5-Methyldeoxycytidine in the *Physarum* minichromosome containing the ribosomal RNA genes

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ABSTRACT

5-Methyldeoxycytidine (5MC) was analyzed by high pressure liquid chromatography (HPLC) and by restriction enzyme digestion in rDNA isolated from *Physarum polycephalum*. rDNA from *Physarum* M₃C strain microplasmodia has a significant 5MC content (about half that of the whole genomic DNA). This rDNA contains many C5MCGG sites because it is clearly digested further by Msp I than by Hpa II. However, most 5MC is in other sites. In particular, alternating CG sequences appear to be highly methylated. HPLC of deoxyribonucleosides shows that most of the transcribed regions contain little or no 5MC. Restriction digestion indicates that there is little or no 5MC in any of the transcribed regions including the transcription origin and adjacent sequences. Over 90% of the total 5MC is in or near the central nontranscribed spacer and most methylated restriction sites are in inverted repeats of this spacer. rDNA is very heterogeneous with respect to 5MC. The 5MC pattern doesn't appear to change with inactivation of the rRNA genes during reversible differentiation from microplasmodia (growing) to microsclerotia (dormant), showing that inactivation is due to changes in other chromatin variables. The 5MC pattern is different between *Physarum* strains. The possible involvement of this 5MC in rDNA chromatin structure and in cruciform and Z-DNA formation is discussed.

INTRODUCTION

5-Methyldeoxycytidine (5MC) is found in the DNA of most eukaryotes with the likely exceptions of yeast, *Tetrahymena* and many arthropods (1-5). At least 90% of this 5MC is in the sequence CpG (6,7). 5MC levels tend to be higher in inverted repeats than in tandem repeats and lowest in unique sequences (8-11). Generally, hypomethylation of genes precedes or accompanies their transcription (2,3,12). However, some genes are transcribed when highly methylated and in some cases not transcribed when less methylated (13). The pattern of 5MC in DNA is heritable and methylation inhibiting drugs such as 5-azacytidine change not only specific gene expression but also the differentiated state of some cells. The exact role of 5MC in control of these functions remains to be determined. For more complete discussions of 5MC in eukaryotes see (2) and (3).

Figure 1. Map of *Physarum* rDNA defining the transcribed regions and Bgl I (B), Sma I (S) and Xho I (X) cleavage sites (adapted from published maps (15) and consistent with our rDNA cleavage patterns). The Bgl I sites of the NTS are within and roughly delineate inverted repetitive regions as described (15). The rDNA is palindromic and transcription is from the inside out.

Physarum polycephalum provides a useful system for studying the functions of DNA methylation by relating the methylation, chromatin structure and gene expression of rDNA chromatin at different stages of differentiation. The ribosomal RNA genes are found in the nucleoli in linear minichromosomes containing the 60 kilobase pair (kbp) rDNA. One to two percent of a haploid genome is rDNA (about 300 rDNA minichromosomes)(14). Figure 1 shows the palindromically arranged genes with outside and central nontranscribed spacers (NTSs). The central NTS contains the rRNA transcription origins, and the rDNA replication origins. These replication origins are flanked by or are part of inverted repeats (15) as has been found for mammalian replication origins (16). Recently an additional small transcription unit has been observed in the outside "NTS" (17). rRNA genes are actively transcribed in *Physarum* microplasmodia. On starvation, microplasmodia undergo reversible differentiation to dormant microsclerotia and rRNA gene transcription ceases. The transcribed regions of *Physarum* microplasmodial rDNA chromatin are more susceptible to micrococcal nuclease digestion than the central NTS (18,19) or either region of microsclerotial (inactive) rDNA chromatin or bulk chromatin (20). From these more susceptible regions, micrococcal nuclease produces extended particles, sedimenting at 5S, called A particles (18,19) or lexosomes (21). Inactive rDNA chromatin does not give lexosomes but releases 11S mononucleosomes (20). In microplasmodial rDNA, the transcribed regions are in an extended conformation which contains stoichiometric histone ratios but a much lower DNA packing ratio than the nucleosomal chromatin of the central NTS (21). On differentiation to microsclerotia, rDNA transcription ceases and the coding regions become nucleosomal and are no longer extended (21). The central NTS is largely in a transcriptionally incompetent chromatin state immediately adjacent to rRNA genes which are either in the active form in microplasmodia or

in the inactive form in microsclerotia. Thus three different transcriptional states of rDNA chromatin can be defined in these stages of differentiation. It has been shown previously that Physarum rDNA is significantly methylated (22).

