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DNase I hypersensitive regions correlate with a site-specific endogenous nuclease activity on the r-chromatin of *Tetrahymena*

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ABSTRACT

A novel nuclease activity have been detected at three specific sites in the chromatin of the spacer region flanking the 5'-end of the ribosomal RNA gene from Tetrahymena. The endogenous nuclease does not function catalytically in vitro, but is in analogy with the DNA topoisomerases activated by strong denaturants to cleave DNA at specific sites. The endogenous cleavages have been mapped at positions +50, -650 and -1100 relative to the 5'-end of the pre-35S rRNA. The endogenous cleavage sites are associated with micrococcal nuclease hypersensitive sites and DNase I hypersensitive regions. Thus, a single well-defined micrococcal nuclease hypersensitive site is found approximately 130 bp upstream from each of the endogenous cleavages. Clusters of defined sites, the majority of which fall within the 130 bp regions defined by vicinal micrococcal nuclease and endogenous cleavages, constitute the DNase I hypersensitive regions.

INTRODUCTION

The Southern blotting technique permits visualization of specific sequences in total genomic DNA. Combined with the classical approach of probing chromatin structure by nuclease digestion, this has become a powerful tool in the elucidation of chromatin organization at defined loci in eukaryotic cells. Using this strategy, several groups have reported on the presence of defined sites or narrow regions along the chromatin fibre with extreme susceptibility to endonucleolytic attack. The phenomenon was originally described in [1,2] and recently reviewed by Elgin[3]. The susceptible sites are commonly referred to as DNase I hypersensitive sites. The hypersensitivity in some cases extends to other endonucleases, such as DNase II, micrococcal nuclease, nuclease S1, endogenous nucleases or appropriate restriction enzymes[4-8]. The bulk of evidence indicates that the presence of DNase I hypersensitive sites correlates with a specific chromatin

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conformation believed to be a prerequisite for transcriptional activity [9-15]. The nature of the regulatory function reflected in the acquisition of a DNase I hypersensitive site upon activation of the gene region is unknown, although the hypersensitive sites are often found at or close to the 5'-ends of genes [10,13,16-18]. The biochemical basis for hypersensitivity is still obscure, but it seems to prevail at sites with an irregularity in the nucleosomal array [16] or at sites devoid of a nucleosome [8,19,20]. It is not yet clear whether specific classes of proteins are associated with the open region.

The knowledge concerning hypersensitive sites mainly originate from class II genes. Therefore, it seems important to examine the chromatin structure of a region flanking the 5'-end of a class I gene. We have chosen the gene coding for ribosomal RNA in Tetrahymena, as it exists extrachromosomally and therefore is amenable to study both on the level of nuclei and isolated chromatin.

The presented data demonstrate a close correlation between three endogenous cleavage sites and corresponding sets of micrococcal nuclease and DNase I hypersensitive sites. We discuss the possibility that interaction of the endogenous nuclease with specific regions is maintaining an open configuration rendering the DNA accessible in these regions.

MATERIALS AND METHODS

Preparation of nuclei. Macronuclei were prepared from early log-phase cells (0.5 - 1 × 10^5 cells/ml) of Tetrahymena thermophila, strain B1868-7 according to our standard procedure[21].

Extraction of macronuclear DNA. Freshly prepared macronuclei were suspended in ice-cold digestion buffer (10 mM Tris-HCl, pH 7.2, 0.1 M sucrose, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 0.2 mM 2-mercaptoethanol) at a density corresponding to 0.1-0.2 mg DNA per ml. Endogenous cleavage of rDNA was induced by addition of SDS and EDTA to final concentrations of 1% and 15 mM to a nuclei suspension equilibrated at 25°C for 2 min. After 1 min of gentle mixing, the lysate was heated to 60°C and adjusted to 800 mM NaCl. Deproteinization was done with 200 µg/ml proteinase K (Merck) for 2 hrs at 60°C followed by extraction with phenol and

chloroform/isoamylalcohol(24:1). Nucleic acids were ethanol precipitated and dissolved in 250 mM Tris-HCl,pH 8.0, 250 mM NaCl,25 mM EDTA, 0.5% SDS and incubated with 10 u/ml RNase A (DNase-free) for 30 min at 37°C. The RNase treatment was terminated by the two-step organic extraction described above. After three rounds of ethanol precipitation, the final DNA pellet was dissolved in 10 mM Tris-HCl, pH 8.0, 5 mM NaCl, 0.5 mM EDTA.

