Protein Tightly Bound near the Termini of the Physarum Extrachromosomal rDNA Palindrome

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ABSTRACT   The genes coding for ribosomal RNA in plasmodia of Physarum polycephalum are arranged palindromically on extrachromosomal rDNA molecules of 61 kb (kilobasepairs). Incubation of mildly extracted rDNA with the ^{125}I Bolton-Hunter reagent results in incorporation of label not removed by SDS, CsCl, or various organic solvents. Labeled protein is preferentially associated with terminal rDNA restriction fragments, as detected after gel electrophoresis of the DNA. Antibody reaction with dinitrophenylated protein-rDNA complexes allows visualization of protein located from 1 to 2 kb from the termini, in a region containing multiple inverted repeat sequences and single-strand gaps. DNase I treatment of either rDNA or rDNA termini releases primarily two labeled protein bands of 5,000 and 13,000 daltons as well as less prominent bands of higher molecular weight. We discuss mechanisms for involvement of terminal protein in replication of 3' ends and chromosomal integration of the rDNA.

In nuclei of the lower eucaryote Physarum polycephalum, the genes coding for ribosomal RNA are episomal in that they are largely, if not exclusively, located on discrete rDNA molecules of 61 kb (kilobasepairs) (1-6). In the plasmodial phase of the life cycle the rDNA is located entirely in the nucleolus during interphase. Each rDNA molecule contains two transcription units of ~13 kb coding for ribosomal RNA. These are arranged palindromically about a large central spacer of 27.6 kb (2-4). In each molecule the most distal coding region, that for 26S rRNA, is interrupted by two intervening sequences (4). A terminal spacer of variable length averaging 5.4 kb lies distal to the 26S gene. We have recently shown that this spacer contains a region of multiple inverted repeats of ~100 bp located near the actual terminus (5). In this region single-strand DNA gaps are selectively located at the 5' end of a satellite-like sequence beginning CCCTA. Sequences in the rDNA terminal repeat are homologous to chromosomal DNA sequences dispersed throughout the genome.

Approximately 150 copies of the rDNA from each nucleus are distributed without loss to daughter nuclei during mitosis (6) together with chromosomal DNA that segregates (in strain a × i) to form 46 chromosomes (7). Although little is known about the mechanisms for replication or distribution of rDNA, it is clear that the termini must play a critical role in these processes. In this study we report that a significant percentage of Physarum rDNA termini possess very tightly, possibly covalently, bound proteins located in a region corresponding to the terminal inverted repeats.

MATERIALS AND METHODS

Isolation of rDNA-Protein Complexes

Nucleoli were isolated from microplasmodia as previously described (4, 5). The rDNA was extracted from nucleoli by a modification of the selective extraction procedure of Braun and Evans (8). Aliquots of 10^9 nucleoli were suspended in 2 ml of 0.15 M Na-citrate, 0.15 M NaCl, and 5 mM EDTA, pH 7.8, containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF); SDS was added to 2%. The mixture was incubated for 60 min at 27°C. After adding NaCl to 1.0 M, the mixture was placed at 4°C for 12 h. After centrifugation at 12,000 g for 30 min, the supernate was dialyzed against 50 mM EDTA, pH 8.0, with 1 mM PMSF; CsCl was added to 56%, and rDNA was banded by centrifugation as previously described (2). Glassware and dialysis bags were treated with a solution of 0.1% Ficoll, 0.1% polyvinyl pyrrolidone, and 0.1% bovine serum albumin to prevent binding and loss of rDNA-protein complexes. Restriction digestion and gel electrophoresis were performed as described for rDNA (4) except that 0.1% SDS was included in electrophoresis buffers to facilitate entry of protein-complexed fragments into agarose gels.