We have used a combination of high pressure liquid chromatography (HPLC) and restriction enzyme digestion to show that methylation of M₃C strain microplasmodial rDNA is largely, if not entirely, in inverted repeats of the central NTS and that the transcribed regions and adjacent sequences contain little or no methylation. The level and pattern of 5MC doesn't appear to change with inactivation of the rRNA genes during differentiation from microplasmodia to microsclerotia. Therefore this inactivation is due to changes in chromatin structure other than DNA methylation. Physarum rDNA is very heterogeneous with respect to methylation such that the central NTS of some rDNA molecules is much more extensively methylated than that of others.

MATERIALS AND METHODS

Physarum polycephalum was grown in semi-defined medium (23) and its nuclei isolated (24). We used two strains, M₃C (a gift from Dr. S. Shall) and Lu648 × Lu688 (LuLu) (a gift from Dr. J. Dee). The microplasmodial and microsclerotial forms we used are normally diploid although some polyploidy may be present after over three years of culture in our laboratory. Nuclear DNA was isolated as described (25) except that in place of dialysis, proteinase K digests were phenol or CHCl₃:isopentanol 24:1(v/v) extracted and the DNA ethanol precipitated.

rDNA was isolated from nuclear DNA by successive equilibrium density gradient centrifugation in CsCl (22), CsCl and bisbenzimidazole (26) or KI and bisbenzimidazole (27) as described elsewhere (28).

DNA was enzymatically hydrolyzed to deoxyribonucleosides (9). These were separated by isocratic elution from a 300 × 4 mm Waters μ Bondapak C₁₈ (reverse phase) HPLC column with 3.2 mM Na₃PO₄, 0.15 mM (Et₃N)₃ PO₄ pH 3.5 at 1 ml/min, 37°C and detected by absorbance at 280 nm. Digests of calf thymus DNA (Sigma) or herring sperm DNA (Boehringer Mannheim) were used for comparison and standard deoxyribonucleosides (Sigma and P-L Biochemicals) for quantitation. Deoxyribonucleosides from DNA were injected in their enzymatic cleavage mixture and standard deoxyribonucleosides in a mock enzymatic cleavage mixture (containing no DNA). Peaks were identified by absorbance ratios (A₂₈₀/A₂₅₄) and elution positions as compared to standards.

Except as specifically noted, we used the suppliers instructions for re-

striction digests except that DNA concentrations and digestion times were often two to five fold greater and enzyme concentrations often two to twenty fold greater. The criterion for complete digestion was an unchanged cleavage pattern after over an hour of additional digestion time with active enzyme. Enzyme activity was verified by cleavage of λ DNA to which an aliquot of the digestion mixture had been added. This criterion was used for all but Hpa II and Msp I digests which were judged complete on the basis of high reproducibility, and for Bgl I and Sma I whose rDNA maps are relatively simple and have been published (15). Restriction enzymes Hha I, Hpa II, Msp I, Sma I and Tha I were from Bethesda Research Laboratories, Bgl I and Bss HII from New England Biolabs and Cfo I from Boehringer Mannheim.

Restriction fragments were analyzed by submerged horizontal agarose gel electrophoresis in 40 mM Tris-HCl, 20 mM NaCH₃COO, 2 mM Na₂EDTA pH 7.8. Gels were photographed after ethidium bromide staining and ultraviolet illumination. Fragments were electroeluted (29) from gel slices by placing slices lengthwise in dialysis tubing and submerging them in the electrophoresis chamber in their original orientation. After further electrophoresis, fragments were recovered from the surrounding buffer by partial lyophilization and ethanol precipitation. DNA to be electroeluted was not stained but was located by staining marker lanes.

For quantitative analysis, photographic negatives of gels were scanned in a Cary 210 spectrophotometer with a scanning attachment controlled by a Hewlett-Packard 9845S computer. HPLC elution profiles were recorded using a Hewlett-Packard 3455A digital voltmeter and the 9845S (30). Quantitation was of peak areas, not heights. rDNA fragment size was determined by interpolation from sizes of Hae III generated PM2 DNA fragments (31).

RESULTS

Physarum rDNA was purified from nuclear DNA using successive equilibrium density gradients with bisbenzimidide. This method gave rDNA in hundred microgram quantities with very reproducible yields. Purified rDNA gave a single peak on an equilibrium density gradient (not shown) and no background of main band DNA in restriction digests (see below).