Intact control rDNA was obtained by incubation of the nuclei suspension with 800 mM NaCl, 15 mM EDTA for 5 min in the cold before being passed through the above procedure.

Nuclease digestion of macronuclear chromatin. Macronuclei suspended in digestion buffer were equilibrated at 25°C for 2 min and digested with micrococcal nuclease (E.C.3.1.31.1) or DNase I (E.C.3.1.21.1) for 6 min at the enzyme concentrations given in the figure legends. Both nucleases were obtained from Worthington Biochemicals. Digestions were terminated by addition of NaCl and EDTA to final concentrations of 800 and 15 mM. After 1 min of gentle mixing, the samples were supplemented with SDS to 1% and heated to 60°C. Further purification of DNA was as outlined in the preceeding section.

Formation of snap-back molecules. S1-Digestion. Formation of snap-back derivatives[22] of rDNA was accomplished by boiling macronuclear DNA in 10 mM Tris-HCl, pH 8.0, 5 mM NaCl, 0.5 mM EDTA for 5 min. Subsequently, the samples were quickly chilled to 0°C and left for 15 min to allow snap-back renaturation of palindromic sequences.

Digestion with nuclease S1 (Sigma, type III; E.C.3.1.30.1) was done after snap-back formation in 50 mM sodium acetate pH 5.0, 150 mM NaCl, 2 mM $ZnSO_4$ with 5 units of S1/µg DNA for 30 min at 37°C. The reaction was stopped by addition of EDTA to 15 mM and adjusting the pH to 8.0. After addition of glycerol, the samples were loaded directly for electrophoresis.

Electrophoresis and hybridization conditions. Electrophoresis of DNA was in 200 × 200 × 4 mm agarose gels run in a horizontal equipment. The electrophoresis buffer has been given before[23]. Following electrophoresis, DNA was transferred to nitrocellulose filters (Schleicher and Schüll, BA 85) according to Southern[24]. Hybridization was done for 16 hrs at 65°C in 6 × SSC

pH 7.0, 0.5% SDS, 50 µg/ml poly U, 100 µg/ml sonicated, heat-denatured salmon sperm DNA, 0.02% Ficoll, 0.02% polyvinyl pyrolidone and approximately 10 ng/ml of ^{32}P-labelled DNA probe with a specific activity of 0.5 - 1 × 10^8 cpm/µg DNA. Filters were presoaked at 65°C in hybridization buffer without labelled probe for 4-6 hrs prior to hybridization. Further processing and autoradiography of the filters was as previously described[25].

DNA-Probes were ^{32}P-labelled by nick translation according to Rigby et al.[26]. The hybridization probes used in our experiments were: i) total rDNA from T.thermophila B1868-7; prepared as described[27], ii) a 2.1 kb Hind III fragment spanning the 17S coding region cloned in pBR322 (pRP4; constructed by R.Pearlman) and, iii) a palindromic 4.19 kb Hind III fragment of the central spacer region of T.thermophila rDNA inserted in pBR322 (pTth1; originally developed by E.Blackburn).

RESULTS

The extrachromosomal, highly amplified rDNA is confined to the nucleoli in the macronucleus of Tetrahymena[28]. The rDNA molecules are giant palindromes [22,29], each containing two coding sequences separated by a central spacer, the centre of which constitutes the symmetry axis of the molecules. Two distal spacers are flanking the 3'-ends of the coding sequences (see fig.1). We consider the analysis of the chromatin structure in the central spacer particularly important, since this region harbours the origin of replication [30,31] as well as the transcriptional promotors[32].