Labeling of rDNA-Protein Complexes with the ^{125}I Bolton-Hunter Reagent

Labeling using the N-hydroxysuccinimide ester of 3,5-[^{125}I]iodo 4-hydroxy phenylpropionic acid (Bolton-Hunter reagent; New England Nuclear, Boston, Mass; 4,000 Ci/mmol) was performed essentially as described by Rekosh et al. (9) for adenovirus DNA protein. Dried Bolton-Hunter reagent (1.0 mCi) was reacted with 200 μg of rDNA-protein in 0.1 ml of $Na_2B_4O_7$, pH 8.5, for 30 min on ice. The reaction was stopped, and unreacted reagent removed as described (9). Labeled rDNA protein was further purified on a 10-ml CsCl gradient overlayered with 1 ml of 20% sucrose solution containing 10 mM Tris-HCl, pH 7.9, 1.0 mM EDTA, and 0.1 mM PMSF. Centrifugation was carried out for 30 h at 25,000 rpm in an SW-41 rotor. DNA fractions labeled with ^{125}I were pooled

and ethanol precipitated for further analysis. In separate experiments, we also banded rDNA protein in similar gradients containing 37% CsCl and 4 M GuCl. The proteolysis inhibitor $NaHSO_3$ was included in all buffers in several experiments. The inhibitor PMSF was present in all dialysis and centrifugation buffers. Nuclease digestions were performed for 2 h at 37°C in buffer containing 60 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM $CaCl_2$, and 10 mM Tris-HCl, pH 7.4. DNase I and staphylococcal nuclease (Worthington Biochemical Corp., Freehold, N. J.) were used at 10 U/ml and 50 U/ml, respectively. Proteinase K (Merck & Co., Inc., Rahway, N. J.) was used at 100 μg/ml for 4 h at 37°C.

Electron Microscope Visualization of rDNA-Protein Complexes

Protein bound to DNA was visualized after treatment with dinitrofluorobenzene (DNFB; Pierce) followed by anti-dinitrophenyl (DNP)-protein antibody essentially as described by Wu and Davidson (10). After dinitrophenylation of protein bound to rDNA as described (10), protein-rDNA complexes were reacted with purified rabbit anti-DNP IgG (prepared against dinitrophenylated bovine serum albumin) as follows. Dinitrophenylated rDNA protein (5 μg) was incubated with 200 μg of rabbit anti-DNP IgG for 1 h at 37° in 80 μl of solution containing 5 mM Na_3 EDTA, 0.2 M NaCl, 0.05 M tricine, pH 8.0, and 1.0 mM PMSF. In a modification of previous procedures (10), we incubated, for a further 20 min at 18°C after addition of S. aureus protein A (Sigma Chemical Co., St. Louis, Mo.), 10 μl at 1.0 mg/ml in the above buffer. Unbound antibody and protein A were removed on 1.5 ml columns of Sepharose 2B. Eluted rDNA-protein complexes were treated with 0.02% glutaraldehyde for 10 min at 18°C and spread for electron microscopy by the formamide procedure previously described (4, 5).

RESULTS

Selective Labeling of rDNA-Protein Complexes

Nucleoli were disrupted in 2% SDS under conditions that did not employ proteolytic enzymes and that were designed to minimize endogenous proteolysis. Upon CsCl density gradient centrifugation, the rDNA thus extracted banded as a broad density fraction rather than as a sharp peak of density 1.713 g/cm^3, as is expected for rDNA purified by more extensive procedures (2). The rDNA pooled from these gradients contains a relatively high percentage of chromosomal DNA fragments. This rDNA fraction was labeled as described with the
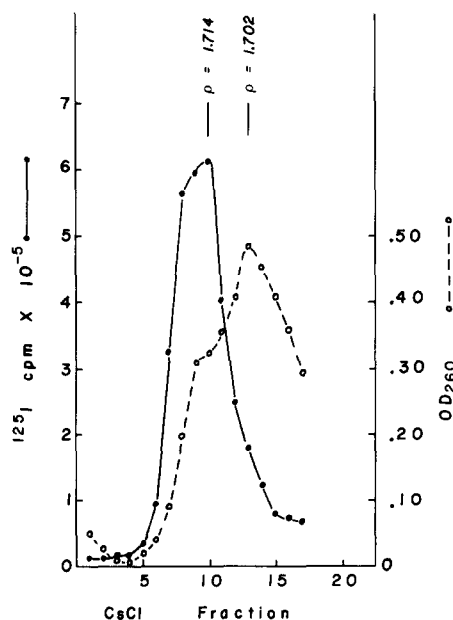

FIGURE 1   CsCl gradient centrifugation of rDNA-protein complexes. DNA extracted from highly purified nucleoli was labeled using the [125]I Bolton-Hunter reagent and centrifuged in a 10-ml CsCl gradient with a 1-ml upper sucrose layer as described. Fractions were analyzed for gamma emission and absorbance at 260 nm.

