

The Mouse Ribosomal DNA Amplification Promoting Sequence 1 is Highly Methylated and Repeated in the Genome

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Upstream of the mouse 45S pre-ribosomal RNA promoter there are regions that are involved in enhancement of pre-rRNA transcription, origin of replication and promotion of amplification. We report that in different mouse strains and cell lines the enhancer region, which overlaps with the origin of replication, is hypo-methylated. Contrary to that the amplification-promoting sequences 1 and 2, identified upstream in the intergenic spacer, are hyper-methylated. Hybridization results also suggest that amplification-promoting sequence 1 is repeated in the genome.

Introduction

In animals the ribosomal rRNA genes are tandemly repeated in lines of hundreds of kilobase pairs (kb), in which each gene is separated from the next by a long intergenic spacer (IGS). Transcription regulation sequences as well as sequences associated with replication and chromosome structure are present in IGS (Reeder, 1990; Moss and Stefanovsky, 1995; Hannan *et al.*, 1998). In mouse rDNA the intergenic spacer contains a repeated region implicated in enhancement of transcription (Pikaard *et al.*, 1990). Further results have shown that the enhancer and the origin of replication region overlap (Gogel *et al.*, 1996). It was reported earlier that mouse IGS contains two short amplification-promoting sequences (APS1 and APS2), which stimulate amplification of cis-linked plasmid DNA in cell culture (Wegner *et al.*, 1989; Zastrow *et al.*, 1989). APS1 and APS2 are located close to the zone of replication initiation, 2.2 kb and 2.7 kb upstream of the enhancer domain (Langst *et al.*, 1997).

Here we report on the methylation of the regulatory regions in IGS using digestion with methylation-sensitive restriction enzymes. We show that the gene enhancer region, which contains many CpG sites, is hypo-methylated in different mouse strains and in cell lines. The 370 bp amplification-promoting sequence (APS1) contains a cluster of CpGs, which makes this sequence a potential site for differential DNA methylation. Our studies

show that the amplification-promoting sequences APS1 and APS2 are methylated. Results demonstrate that in most rDNA repetitive units there is a distinct ~ 2 kb zone, which is a boundary between methylated and non-methylated domains in IGS. Southern hybridizations also show that sequences homologous to APS1 are present elsewhere in the genome, outside the sequenced part of rDNA.

Materials and Methods

Mouse strains and cell lines

We used 60 day old inbred Balb/c and C57 black mice. Mouse Friend erythroleukemia cells (clone F4 N) and mouse 3T3 fibroblasts were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum and maintained at 37 °C in a 5% CO₂.

DNA isolation

Liver nuclei were purified by a modified two step hyper-osmotic sucrose/detergent procedure (Karagyzov *et al.*, 1980). Briefly, mouse tissue was homogenized in 1.2 M sucrose in buffer A (10 mM Tris[hydroxymethyl]aminomethane-HCl, pH 7.5), 10 mM MgCl₂, 0.5 mM EDTA), nuclei were pelleted, suspended in buffer B (0.25 M sucrose, 0.2% Triton X-100 in buffer A) and centrifuged through a 1.2 M sucrose cushion in buffer A. For DNA preparation nuclei were lysed in buffer D (100 mM NaCl, 10 mM Tris-HCl,

pH 7.5, 10 mM EDTA, 0.4% SDS) and treated with RNase A and proteinase K, followed by phenol-chloroform extraction. Mouse Friend erythroleukemia cells and mouse 3T3 fibroblasts were directly lysed in buffer D and DNA was purified as described.

DNA restriction analysis

DNA was digested with restriction enzymes under conditions recommended by the supplier. Digested DNA (10–20 µg) was size fractionated on 1% agarose gels, denatured in 0.5 M NaOH, 1.5 M NaCl and transferred to Hybond N+ membrane (Amersham-Pharmacia-Biotech). For control of genomic DNA digestion each blot was hybridized with pTT4 or pMr100, probes complementary to sequences present once in each repeating unit. Prior to rehybridization blots were stripped in 0.2 M NaOH, 1% SDS for 30 min, room temperature.