When isolated macronuclei are exposed to a strong ionic detergent in a low ionic strength buffer, a fraction of the rDNA molecules receives double-stranded cleavage at specific sites. An example of this phenomenon is given in Figure 2A, lane 1. Isolated macronuclei were lysed with SDS. The DNA was subsequently isolated and subjected to agarose gel electrophoresis and Southern blotting. rDNA sequences on the blot were visualized by hybridization with nick translated rDNA (probe I, see fig.1). In addition to the 20 kb band corresponding to intact, palindromic rDNA, a series of distinct bands of lower molecular weight emerges. The sizes of these fragments cluster around half the

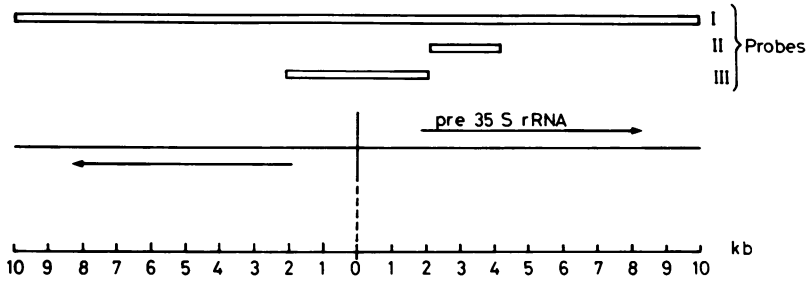


Figure 1. Physical map of macronuclear rDNA from T.thermophila, based on Engberg et al.[29]. Horizontal arrows indicate the coding regions. The open, horizontal bars symbolizes the hybridization probes. Probe I: full-size rDNA, probe II: a 2.1 kb Hind III fragment of rDNA, probe III: a 4.19 Hind III fragment of rDNA (see Methods for details).

size of full-size palindromic rDNA, indicating that a fraction of the rDNA has undergone cleavage near the centre of the molecule, probably within the central spacer. The presence of such rDNA fragments does not reflect the native state of the rDNA in the r-chromatin as the cleavages can be avoided by extraction of the nuclei with salt prior to the addition of SDS. Figure 2A, lanes 2 through 5 clearly demonstrate an inverse relationship between the concentration of NaCl present during preincubation and the fraction of rDNA getting cleaved. The induction of specific cleavages by SDS is instant and independent upon divalent metal ions and high energy compounds. Neither do the protease, RNase or high temperature treatments used in purification of DNA from the nuclear lysates influence their formation. The fraction of rDNA molecules cleaved in response to SDS-treatment of chromatin is routinely in the order of ∿25%. Panel B of Figure 2 shows that addition of an intercalating compound such as ethidium bromide to isolated nuclei has the same effect as SDS-treatment. Thus, interference with either protein or DNA structure leads to the generation of the specific cleavages.

We have taken advantage of the palindromic structure of the rDNA in mapping the specific cleavages induced by SDS. Upon denaturation and rapid renaturation, the separated strands of a palindromic sequence will reanneal on themselves forming "snap-back" molecules[22] with a molecular weight corresponding to half

