Supercoil induced S1 hypersensitive sites in the rat and human ribosomal RNA genes

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Received 21 January 1986. Revised and Accepted 19 March 1986

ABSTRACT

Rat and human ribosomal RNA gene fragments in supercoiled plasmids were examined for S1 nuclease hypersensitivity. In the transcribed portion of genes the number and distribution of S1 sites were found to be species specific. No S1 sites were detected in the promoter regions. In the nontranscribed spacer (NTS), downstream of the 3' end of 28S RNA gene, S1 sites appear to be conserved in rat and human rDNAs.

A rat NTS fragment (2987 nucleotides long), containing three S1 sites was sequenced and the S1 sites in this region were localized in polypyrimidine-polypurine simple repeat sequences. Other types of simple sequences, two type2 Alu repeats and an ID sequence were also found in the sequenced region. The possible role of simple sequences and S1 sites in transcription and in recombination events of rDNA is discussed.

INTRODUCTION

Transcriptionally active chromatin is often, but not always, characterized by the presence of DNAse I and/or S1 nuclease hypersensitive sites (SHS) in the 5' or 3' end of genes, transcribed by RNA polymerase II (reviewed in 1) and III (2). The DNAse I and S1 hypersensitive sites in many cases map very close to each other, or are overlapping (3, 4, 5) and the two enzymes may recognize different aspects of the same structure (6). The SHS detected in chromatin were also detectable in most, but not in all, cases in supercoiled plasmids (3, 6, 7). In the formation of the nuclease hypersensitive sites in plasmids or in chromatin, DNA conformation changes (7, 8) specific proteins (9, 10) or the torsional stress present in activ chromatin (11) may be involved. Although the DNAse I and S1 sites were often correlated with the expression state of genes, many of them are believed to be
involved in processes other than transcription (e.g. 12, 13, 14).

DNAse I hypersensitive sites in rDNA chromatin, probably correlated with gene activity, have already been detected (15, 16, 17). To date SHSs in rDNA fragments in supercoiled plasmids only of three Drosophila species have been examined (18). The conservation of S1 sensitivity, that was detected in Drosophila NTS regions that are divergent in sequence, might indicate the conservation of a specific function, this function, however, is not clear (18).

Neither DNAse I nor S1 hypersensitivity of mammalian rDNAs have yet been examined. In the present work rat and human rDNA fragments in supercoiled plasmids were examined for S1 hypersensitivity. SHSs in the NTS of the two genes, downstream of the 28S RNA genes seem to be conserved, as in Drosophila (18). A species specific distribution of SHSs in the transcribed part of the genes was also observed. No SHS were found at or near the promoter regions. The nucleotide sequence of a ~3 kb rat NTS 3' end fragment, that contains three SHSs was determined and found to contain a number of different simple repeat sequences. The possible involvement of these sequences in recombination processes is discussed.

MATERIALS AND METHODS

Materials

Restriction endonucleases, S1 and Bal31 nuclease were purchased from Bethesda Research Laboratories and were used according to the supplier’s recommendation.

Plasmid DNAs were prepared according to the method of Birnboim (19).

E.coli DNA polymerase was supplied by Boehringer-Mannheim, \( \alpha^{32} \) P-dATP by the Institute of Isotopes, Hungary, and the nick translation was made by the method of Rigby (20). \( \gamma^{32} \) P-ATP and \( \alpha^{32} \) P-dCTP were obtained from Amersham. The DNA sequencing was performed as described by Maxam and Gilbert (21).

S1 nuclease digestion

Plasmid DNAs were digested with S1 nuclease essentially as described by Larsen and Weintraub (3). One unit of S1 enzyme
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was used per microgram DNA and the digestion was performed in high salt buffer, containing 300 mM NaCl, 30 mM Na-acetate, pH 4.5, 3 mM ZnCl₂ and 0.1 mM EDTA, or in low salt buffer containing 30 mM NaCl, 30 mM Na-acetate, pH 4.5, 3 mM ZnCl₂, 0.1 mM EDTA at 25 or 37°C for 15 min. The samples after S1 digestions were extracted with phenol, several times with chloroform, precipitated with ethanol and then digested with restriction enzymes. Bal31 nuclease digestions were carried out as described (18).