DNA probes and hybridization

The 45S pre-ribosomal RNA transcription unit and the adjacent IGS regions are sequenced (EMBL accession number X82564 MM45SRRNA). The probes used were obtained as follows. Probe p370 (APS1 or muNTS1, EMBL accession number X52413 MMMUNTS1) is sub-cloned from pSala 1.2 (Wegner *et al.*, 1989) in the EcoRV site of pBS (pBluescript SK+, Stratagene). It contains a 370 bp region of the IGS (positions from –4945 to –4575) (+1 is the 45S pre-rRNA transcription start site). Probe p420 (APS2 or muNTS2, EMBL accession number X52412 MMMUNTS2) is sub-cloned from pSala 1.0 (Wegner *et al.*, 1989) in the BamHI site of pBS. It contains 425 bp of the IGS (positions from –4530 to –4105). Probe pTT4 contains a 1632 bp TaqI–TaqI fragment, obtained from p5'–1800 (Pikaard *et al.*, 1990) and sub-cloned in SalI site of pBS (pTT4 is the mouse rDNA enhancer domain, position from –1800 to –168). pMr100 contains a 6.7 kb EcoRI–EcoRI fragment from mouse 45S pre-rRNA genes. It is complementary to parts of 18S and 28S, and the entire 5.8S rRNA (Grummt *et al.*, 1979). The identity of all clones used in this study was verified by sequencing. Probes were labeled by random priming and hybridized in 5X SSC, 5% SDS, 50 mM Na₂HPO₄/NaH₂PO₄, pH 7.0, 50% formamide for

16–18 h. Membranes were washed in 2X SSC, 1% SDS, followed by washing in 1XSSC, 1% SDS, and finally in 0.1X SSC, 1% SDS. Membranes were exposed to an X-ray film Kodak X-Omat, AR.

Results and Discussion

Methylation pattern of the enhancer elements

The mouse rRNA gene repeating unit is 44 kb long (Cory and Adams, 1977). It consists of the pre-rRNA transcription unit (13,400 bp), and the intergenic spacer (IGS), which contains the enhancer (Moss and Stefanovsky, 1995). The mouse the pre-rRNA transcription unit, the enhancer and the adjacent regions of IGS are sequenced (Gogel *et al.*, 1996). Fig. 1 shows the 5'-end of the 45S pre-rRNA gene and the upstream part of IGS. Previous investigations have mapped the region of replication initiation in a ~3 kb long zone centered 1.6 kb upstream of the pre-rRNA transcription start site (Gogel *et al.*, 1996). Thus regions of transcription enhancement and replication initiation in mouse rDNA overlap.

The mouse rDNA enhancer consists of tandemly repeated 140 bp elements (Pikaard *et al.*, 1990). Enhancer regions differ and fall into several defined size classes, each composed of various number of 140 bp elements (Kuehn and Arnheim, 1983; Pikaard *et al.*, 1990). Each mouse enhancer 140 bp element possesses ten CpG dinucleotides and three HpaII/MspI sites. We investigated enhancer methylation by digestion with TaqI endonuclease, (TaqI sites boarder the enhancer) followed by digestion with the methylation sensitive enzyme HpaII. Results (Fig. 2, panels A and B) show that enhancers in mouse strains and in cell lines are hypo-methylated. Autoradiography scanning shows that in cell lines enhancer methylation is stronger than in liver (although methylation does not exceed 5–15%), and that some size classes are more methylated (Fig. 2, panel A). It was reported earlier that in mice enhancer methylation increases with age (Swisshelm *et al.*, 1990).

In rodents enhancer sequences are transcribed, in mice, however, enhancer transcription is very low (Paalman *et al.*, 1995). Furthermore, in mouse cells only half of the rRNA genes are transcriptionally active (Conconi *et al.*, 1989). Apparently, the hypo-methylation of enhancer rDNA does not depend directly on transcription. It might be spec-