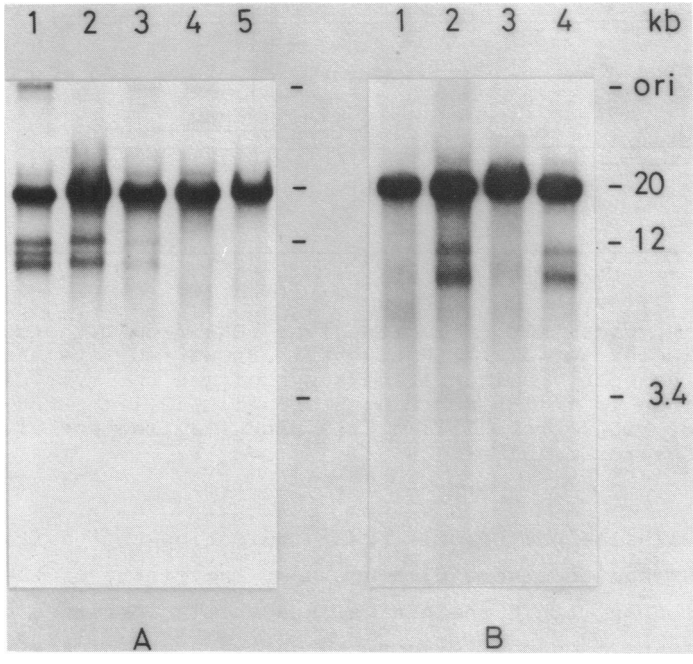


Figure 2. Southern blot analysis of rDNA with specific cleavages induced by SDS or ethidium bromide. Panel A: macronuclei incubated with NaCl at concentrations 0 mM (lane 1), 100 mM (lane 2), 200 mM (lane 3), 300 mM (lane 4), and 500 mM (lane 5) were lysed with 1% SDS. All samples were brough to 800 mM NaCl before being processed for electrophoresis. Panel B: SDS added after preincubation in 800 mM NaCl (lane 1), SDS added before NaCl (lane 2), ethidium bromide (100 µg/ml) added after preincubation in 800 mM NaCl (lane 3), ethidium bromide added before NaCl (lane 4). The hybridization probe was in both panels probe I (full-size rDNA).

of the original molecule. If the original molecule has a double-stranded cleavage, the two snap-back derivatives will be single-stranded from the cleavage site to the distal terminus. Subsequent removal of the single-stranded tail by S1-treatment generates a double-stranded fragment measuring the distance from the symmetry axis to the cleavage site (see fig.3A). In Figure 3B, DNA from nuclei treated with: (i) salt before the addition of SDS (lane 1), or (ii) SDS before addition of salt (lane 2), were subjected to snap-back/S1 treatment. Electrophoretic analysis of the products shows that the control DNA (NaCl added before SDS) gives rise to a single snap-back fragment of rDNA with the ex-