[125]I Bolton-Hunter reagent under conditions known to result in labeling of protein of DNA-protein complexes (9). Subsequent CsCl gradient centrifugation employed an upper sucrose layer as described to retain unbound labeled protein. Fig. 1 shows that most label bands in the CsCl gradient with the same density as the rDNA satellite shoulder. Little or no label bands with chromosomal DNA, the prominent absorbance peak at ρ = 1.702 g/cm^3. Care must be taken to use highly purified nucleoli for these extractions: slight contamination with mitochondrial DNA, which is less dense than chromosomal DNA, results in significant contamination of gradients with ^{125}I-labeled mitochondrial protein. DNA pooled from the denser side of the labeled peak, >90% pure rDNA as determined by analytical ultracentrifugation, was used for further analyses. Specific activities of ^{125}I incorporation varied from 1 to 8×10^3 cpm/μg DNA in different experiments. More than 80% of this label is retained after either ethanol precipitation, treatment with 80% formamide, 9 M urea, 4 M GuCl, 2% SDS, or further CsCl gradient centrifugation. Phenol extraction removes ~35% of label. However, after phenol extraction, unlabeled rDNA could be labeled to high specific activity with the Bolton-Hunter reagent, suggesting that phase distribution of rDNA-protein in aqueous phenol mixtures may be complicated. Treatment with 0.5 M NaOH removes >80% of label, and digestion with proteinase K removes >90% of label from the rDNA.

Labeled Proteins Are Present at the rDNA Termini

Labeled rDNA-protein complexes were digested with restriction endonucleases and subjected to electrophoresis on 1.4% agarose gels containing 0.1% SDS. Restriction fragment bands were excised from gels, and individual fragments were rerun on agarose gels as described in Fig. 2. This rerunning procedure reduced background from labeled mitochondrial DNA-associated proteins that occasionally trailed through CsCl gradients. Fig. 2 shows an autoradiograph of the three rDNA Bam HI fragments separated as described. The Bam c fragment of 2.7 kb, visibly stained with ethidium in Fig. 2A, is derived from the rDNA termini (4). This fragment varies in size by ±400 bp and appears as a heterodisperse band on gels (5). The Bam a and b fragments (21.4 kb and 13.2 kb) are derived primarily from rRNA coding regions and nontranscribed central spacer, respectively. As seen in Fig. 2A and B, the rDNA termini are prominently labeled with [125]I. In some experiments significant label could be detected in nonterminal fragments, but the terminal fragments are always labeled in great disproportion to their percentage of the rDNA. This is seen in Fig. 2B, in which rDNA fragments are present in equimolar quantities. Terminal rDNA-protein fragments have a high tendency to aggregate and a high affinity for agarose. SDS was necessary for labeled fragments to enter the gel, and electroelution was inefficient. In separate experiments rDNA prepared as described was nick translated using α-^{32}P-deoxynucleotides, digested with various restriction enzymes, and subjected to electrophoresis both before and after treatment with proteinase K. No shift in position of the already heterodisperse terminal restriction bands could be detected. One explanation for this could be that many termini do not have tightly attached protein and that any shift that might occur is masked by band heterogeneity. Alternatively, bound protein could retard labeling, which occurs selectively at terminal single-strand gaps as previously reported (5).
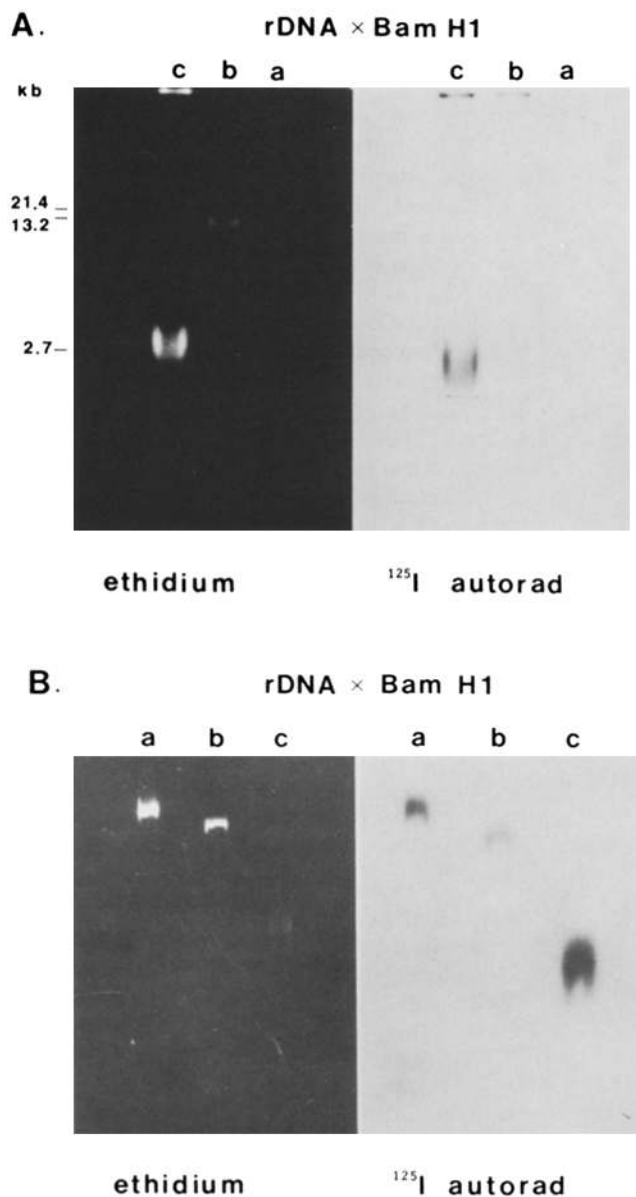
A.