The total 5MC content of pure rDNA was determined directly by digesting it to deoxyribonucleosides and separating these by reverse phase HPLC. A typical elution pattern is shown in Figure 2. Table 1 shows the 5MC contents of microplasmodial and microsclerotial rDNAs, separated Sma I fragments of rDNA, microplasmodial whole genomic DNA, LuLu rDNA and, for comparison, calf

Figure 2. Typical reverse phase HPLC elution pattern of deoxyribonucleosides from M₃C microplasmodial rDNA. Inset shows detail of 5MC, G, T and A peaks. Arrow at zero volume is the injection point.

thymus and herring sperm DNAs. We used about 0.5 microgram of rDNA per determination. The three rDNA fragments generated by Sma I (the 26 kbp or central fragment, the 3 kbp fragments and 13 kbp or outside fragments)(Figure 1) were separated by preparative agarose gel electrophoresis. The measured values of 5MC for the 13 kbp and 26 kbp Sma I fragments were corrected for cross contamination (Table 1)(32). Cross contamination of the 3 kbp fragment may be responsible for its small apparent 5MC content (see below). This shows that over 90% of the 5MC in rDNA is in the central Sma I fragment and that the outside NTS and the rRNA coding regions in the 13 and 3 kbp Sma I fragments

Table 1. Percent 5MC of Physarum and other DNAs.

DNA type	Number of preparations (determinations)	$100 \times 5MC / (C + 5MC)$
M ₃ C microplasmodial rDNA	3 (8)	2.6 ± 0.2
M ₃ C microsclerotial rDNA	2 (3)	2.6 ± 0.3
LuLu microplasmodial rDNA	1 (2)	2.9 ± 0.2
M ₃ C microplasmodial rDNA 3kbp fragment	1 (1)	0.7
M ₃ C microplasmodial rDNA 13kbp fragment	1 (1)	1.4 *
M ₃ C microplasmodial rDNA 26kbp fragment	1 (1)	4.9 *
M ₃ C whole genomic DNA	2 (3)	5.7 ± 0.2
Calf thymus DNA	2 (2)	6.1 ± 0.1
Herring sperm DNA	1 (2)	8.0 ± 0.1

Note: Determinations were made from multiple digestions and/or chromatograms of the same sample.

* When corrected for cross-contamination between the 13 kbp and 26 kbp fragments, these 5MC contents are 0.0% and 5.7% respectively.

Figure 3. Cleavage patterns of whole M₃C microplasmodial rDNA with Msp I (lane 3) and Hpa II (lane 14) and its Sma I fragments: 3 kbp with Msp I (lane 5) and Hpa II (lane 6), 13 kbp (outside) with Msp I (lane 8) and Hpa II (lane 9) and 26 kbp (central) with Msp I (lane 11) and Hpa II (lane 12). Markers are PM2 DNA digested with Hae III (lanes 2 and 15) and λ DNA digested with Hind III (lanes 1 and 16). Sizes of selected fragments are in kbp. The 13 kbp and 26 kbp fragments digested here were contaminated with small amounts of each other (see text).

contain little or no 5MC. We find no significant difference between the 5MC contents of microplasmodial and microsclerotial rDNAs.

C5MCGG sites in rDNA were studied with the restriction enzymes Hpa II (cleaves CCGG but not C5MCGG) and Msp I (cleaves both)(33). In our initial results (not shown), rDNA digestion patterns with Hpa II and Msp I were identical implying the absence of C5MCGG sites in rDNA as reported by Reilly et al. (34). Later we discovered that C5MCGG sites could be cleaved by lowering the DNA concentration in the Msp I digestion mixture suggesting that Msp I digestion is affected by rDNA concentration. This phenomena was not always "all or none". Extremely slow digestion of C5MCGG sites, resulting in their incomplete cleavage in an otherwise complete digest, was sometimes observed in

Figure 4. Cleavage patterns of whole rDNAs with Hpa II, Msp I and Cfo I. M₃C microplasmodial rDNA with Hpa II (lane 6), Msp I (lane 5), Hpa II and Bgl I (lane 4), Msp I and Bgl I (lane 3), and Cfo I (lane 11). M₃C microsclerotial rDNA with Hpa II (lane 8), Msp I (lane 7), and Cfo I (lane 12). LuLu rDNA with Hpa II (lane 9), Msp I (lane 10) and Cfo I (lane 13). Markers are PM2 DNA digested with Hae III (lanes 2 and 14) and λ DNA digested with Hind III (lanes 1 and 15).