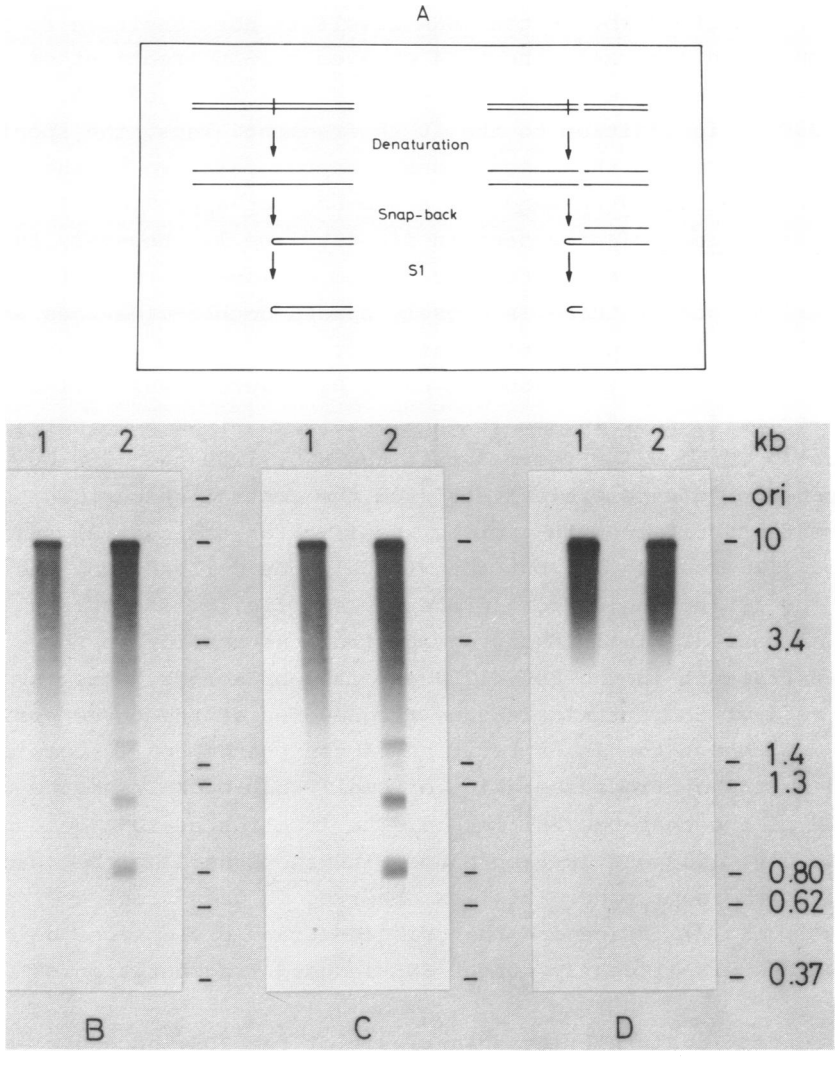


Figure 3. Mapping of the endogenous cleavage sites in rDNA by the snap-back/S1 method. Panel A shows a general outline of the method; left part: intact rDNA, right part: cleaved rDNA. DNA from macronuclei incubated with 800 mM NaCl prior to addition of 1% SDS (control DNA, lane 1) or from macronuclei lysed with SDS before addition NaCl (specifically cleaved DNA, lane 2) was subjected to snap-back/S1 treatment. The resulting fragments were analysed by Southern hybridization with different probes. Panel B: probe I, Panel C: probe III, Panel D: probe II.

pected size of 10 kb, as the rDNA itself is not sensitive to S1 treatment. In contrast, the rDNA cleaved by SDS-treatment of chromatin gives rise to three fragments with sizes of 800, 1250 and 1900 bp in addition to the 10 kb fragment. Thus, the specific cleavages occur at these positions measured relative to the symmetry axis. The 800 bp band has a sub-band at approximately 830 bp (this is most clearly seen in fig.4B, lane 1). However, the resolution of the electrophoretic system employed here is too low to clarify whether there is closely spaced double-cleavages at the 1250 and 1900 bp positions as well.

The hybridization probe in panel B, Figure 3 was full-size rDNA (probe I, see fig.1). It can be inferred from a comparison of panels C and D in Figure 3 that the 800, 1250 and 1900 bp fragments originate exclusively from the central spacer. The fragments hybridize with a probe specific for the central spacer (probe III, see fig.1), but not with a probe derived from the flanking 17S coding region (probe II, see fig.1). There is a gradual decrease in intensity going from the smaller to the larger fragment in the 800, 1250 and 1900 bp series. This may in part reflect the actual cleavage frequencies at the three positions, although the interpretation of the phenomenon is complicated by the overrepresentation of small snap-back fragments inherent in the snap-back/S1 method. The fraction of rDNA detected in the 800, 1250 and 1900 bp bands with the central probe (panel C) is of the same order (25%) as observed in the direct analysis (lane 1, fig.2), indicating that single-stranded cleavages do not contribute significantly to the SDS-induced fragmentation of rDNA.