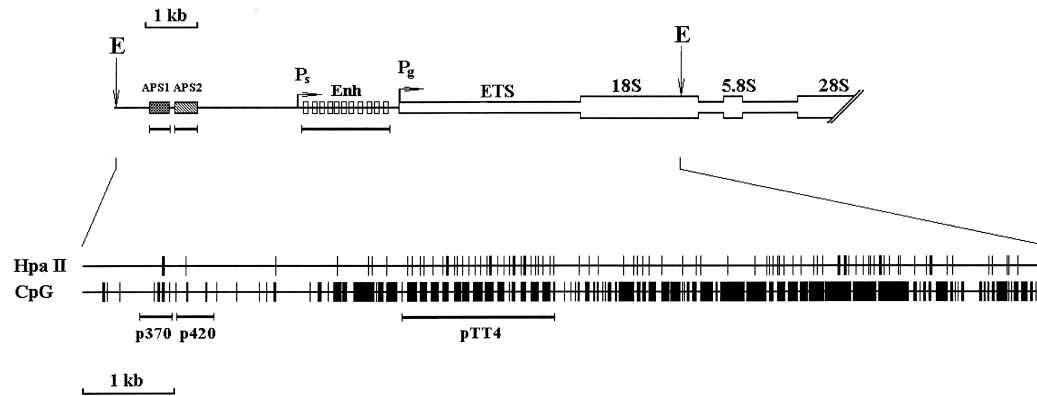


Fig. 1. Map of the 5'-end of the mouse ribosomal RNA gene repeating unit (accession number X82564). E – sites for EcoRI; Pg – gene promoter; Ps – spacer promoter; APS1 – amplification promoting sequence 1; APS2 – amplification promoting sequence 2; Enh – repetitive enhancer elements; ETS – external transcribed spacer; 18S and 5.8S – genes for 18S and 5.8S rRNA. Vertical lines – CCGG (HpaII/MspI sites) and CpG dinucleotides. The position of probes for APS1 (p 370), APS2 (p420) and the enhancer (pTT4) are also indicated.

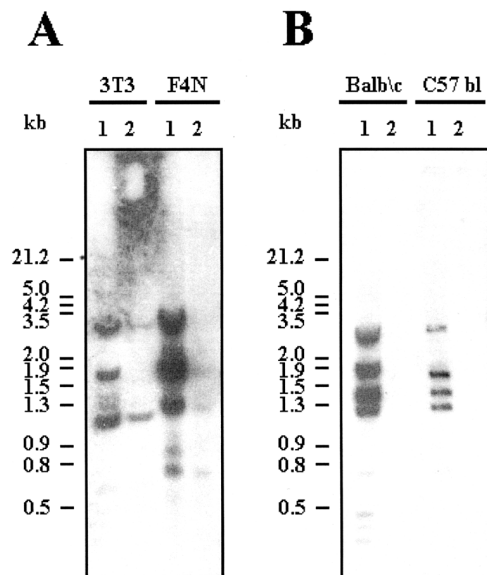


Fig. 2. Methylation of enhancer in mouse strains and cell lines. Hybridization is with pTT4 (enhancer probe, see Materials and Methods). Genomic DNA digested with TaqI only (lane 1). Additional digestion with HpaII (lane 2). Molecular marker sizes are given in kb.

ulated that it is related to initiation of replication that takes place in this region (Gogel *et al.*, 1996). Recently it was proposed, and experimental evidence was presented that regions rich in unmethylated CpG dinucleotides are associated not only with house-keeping gene promoters but also with origins of replication (Delgado *et al.*, 1998). It ap-

pears that rDNA replication in the mouse also starts in a region rich in unmethylated CpGs.

The amplification-promoting sequences in the mouse rDNA are repeated

Amplification-promoting sequences, APS1 and APS2 (Fig. 1) are positioned near the enhancer (Wegner *et al.*, 1989). Southern analysis of EcoRI digested genomic DNA shows two or three prominent fragments hybridizing to p370 (Fig. 3). Hybridization of the same blots with probe TT4 reveals that only the longest fragment (~16 kb) is complementary both to APS1 and the enhancer. An EcoRI fragment with similar length, hybridizing to 18S rRNA, was previously reported in the mouse genome (Cory and Adams, 1977). It should be noted that the sequenced EcoRI fragment is shorter (~11 kb, see Fig. 1). This confirms previous conclusion that the sequenced clone represents a minor fraction of mouse rDNA or it is the result of host-mediated rearrangements (Grummt *et al.*, 1979).