pH 7.4 (10 or 20 mM Tris·HCl) but not in pH 8.0 (50 mM Tris·HCl) digestion buffer. We have not analyzed these phenomena in detail nor determined exactly what factors are responsible. rDNA digestion patterns with Hpa II and Msp I are shown in Figures 3 and 4.

rDNAs from microplasmodia of different Physarum strains give different Hpa II digestion patterns (Figure 4). The reversible differentiation from microplasmodia to microsclerotia was not accompanied by a change in the Hpa II digestion pattern of the rDNAs (Figure 4). The digestion patterns and amounts of each fragment in them, were constant from preparation to preparation of M₃C rDNA. The amounts of the several fragments in each digest vary from 2.8 to less than 0.1 copies per rDNA showing that some rDNA molecules are much more extensively methylated than others. As shown below, this heterogeneity of

rDNA molecules with respect to methylation is indicated by digestion with other restriction enzymes.

The approximate location of C5MCGG sites in rDNA was determined by Msp I and Hpa II digestion of its isolated Sma I fragments (Figure 3) and by double digestion with Bgl I and Hpa II (Figure 4). The Hpa II digest of the central Sma I fragment contains fragments corresponding to all the C5MCGG containing fragments in Hpa II digests of whole rDNA. Analysis of the gel scans locates all of the C5MCGG sites in the central Sma I fragment and shows that contamination with this fragment is responsible for what look like C5MCGG sites in digests of the outside fragment. The original negative of the 3 kbp fragment Hpa II and Msp I digests show extremely faint bands corresponding to smaller fragments in digests of the rest of the rDNA. This small amount of contamination may be responsible for the 5MC content of the 3 kbp fragment as determined by HPLC (above).

Double digestion of rDNA with Bgl I and Hpa II (Figure 4) shows that all of the C5MCGG containing fragments except for the faint 540 bp fragment are cleaved by Bgl I. Since none are cleaved by Sma I, all but the 540 bp fragment overlap one or more of the Bgl I sites in the inverted repeats of the central NTS (Figure 1 and ref. 15). The only Bgl I site outside of these inverted repeats is less than one hundred base pairs from, and on the outside of, the innermost Sma I site (15). Cleavage of much over 100 bp by Bgl I from a large, for example a 760 bp Hpa II fragment, cannot occur without its cleavage by Sma I in Sma I/Hpa II double digests (eg. Hpa II digestion of Sma I fragments).

From the sizes and copy numbers of rDNA Hpa II and Msp I fragments (not shown) we estimate that, on the average, each rDNA has roughly 20 C5MCGG sites (or roughly 40 5MC assuming no hemimethylation). When compared to about 870 5MC per rDNA (0.026 of 0.28 C of 60,000 base pairs (120,000 bases)) from our HPLC data, it is obvious that most 5MC is in other than C5MCGG sites.

rDNA was digested with the restriction enzymes Hha I (GCGC), Cfo I (GCGC), Tha I (CGCG) and BssH II (GCGCGC) resulting in very heterogeneous digestion patterns (Figures 4 and 5) as observed for Hpa II. Most of the heterogeneous fragments generated by Hha I, Tha I and Bss HII are digested by Bgl I but not by Sma I, showing that they are in or encompass the inverted repeats of the central NTS (Figure 5). These heterogeneous fragments show considerable size range. Some fragments cover one or more inverted repeats (in Hpa II and Hha I digests) or most or all of the central NTS (in Tha I and Bss HII digests). All of these digests were complete with the possible excep-

Figure 5. Cleavage patterns of whole rDNAs with Hha I, Tha I and Bss HII. M₃C microplasmodial rDNA with Hha I (lane 3), Hha I and Bgl I (lane 2), Hha I and Sma I (lane 1), Tha I (lane 8), Tha I and Bgl I (lane 7), Tha I and Sma I (lane 6), Bss HII (lane 13), Bss HII and Bgl I (lane 12), Bss HII and Sma I (lane 11). M₃C microsclerotial rDNA with Hha I (lane 4), Tha I (lane 9), Bss HII (lane 14). LuLu microplasmodial rDNA with Hha I (lane 5), Tha I (lane 10), Bss HII (lane 15). Markers are PM2 DNA digested with Hae III and λ DNA digested with Hind III (lane 16). Sizes of selected fragments in lane 16 are in kbp.

tions of the Tha I digest of microsclerotial rDNA and the Bgl I/Bss HII double digest which appear to be complete even though this could not be verified for technical reasons (35).