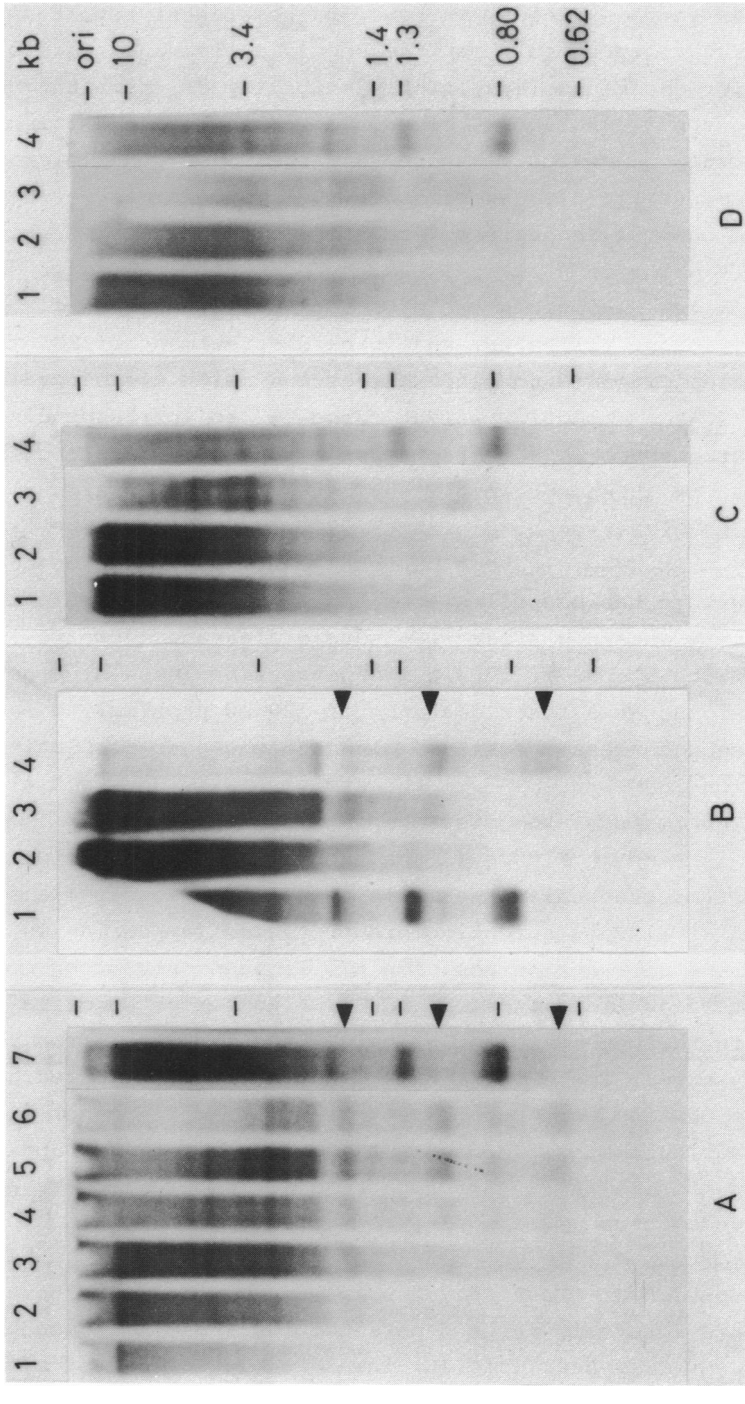
Conventional restriction analysis of the SDS-induced cleavage sites has confirmed the results obtained in fig.3, demonstrating that the appearance of these bands are not an artefact of the snap-back/S1 mapping technique (see also lane 1, fig.3B). In addition, the analysis showed that cleavages can occur at several of the sites on each rDNA molecule (Gocke, Bonven and Westergaard, unpublished).

We have previously described a DNase I and micrococcal nuclease hypersensitive region in the central spacer of rDNA[25]. Since the snap-back/S1-mapping is particularly sensitive to

cleavages in this region, we have in the present report applied this method to precisely map the specific cleavages made by DNase I and micrococcal nuclease. The possibility of inhibiting the endogenous nuclease activity by NaCl enables us to distinguish between the endogenous cleavages and the DNase I and micrococcal nuclease-specific cleavages, since rDNA cut exclusively by one of these enzymes can be recovered from digestion terminated with NaCl/EDTA prior to nuclear lysis by SDS (see Methods for details). Figure 4A, lane 1-6 shows the electrophoretic pattern of rDNA (detected by probe III) after snap-back/S1-treatment of DNA from macronuclei digested to different extends with micrococcal nuclease. In the molecular weight range 0-2 kb (corresponding to the central spacer), a characteristic pattern of distinct bands is seen. The three more prominent bands (marked by arrows) correspond to cleavages at distances 670, 1120 and 1800 bp from the symmetry axis. The cleavage pattern generated by micrococcal nuclease within the central spacer shows a striking similarity to the pattern obtained after SDS-induced cleavage (compare for instance lanes 5 and 7). Thus, a micrococcal nuclease hypersensitive site is found at a fixed distance of 130 bp proximal to each of the endogenous cleavage sites, although the poor resolution in the region 1800-1900 bp from the symmetry axis does not allow a precise estimate of the distance in this region.

It is seen in Figure 4A that position 1800 is the primary target of micrococcal nuclease in the central spacer (lane 2). The site at 1120 is slightly more protected (appearing in lane 3), whereas cleavage at position 670 is seen only after more extensive digestion (lanes 5 and 6). A number of specific bands appear in the higher molecular weight region (>2 kb from the symmetry axis). These intragenic cleavage sites appeared also on naked DNA and were not investigated further. A minor micrococcal nuclease-specific band with a size of 900 bp, apparently not conforming the general pattern of localization relative to the endogenous cleavage sites, appears after extensive digestion.

