of the full-sized fragment C intensity is observed. Exhaustive treatment with *Sau3A* gives rise to the expected 3.1-kb fragment (Fig. 3A, lane 9). Figure 3B shows similar mode of noncloned genomic rDNA hydrolysis when *Mbo*I (non sensitive to methylation) is used instead of *Sau3A*. One can see that the fragments 10.5, 7.5, 7, and 4.5 kb appear first (Fig. 3B, lanes 5 and 6). The more early appearance of the 4.5-kb fragment can be recorded as insignificant difference between the results of the two isoshizomer actions.

In other experiments, rDNA of the cosmid clone (i.e., unmethylated rDNA) has been used for the study of *Sau3A* restriction kinetics (Fig. 3C). Since this cosmid contained a truncated variant of the fragment C (about 6.5 kb), it was difficult to make direct comparison of its restriction kinetics with that obtained for the 12-kb fragment C in the total genomic DNA. Nevertheless, primary appearance of size characteristic fragments (for instance, 5 and 4.5 kb) in this case can also be recorded. It is important that a change in the order of *Eco*RI and *Sau3A* treatment of cosmid DNA has no effect on the kinetics pattern (Fig. 3D). It means that similar restriction fragments are generated from linear or superhelical cosmid DNA substrates. Thus, the results let to conclude that neither supercoiled state, nor rDNA methylation, or different physical properties of isoshizomer enzymes has influence on enzyme recognition sites sensitivity. We also could not find any correlation between distribution of hypersensitive *Sau3A*

sites and unwinding human rDNA elements recorded by Dr. C. Benham with the use of his stress-induced duplex destabilization (SIDD) program (21). Earlier, nonrandom hypersensitive cleavage sites produced by micrococcal nuclease on the naked SV40 DNA were described by Nedospasov and Georgiev (22). More recently, Vernick and McCutchan described supercoil-independent nuclease hypersensitive sites that reproducibly flank and delineate the coding regions of some eukaryotic genes tested under defined *in vitro* conditions (23–25). Although detailed description of the shared structural characteristics of these gene-excision cleavage sites must await further work it is suggested that they represent a novel class of genetic sites with particular sequence information that make the boundaries of at least some genes.

In this context we examined the environment of all hypersensitive *Sau3A* sites and found them to be adjacent to or entering into *Alu* retroposons (sites 12, 13, 32, 33) or the retroposed pseudogene of the cell cycle protein gene Ψ cdc27 (sites 19, 20) (10). So, we can postulate a possible role of these retroposons or other surrounding sequences in nuclease accessibility. We also compared the positions of the hypersensitive *Sau3A* areas with the known maps of human *ori* of replication. The results obtained by the nascent-strand abundance analysis (26) suggest that major initiation sites of replication are located a few kb upstream of the transcribed region not far from hypersensitive Sau3A sites 19 and 20 in the Ψ cdc 27 pseudogene and 32 and 33 sites adjacent to *Alu*. Thus, a certain correlation can be noted between hypersensitivity of *Sau3A* recognition sites and their position relatively to retroposons in the *ori* of replication area. Although structural characteristics of the regions responsible for *Sau3A* hypersensitivity in human rIGS remains unclear, we anticipate that obtained results explain a reproducible loss of definite rIGS parts in the DNA library used in this study.

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