The existence of shorter (~6 kb and ~3.4 kb) genomic EcoRI fragment(s), hybridizing to p370, but not to pTT4, is unexpected. In the mouse about 80% of IGS is not sequenced, so maybe in rDNA there are two or three additional EcoRI fragments that contain APS1 homologous sequences. However, the possibility that sequences similar to APS1 are present elsewhere in the genome should not be excluded. It is likely that the

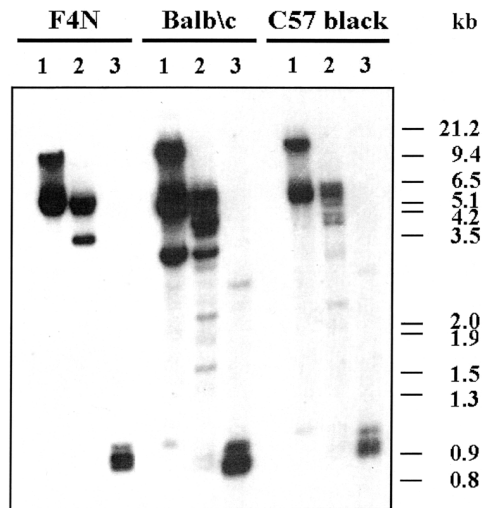


Fig. 3. Methylation of APS1 element in different mouse strains and cell lines (see top). Hybridization with p370 (APS1 probe, see Materials and Methods). In all lanes genomic DNA is digested with EcoRI. Lane 2 – additional digestion with methylation sensitive HpaII; Lane 3 – additional digestion with MspI. Molecular marker sizes are given in kb.

adjacent APS2 sequences are also present in other parts of the genome. However, blot hybridization with probe p420 gave confusing results, as APS2 contains the mouse repetitive B1 element (Stolzenburg *et al.*, 1994).

Methylation pattern of the amplification promoting sequences 1 and 2

APS1 contains a short cluster of CpG dinucleotides (200 bp, nine CpGs) with two HpaII/MspI sites. The APS2 sequence contains few CpGs and one HpaII/MspI site (Fig. 1). To investigate methylation of APS1 and APS2 sequences we examined bands hybridizing to p370 after EcoRI and HpaII or MspI digestion. Specific attention is on the longest EcoRI fragment, which originates from rDNA (Fig. 1). As seen on Fig. 3 hybridization after digestion with EcoRI and methylation insensitive MspI results in EcoRI-MspI fragments 0.9 kb long. If CCGG sites in APS1 or APS2 are not methylated this will result in the appearance of the same recognizable short EcoRI-HpaII frag-

ment(s). However this is not the case (Fig. 3). This reveals that contrary to the hypo-methylation of the enhancer segments, the amplification promoting sequences in mouse rDNA are highly methylated in all cell lines and strains tested. The size reduction of the longest fragment after HpaII treatment is apparently due to digestion of the enhancer, which contains numerous unmethylated sites (see Fig. 2). The size and number of the HpaII resistant fragments hybridizing to p370 may prove useful to locate the boundary between methylated and non-methylated regions in rDNA. Results show that in different repetitive units unmethylated HpaII sites may vary in position. Apparently, the boundary between methylated and unmethylated domains in mouse IGS is not a rigid and uniform one, but rather a broad zone of about 2 kb, between the enhancer and the amplification-promoting elements.

It was shown previously that the mouse rDNA sequences APS1 and APS2 (previously designated as muNTS1 and muNTS2) mediate head-to-tail reiteration of the vector DNA in chromosomes of transfected mouse cells as well as activate SV40 DNA replication in vivo (Hemann *et al.*, 1994; Staib *et al.*, 1998). However, rDNA is not amplified in mouse oocytes (Tian *et al.*, 2001) and despite indirect evidence it is unclear whether the amplification promoting sequences are required for activity of the origin of replication. In view of our results it may be speculated that APS1 methylation is relevant to matrix binding. It has been reported that ^mCpGs are binding sites for the Methyl CpG Binding Protein -2, which also binds to the nuclear matrix (Weitzel *et al.*, 1997). It should be noted that APS1 and APS2 contain also nineteen ATTA and ATTTA sequences. These sequences are reported to be nuclear matrix-attachment motifs (Zannis-Hadjopoulos and Price, 1998).

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