Hybridization with nick translated probes

Hybridizations were performed in 3 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M Na-citrate), 0.1% SDS, 20 mM Tris, pH 7.4, 1 mM EDTA, 10 x Denhardt’s solution (1 x Denhardt solution is 0.02% Ficoll, 0.02% Bovine serum albumine, 0.02% Polyvinyl pyrrolidone), 200 µg/ml carrier DNA, at 65°C for 12-14 hours. The filters were washed with several changes of 0.1 SSC, 0.1% SDS at 65°C for 3 hours.

rDNA clones

The pNH10, p4.8 and p5.6 rat rDNA EcoRI clones were described previously (22, 23). The pNH8 SalI clone has recently been isolated. The map position of the clones in the rat rDNA repeat are shown in Fig.1.

Human rDNA clones used in this study were isolated by Wilson et al. (24).

RESULTS

S1 hypersensitive sites in rat rDNA clones

In supercoiled p4.8 plasmid, containing the promoter region and the 3' end of NTS of rat rDNA, three SHSs were detected (Fig.2A). In the samples without S1 treatment, only the bands expected from the restriction map were seen (Fig.2A lanes 5, 6). The three prominent bands, resulted from the S1 digestion indicated the presence of three major S1 cutting sites in supercoiled p4.8 plasmid (Fig.2A lanes 7-14). The positions of these S1 sites were also determined in the p3.3 and pNH10 plasmids (not shown). From the data obtained in these experiments the S1 sites were localized at 1.2, 1.6 and 2.1 kb from the EcoRI site in p4.8 i.e. -2.7, -3.2 and -3.6 kb upstream of the
Figure 1. Structure of the rat rDNA repeat and map position of clones used in this work.

A. The pNH10, pNH8 and pNH5.6 are Novikoff hepatoma rDNA clones and p4.8 and p5.6 are rat liver rDNA clones (22, 23). From p4.8 the variable region (Vr-hatched box) was deleted to obtain p3.3.

B. Restriction map and sequencing strategy (arrows) of the EcoRI/SalI rat NTS fragment. From the HinfI and DdeI sites the sequencing was performed on both DNA strands.

transcription initiation site. In the promoter region no S1 cutting occurred. The lack of SHS in the promoter region was not the result of masking effect of the strong SHSs in the NTS,
since SI cutting could not be demonstrated in a HindIII/Aval subclone, containing only the promoter and variable regions (not shown).

We examined the supercoiled p5.6 and pNH5.6 plasmids containing the whole ETS and 1.7 kb of the 18S RNA coding region for SI sensitivity. Two SI sites were detected in these plasmids near the 5' end of the 18S RNA gene in the ETS. Since the pNH8 plasmid overlaps with p5.6 and pNH5.6 in the region that contains the SI sites, only the results obtained in pNH8 are shown (Fig.2B). The two SI sites in the ETS were 1.7 and 2.0 kb from the EcoRI site. In the rat ETS there is a polypyrimidine tract, containing multiple CT repeats just upstream of the 5' end of 18S RNA gene (25). Because of the low resolving power of agarose gel (±50 nucleotides) we analyzed the digestion products in polyacrylamide gel. The SalI site in pNH8 is 450 bp from the 5' end of 18S RNA gene. In SI treated pNH8 plasmid the SalI digestion generated 430-440 bp fragments (Fig.5B) indicating that SI cutting in the ETS occurred in the polypyrimidine tract at the 5' end of the 18S RNA gene (site 5 in Fig.6).

In pNH8 plasmid besides the two SI sites in the ETS there were additional SI sites (Fig.2B lanes 10, 11, 12) in the internal transcribed spacers. Both ITS 1 and 2 in rat rDNA contain a number of polypyrimidine.polypurine sequences (26). One of them starts immediately upstream of the 5' end of 28S RNA gene. It is possible that SI cutting in ITS 1 and 2 occurred in the polypyrimidine tracts, but further mapping experiments are necessary for the exact localization.