Snap-back/S1-treatment of DNA from macronuclei digested with DNase I reveals three hypersensitive regions in each half of the central spacer (fig.4B, lanes 2-4). The DNase I hyper-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10
3.4
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A

sensitive regions fall primarily within the 130 bp regions defined by the endogenous and the micrococcal nuclease specific cleavages. A hypersensitive region spanning the distance from position 690 to position 730 is flanked by minor cleavage sites at positions 630 and 780. A second hypersensitive region covers the stretch between positions 1130 and 1170 with a minor cleavage site at position 1050. The third region is found between position 1850 and 1900. The presence of minor, specific cleavage sites in this region would not be detected in this low-resolution range of the gel. No prominent, specific cleavages were registered within the coding region after DNase I digestion of chromatin. The levels of DNase I susceptibility at the three regions resembles that of the micrococcal nuclease.

The gel pattern obtained after digestion of naked rDNA with micrococcal nuclease or DNase I are shown in Figures 4C and D. The cleavage patterns are in both cases considerably more complex and diffuse than the patterns obtained after digestion of chromatin. This demonstrates that several of the highly sensitive sites on the rDNA are protected on the r-chromatin. The appearance of some weaker bands apparently similar to those created in digestion of chromatin suggests some sequence specificity at the hypersensitive sites.

Figure 5 summarizes the data from Figures 3 and 4.

Figure 4. Mapping of hypersensitive sites in r-chromatin. Macronuclei were digested at 25°C for 6 min with micrococcal nuclease (Panel A) at 0 u/ml (lane 1), 0.05 u/ml (lane 2), 0.1 u/ml (lane 3), 0.25 u/ml (lane 4), 0.5 u/ml (lane 5), 1.0 u/ml (lane 6) or DNase I (Panel B) at 0.01 u/ml (lane 2), 0.1 u/ml (lane 3), 0.5 u/ml (lane 4). The DNA was purified and subjected to snap-back/S1 treatment before Southern blot analysis. For comparison, snap-back/S1 treated DNA from SDS-treated macronuclei was run in lane 7, Panel A and lane 1, Panel B. The bands resulting from hypersensitivity within the central spacer are marked by arrows. In a similar analysis, macronuclear DNA purified without activation of the endogenous nuclease was dissolved in digestion buffer and digested to similar extends as in the chromatin digestions. Panel C, lanes 1-3: micrococcal nuclease; Panel D, lanes 1-3: DNase I. Lanes 4 in Panels C and D are markers similar to lane 1, Panel B. All blots were hybridized with probe III.

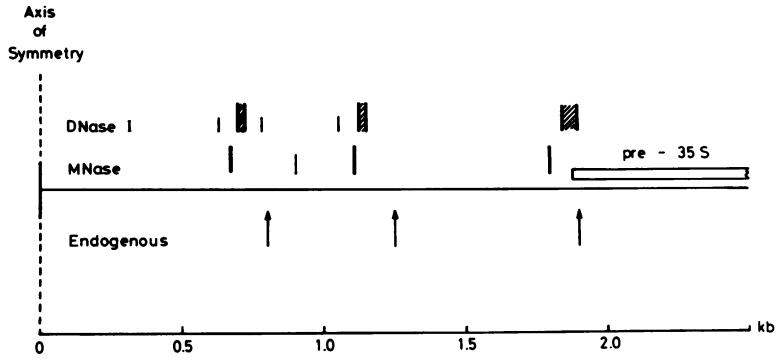

Figure 5. Map of the central spacer of T.thermophila rDNA showing the precise locations of the endogenous cleavage sites (arrows), the micrococcal nuclease hypersensitive sites (vertical lines labelled MNase) and the DNase I hypersensitive sites and regions (vertical lines and hatched areas labelled DNase I).