Heterogeneity such as that observed here can be due to methylation or sequence or both. The availability of 5MC for these sites and the location of the heterogeneous fragments suggests that this heterogeneity is largely, if not entirely, due to methylation. Although digestion patterns of M₃C and LuLu rDNAs are quite similar with Hpa II, Hha I, Cfo I, Tha I and Bss HII, clear differences can be observed between these two rDNAs in the proportion and

positions of some heterogeneous fragments. M₃C microplasmodial and micro-sclerotial rDNAs give the same digestion patterns except with Tha I where there appears to be an overall decrease in the proportion of heterogeneous fragments compared to the lower fragments in the later. This loss of large fragments may be due to the greater degree of shearing of micro-sclerotial rDNA as a result of the nuclear isolation procedure. Note that Cfo I and Hha I are isoschizomers that give the same digestion pattern with methylated eukaryotic DNA (36,37).

DISCUSSION

Using HPLC we have determined the percent 5MC in rDNA, its isolated fragments and whole genomic DNA from Physarum. Our values for the 5MC contents of Physarum microplasmodial whole genomic DNA (38) and calf thymus DNA (2,39, 40) are similar to published values. We have further defined the location and site specificity of at least some of this methylation using restriction enzymes. Our results show the following: 1) rDNA is hypomethylated compared to whole genomic Physarum DNA. 2) At least 90% of the total 5MC in rDNA is in the central Sma I fragment whereas the outside NTS and most of the transcribed regions contain very little or no 5MC. 3) Restriction digestion indicates that most of this methylation is in inverted repeats of the central NTS which are over 2 kbp from the transcription origins. This indicates that there is little or no 5MC in any of the transcribed regions including the transcription origins and adjacent sequences. The methylation of Physarum rDNA is thus correlated with nontranscription and inverted repeats. 4) rDNA is very heterogeneous with respect to methylation. 5) The pattern of methylation doesn't appear to change on reversible differentiation between microplasmodia and micro-sclerotia. 6) This pattern is different between strains.

Evans and Evans (22) analyzed the total 5MC content of Physarum plasmodial rDNA and found it to be significantly methylated. However, Reilly *et al.* (34) found no difference between rDNA digests with Hpa II and Msp I. More recently, Ferris and Vogt (15) reported a very small difference between Hpa II and Msp I digests of a portion of the central NTS from M₃C Physarum and large differences between Hha I digests of parts of the central NTS and their cloned DNAs suggesting that some GCGC sites are methylated. Large differences were observed between Hpa II and Msp II digests of inverted repeats of the central NTS in Physarum strain ATCC 24466 (15).

We show substantial methylation as C5MCGG in these inverted repeats (analyzed collectively) of M₃C rDNA. GGC5MCGG and C5MCGGCC sites have been shown

to be resistant to Msp I cleavage, requiring a 150 to 200 fold Msp I excess to cleave some or all such sites (7,41). C5MCGG sites flanked by other C and G containing sequences are also somewhat resistant to cleavage (7). Each of our C5MCGG sites seem digest at about the same rate and do not appear to be part of GGC5MCGG or C5MCGGCC sites (15 and our unpublished results). They could easily be flanked by other C and G containing sequences. The differential treatment of these sites by Msp I under slightly different conditions makes them obvious candidates for differential recognition by other proteins in vivo. The inhibition of late gene expression in SV40 by in vitro methylation of SV40's only CCGG site (42) and similar results (43) provide a functional basis for suggesting such specific recognition by proteins (42,43).

Of the sites we studied, CGCG (Tha I) and GCGCGC (Bss HII) sites appear to be the most heavily and heterogeneously methylated. Several other restriction enzymes, whose sites contain CG sequences, do not cleave the central NTS. These include Xho I (CTCGAC), Sma I and Xma I (CCCGGG), Pvu I (CGATCG) and Sac II (CCGCGG) (15, 44 and our unpublished observations). This suggests that the alternating CG sequences of Tha I and Bss HII sites in the central NTS are both preferentially methylated and have been selected for over other equally or similarly probable sites during evolution. Methylation of alternating CG sequences greatly promotes their transition from right handed B-DNA to left handed Z-DNA such that it occurs near physiological conditions (45,46). The formation of Z-DNA in the central NTS of rDNA appears likely although direct evidence is needed to demonstrate this.