SI hypersensitive sites in human rDNA fragments

We wished to determine whether SI sensitive DNA sequences were also present in supercoiled plasmids of human rDNA. The pE2 human clone contains the transcription initiation site, the whole ETS and 1.7 kb of the 18S RNA gene. There was no difference between the samples with and without SI treatment in the pE2 clone (Fig.3A), indicating that SHSs neither in the ETS nor in the promoter region were present in this human clone.

Results obtained in the pE6 plasmid, that contains the 3'
end of 18S RNA gene, both ITS 1 and 2, the 5.8S RNA gene and about 3.6 kb of the 28S RNA gene are shown in Fig. 3B. Two SHSs were detected in this plasmid both in the 28S RNA coding region. There were no SHS in the internal transcribed spacers.

Superhelicity requirement, salt and pH effects on SI sensitivity

No specific SI cleavage occurred if the plasmids were first linearized and then treated with SI nuclease. Results obtained with p3.3 are shown in Fig. 3C. Similar experiments with the other plasmids gave identical results. The specificity of SI cutting was only slightly decreased at 25°C if low salt conditions (see Methods) were used. Under low salt conditions at 37°C, however, besides the specific bands the appearance of a smear was observed (Fig. 3D).

The SI sensitivity of supercoiled plasmids was not caused by the low pH used in the SI digestions. Using Bal31 nuclease at pH 8.0 the prominent bands, detected in SI experiments were also observed. Results obtained in pNH5.6 are shown in Fig. 3E.

Nucleotide sequence of the SI hypersensitive region in the rat NTS

The nucleotide sequence of the EcoRI-Sall fragment (the 3' end of the rat NTS) is shown in Fig. 4. A 110 bp long fragment

Figure 2. Localization of major SHS in rat rDNA clones

A. Supercoiled p4.8 plasmid was SI treated as described in Methods and then digested with restriction enzymes. The resulted fragments were probed with the end labeling EcoRI/PvuII fragment. In this and in the subsequent experiments the molecular weight markers (lanes 1, 2 and 15) were λDNA EcoRI and pBR322 HindIII fragments, the lengths are given in kilobases. Supercoiled p4.8 lane 3; SI treated p4.8 lane 4. Plasmid without SI treatment, digested with Aval/EcoRI lane 5; EcoRI/HindIII lane 6. SI treated plasmid digested with Aval lane 7; Aval/EcoRI lane 8; EcoRI lane 9; EcoRI/HindIII lane 10; PstI lane 11; EcoRI/PstI lane 12; PvuII lane 13; EcoRI/PvuII lane 14. The map below shows the relevant restriction sites and the probe. Arrowheads indicate the SI sites. Symbols: A-Aval; E-EcoRI; H-HindIII; P-PstI; Ps-PvuII; S-Sall; X-XbaI.

B. Supercoiled pNH8 with and without SI treatment was digested with the enzymes indicated and probed with the SalI/EcoRI fragment. Supercoiled pNH8 lane 1; pNH8 without SI treatment digested with BamHI lane 2; EcoRI lane 3; PstI lane 4; SalI lane 5; XhoI lane 6. Supercoiled pNH8 lane 7; SI treated pNH8 lane 8; SI treated pNH8 digested with BamHI lane 9; EcoRI lane 10; PstI lane 11; SalI lane 12; XhoI lane 13. In the map below: B-BamHI; E-EcoRI; P-PstI; S-Sall; Xh-XhoI. Arrowheads indicate the SI sites.
in this region, starting from the EcoRI site, has recently been sequenced (27).