DISCUSSION

Tetrahymena rDNA features three defined regions with micrococcal nuclease and DNase I hypersensitive sites in each half of the central spacer. Specific cleavages resulting from activation of an endogenous nuclease activity are associated with these hypersensitive regions. The endogenous cleavages are located 800, 1250 and 1900 bp from the symmetry axis. As the 5'-end of the pre-35S rRNA has been mapped approximately 1850 bp from the symmetry axis (N.Din & J.Engberg, personal communication), the endogenous cleavages map at positions +50, -650 and -1100 relative to the promotor. An approximately 40 bp broad DNase I hypersensitive region flanked by minor specific cuts is centred 90 bp upstream from each endogenous cleavage. A defined micrococcal nuclease cut occur 130 bp upstream from the endogenous cleavages. Thus, it seems plausible that similar structures are present in the three regions, although the experimental resolution in the promotor region is too low to rule out minor differences in the organization around this site. It is tempting to speculate that the action of the endogenous nuclease causes local opening of the chromatin structure similar to the accessible regions reported near the 5'-end of the chicken adult β-globin gene [8]. An alternative explanation for the hypersensitivity might be that the sequence specific binding of the endogenous nuclease sensitizes the DNA

within the binding regions. Thus, it has for several sequence specific proteins been shown that binding of the protein enhances the DNase I sensitivity within the binding region relative to naked DNA [36-39]. The promotor region is more frequently attacked by micrococcal nuclease and DNase I than the region between -650 and -800, which is in turn more sensitive than the -1100 to -1250 region. Whether this merely reflects differences in the surrounding chromatin structure or is the result of truly unrelated phenomena remains to be seen. It is interesting that sequence repeats (recognized as multiple cleavages by the restriction enzyme Dde I) are found adjacent to the endogenous cleavages (R. Pearlman, personal communication). Similar Dde I-repeats have been found in T.pyriformis[32], suggesting that this motif is conserved.

The endogenous nuclease activity apparently associated with the hypersensitive regions is detected by induction of site specific cleavages upon disruption of either the protein or the DNA structure. A similar behaviour has been observed with the proteins involved in transfer replication of bacterial plasmids[33,34] and DNA topoisomerases from both pro- and eukaryotic sources[35]. Exposure of a complex between one of these proteins and DNA to a strong denaturant results in a cleaved DNA molecule with a polypeptide covalently attached to one or both of the free ends created in the process. In the case of topoisomerases, the DNA-protein complex represents a trapped intermediate in the normal catalytic cycle of the enzymes[35]. At present we do not know whether the products resulting from SDS-activation of the endogenous nuclease in r-chromatin represent a similar kind of intermediate. So far, we have not been able to assign true enzymatic activity to the endogenous nuclease. However, the possibility of isolating the cleavage products as covalent protein-DNA complexes would provide a useful approach in the identification of the protein(s). This is a prerequisite for assessment of whether the activity/activities responsible for SDS-induced cleavages at the three sites are in fact identical and may help to further establish the tentative relationship to topoisomerases.

The putative regulatory functions reflected in the presence of hypersensitive sites in chromatin are unknown, but their pre-

valence at key regulatory sites indicates that the phenomenon is non-trivial. The localization of the three hypersensitive sites in front of the class I gene described in this report seems to conform to the general pattern observed with class II genes [3,10,13,16-18]. When the localization of hypersensitive sites is considered together with their presence in activated chromatin, it seems plausible that they participate in the transcriptional control on the chromatin. Based on the presence of a hypersensitive region around the origin of replication in viral systems [4,6,7,19,20] it has been suggested that such regions may also be involved in initiation of replication. The DNase I hypersensitive region associated with the endogenous cleavage at -1100 coincides with the origin of replication in T.thermophila[31]. Hence, the positions of the hypersensitive regions in r-chromatin are compatible with models involving these regions in either transcription or replication or both of these processes.

The studies of the developmentally programmed activation of the chicken globin genes have suggested that acquisition of DNase I hypersensitive sites as well as additional events are necessary but not sufficient conditions for transcriptional activity. Evidently, a transcriptionally inactive gene can exist in a domain exhibiting structural characteristics of active chromatin. In a similar manner we find that transcriptionally inactive r-chromatin from starved Tetrahymena cells has both the endogenous nuclease and the hypersensitive regions (unpublished), indicating that r-chromatin is present in the activated conformation in these cells. This notion is in accordance with the observation[40] that starved cells in response to refeeding are able to resume rRNA synthesis without dependence on the replication cycle(s) believed to be required for transition to the activated chromatin conformation[14].

The site-specific nuclease described in this report is intimately associated with the r-chromatin. It is therefore possible to co-isolate the activity with the nucleolar structure, permitting a detailed characterization of the phenomenon at the level of purified r-chromatin.

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