Eukaryotic DNA contains a variety of repetitive sequences, both in tandem and as inverted repeats. As demonstrated in vitro, inverted repeats can form cruciforms (47,48). In eukaryotes, inverted repeats also tend to be highly methylated. Even after in vivo trimethyl psoralen crosslinking, cruciforms are not observed in the methylated inverted repeats in the central NTS of isolated, native Physarum rDNA (27,49). There are several ways that 5MC may discourage cruciform formation. The 5MC promoted B to Z transition (45,46) absorbs supercoiling that would otherwise promote cruciform formation (47,48). Increased cation concentration and other factors that favor duplex DNA over single stranded DNA slow the transition between cruciforms and linear DNA (50,51). Presumably, the increased strength of base pairing between 5MC and G (compared to C and G)(52-54) could have similar effects. The sequence symmetry of inverted repeats also raises questions about their methylation symmetry. Cruciforms in asymmetrically methylated inverted repeats would have hemimethylated sites and be substrates for maintenance methylases (55) leading

to full methylation of the sites and eventual symmetry of methylation. Thus, methylation must be symmetrical unless cruciforms are rare (*in vivo*) and/or protected from maintenance methylases. In an asymmetrically methylated inverted repeat the fully methylated and unmethylated sites of the linear form may have different base pairing strengths and protein binding capacities than the hemimethylated sites of the cruciform. Asymmetry would then also affect cruciform formation. Further experiments are needed to determine the symmetry of methylation and its effect on cruciform formation in eukaryotic inverted repeats.

In addition to Physarum, heterogeneity of rDNA with respect to 5MC is observed in a variety of organisms and cell lines (1,8,56,57). In some of these, hypermethylation of rDNA is correlated with higher rDNA copy number (8,56) but inversely correlated with rDNA transcription (56,58). This and the proximity of the replication origins to the methylated inverted repeats suggests that, based on methylation, physiological conditions affecting DNA conformation and varying with the Physarum cell cycle or life cycle may determine what population of rDNAs are transcribed or replicated at a given point in a cycle. We've observed no change in the Hpa II digestion pattern or total 5MC content of M₃C rDNAs isolated at different times over several months (over 500 Physarum doublings) suggesting that the rDNA 5MC pattern, like the copy number, is well maintained. This does not rule out the possibility that the methylation pattern diverges in different isolates of the same strain in culture, as it has in different strains.

The low levels of 5MC in the transcribed regions and high levels in the central NTS that we observe, very clearly agree with the general correlation between hypomethylation and transcriptional activity. Of other rDNAs studied, 5MC is, or appears to be, present in comparable amounts in the transcribed and nontranscribed regions of specific groups of rDNA repeats although in some cases certain positions within these regions are hypomethylated (57,59,60). The lack of change in rDNA methylation between microplasmodia and microscle-rotia shows that methylation is not needed to bring the coding regions into an inactive conformation. This lack of methylation in coding regions of micro-sclerotial rDNA may retain them in a potentially active conformation. Some mammalian genes that are expressed in all tissues do not show the tissue specific changes in methylation observed in tissue specific genes (61). In one such gene, undermethylation of its 5' end is observed in several tissues and appears to be necessary for expression (61). Further work is needed to determine if changes in rDNA methylation accompany changes in transcription follow-

ing irreversible differentiation.

The central NTS of Physarum rDNA is relatively inaccessible to micrococcal nuclease in isolated nuclei (18,19) or nucleoli (21). In electronmicrographs of histone depleted nucleoli, it appears that the ends of the rDNA molecules are free and the centers are bound in the nucleolar scaffold (62). In the metaphase chromosomes of numerous organisms, the heterochromatic regions, especially those of, or surrounding, centromeres, contain highly methylated, highly repetitive sequences. Loss of methylation in these regions has been shown to result in their decondensation and breakage (63-65). It is hypothesized that chromosome scaffolding or folding proteins bind regions of DNA that have a high concentration of methyl groups (from 5MC or thymidine) (65). The ability to isolate very pure nucleoli from Physarum (21,66) should allow determination of whether or not interaction of methylated rDNA and specific binding proteins is responsible for the inaccessibility of the central NTS and the arrangement of rDNA chromatin in the nucleolus.

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