Hybridization experiments suggested (28) the presence of type2 Alu repeats in the NTS of the rat rDNA. In the sequenced region two type2 Alu repeats and an ID sequence were found (underlined in Fig.4). The ID sequence is believed to play a specific role in the transcription or processing of brain-specific mRNAs (29). The significance of the ID sequence and the Alu repeats in the 3' end of the rat NTS and also in the 5' end of NTS (30) is not known at present. The most striking feature of the sequenced region is the presence of a number of simple sequences. There are (TAAG)$_7$ repeats at the 5' end and (TAAG)$_8$ and (TAAA)$_8$ repeats at the 3' end of the ID sequence. A GT$_{23}$ cluster (between nucleotides 681 and 726) a GA$_{15}$ repeat (between nucleotides 1215 and 1244) and two long polypurine tracts (between 1491 and 1597 and between 1961 and 2052 nucleotides, respectively) were found in the sequenced NTS fragment. In the polypurine tracts there are multiple CT and CCCT repeats that were shown to be S1 sensitive in supercoiled plasmids in a number of cases (6 and references therein). If the S1 treated p4.8 plasmid was digested with Xbal enzyme,(the cleavage site

**Figure 3A., B. S1 hypersensitivity of human rDNA clones**

A. The pE2 plasmid without S1 treatment digested with BamHI lane 1; EcoRI lane 2; HindIII lane 3. S1 treated pE2 digested with BamHI lane 4; EcoRI lane 5; HindIII lane 6.

B. The pE6 plasmid was S1 treated in low salt (lane 2) or in high salt (lane 3) conditions and then digested with EcoRI. Plasmid without S1 treatment, digested with EcoRI lane 4; S1 treated pE6 lane 5. Lane 1, markers. Map below: E-EcoRI; B-BamHI. The arrowheads indicate the S1 sites.

**3C., D., E. Superhelicity requirement salt, and pH effects on S1 hypersensitivity**

C. S1 treated p3.3 digested with EcoRI/SalI lane 1; p3.3 linearized with EcoRI lane 2; p3.3 digested with EcoRI then S1 treated lane 3. Markers lane 4.

D. Supercoiled p4.8, S1 treated in 300 mM NaCl at 37°C lane 1; at 25°C lane 2; samples 3 and 4 are part of another experiment. S1 treatment in 30 mM NaCl at 37°C lane 5, or at 25°C lane 6. In low salt the specificity of S1 cutting decreased.

E. Supercoiled pNH5.6 was digested with Bal31 nuclease at pH 8.0 and then with EcoRI/HindIII (lane 2). Plasmid without Bal31 treatment, digested with EcoRI/HindIII (lane 1). In the Bal31 digested sample, 1.7 and 2.1 kb bands are seen (as in S1 treated pNH8).
**Figure 4. Nucleotide sequence of the rat NTS EcoRI/SalI fragment (p4.8 plasmid)**

The type2 Alu repeats and the ID sequence are underlined and the arrowheads indicate the direction of possible transcription. Simple repeat sequences are shown by dashed lines. Since the exact number of nucleotides of the variable region (between the transcription initiation site and the SalI site) is not known, the sequence is numbered from the EcoRI site. The map position of the sequenced NTS region is shown in Fig.1.

is localized between the polypyrimidine tracts at nucleotide 1858) two broad bands,120-150 and 280-330 bp long respectively were observed (Fig.5A), indicating that S1 cutting occurred in the long polypyrimidine tracts.

**DISCUSSION**

The SHSs found in rat and human rDNA fragments in supercoiled plasmids are summarized in Fig.6. Sites 1 to 7 in the
Figure 5. Localization of SHSs in polypurimidine tracts
A. SI treated p4.8 plasmid was digested with XbaI, and the resultant fragments were electrophoresed in 5% polyacrylamide gel, lane 2. Marker: pBR322 HindIII fragments, lane 1. The broad bands 120-150 and 280-340 nucleotides long respectively are the results of SI cutting in the 92 and 106 bp polypurimidine polypurimidine tracts in the NTS (sites 2 and 3 in Fig. 6).
B. The supercoiled pNH8 plasmid without (lane 1) and with (lane 2) SI treatment, was digested with SalI enzyme. The SalI digestion generated 430 bp long fragments, indicating that SI cutting occurred in the polypurimidine cluster at the 5' end of 18S RNA gene (site 5 in Fig. 6).

rat, and sites 1 and 2 in the human gene were determined experimentally. The presence of SHSs downstream of the 28S RNA gene are predicted in both species (site 8 in rat and site 3 in the human rDNA in Fig. 6). There are long (100 bp or longer) polypurimidine.polypurimidine tracts, containing multiple CT and CCCT repeats in the sequenced 5' end regions of the two mammalian NTSs (30, 31). Since a (CT)_{12} repeat is sufficient to generate SHS in pBR322 plasmid, we may predict that the rDNA NTS
Figure 6. Summary map of the SHSs in rat and human rDNA
In the transcribed part of genes (45S RNA) both the number and
distribution of SHSs are different in the two species. The SHSs
downstream of the 28S RNA gene appear to be conserved in the
two mammalian rDNAs. Further details are in DISCUSSION.

sequences, containing long CT clusters would be also S1 sensitive
in supercoiled plasmids.

The promoter regions of neither the rat nor of the human
rDNAs were S1 sensitive. Similar results were obtained in
Drosophila rDNA where the promoter region showed only weak S1
sensitivity (18). These results are in contrast with the S1
hypersensitivity of promoter regions of genes, transcribed by
polymerase II, and may reflect the differences in transcription
requirements of polymerases I and II. It is possible, however,
that S1 hypersensitive sites in the mammalian rDNA promoters can
only be detected in chromatin and not in supercoiled plasmids.

Sites 1 and 2 in the human 28S RNA gene may simply reflect
the foldback capability of the rRNA (18). Since sequence
differences exist even among the well conserved 28S RNAs (32)
the lack of SHS in the rat 28S RNA gene is not surprising. The
difference in S1 sensitivity of the transcribed spacers (ETS
and ITSs) may also be the result of sequence divergence. In the
rat rDNA there are long polypyrimidine/polypurine sequences
immediately upstream of the 18S and 28S RNA genes (25, 26), that
may have a role in the processing of the rRNAs (25). Site 5
certainly and site 7 probably are localized in these tracts. In the human gene the ETS and ITS are not SI sensitive.

SHSs in the NTS, downstream of the 28S RNA gene are apparently conserved in the rat and human rDNAs. The conserved SHSs found in similar position in three Drosophila species, were localized in AT rich cruciform structures (18). Sequence divergence of SI sites is not unexpected, since the SI enzyme can recognize, and cut, many different features in DNA (8, 13, 33, 34, 35). Further studies with the rDNA of different species will reveal if conserved SHS in the 5' end of the NTS are present in all eukaryotes. The conservation of SHSs in the rDNA NTS may or may not be correlated with the conservation of a specific function.

One may only speculate regarding the possible function of the SI hypersensitive NTS regions of mammalian rDNAs. In the sequenced rat NTS fragment, besides the SI sensitive (simple) sequences, a number of other simple sequences are also present. Simple sequences were suggested to have a role in the regulation of rDNA transcription, by influencing the positioning of nucleosomes in rDNA (27). This effect, however, is not specific for the rDNA chromatin, since simple sequences occur, on the average, once in every 6-8 kb DNA in the eukaryote genome (36).

Simple sequences are present not only in mammalian rDNA spacers but also in the spacers of the histone gene cluster in sea urchin (37, 38). They also occur between immunoglobulin genes (39, 40). There are a number of suggestions that different types of simple sequences are hot spots for recombination (41, 42, 43, 44). Interestingly these sequences are SI sensitive (42, 44). It is tempting to speculate that simple sequences in the spacers of multigene families are involved in recombination processes (as hot spots). Simple sequences may allow localized exchange between members of multigene families, localized on homologous or on nonhomologous chromosomes (43) or may combine DNA segments that otherwise share no homology (45). It remains to be seen if recombination events, responsible for the maintenance of homogeneity within multigene families are
correlated with the presence of (S1 sensitive) simple sequences in the multigene family spacers.

At present there are a number of open questions regarding the exact role of SHSSs and simple sequences in rDNA. Some of the questions may be better answered if data concerning the S1 and/or DNAse I hypersensitivity of mammalian rDNA chromatin will be available. Preliminary results, obtained in isolated nuclei, indicate that SHSSs described in the present work are not correlated with gene activity.

ACKNOWLEDGEMENTS

The authors thank M. Frigyesi for expert technical assistance and E. Fekete for typing of the manuscript. This work was supported by a grant No. 390/82/1.6 of the Hungarian Academy of Sciences.

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