rDNA × Bam H1

ethidium ^{125}I autorad

B.

rDNA × Bam H1

ethidium ^{125}I autorad

FIGURE 2 ^{125}I-labeled protein bound to *Physarum* rDNA restriction fragments. Labeling and purification of rDNA on two CsCl gradients was as described in Materials and Methods. The rDNA (5–10 μg) was digested with Bam HI as previously described (4) and subjected to electrophoresis on 1.4% agarose gels containing 0.1% SDS. For panel A, bands were excised, and DNA extracted by electroelution was again subjected to electrophoresis on a 1.5-mm-thick agarose gel. On this gel, band c is overrepresented to enhance visibility. For panel B, excised bands were placed directly onto a second 1.4% agarose gel, and rDNA fragments are thus represented in equimolar quantities. Gels were dried and autoradiographed using Dupont Cronex film and an intensifying screen for 7 d at −80°. Note that the bulk of protein label is in the Bam c terminal restriction fragment even though this fragment contains <10% of the total rDNA.

Release of Labeled Proteins from rDNA by Treatment with DNase I or Staphylococcal Nuclease

Aliquots of labeled rDNA, isolated after two CsCl gradients as previously described, were treated with DNase I and subjected to SDS gel electrophoresis followed by autoradiography to visualize labeled protein bands. Fig. 3 *A* shows that DNase I releases ^{125}I in primarily two protein bands. These do not correspond to the electrophoretic positions of any of the *Physarum* histones, which were run on the same 15% polyacrylamide gel. The molecular weights of the labeled rDNA proteins released by DNase I are ~5,000 and 13,000 daltons (measurements not shown); these are labeled *I* and *II*, respectively, in Fig. 3. In Fig. 3 *B*, it can be seen that brief treatment with either DNase I or staphylococcal nuclease releases protein II. This band appears very diffuse, possibly as a doublet, after DNase I treatment. Protein I is not significantly released from rDNA by staphylococcal nuclease but is released by DNase I. Diffuse labeled material is seen at molecular weights >40,000. This label and label at the top of the gel may represent other proteins on the rDNA but could also be the result of incomplete digestion of DNA protein by the nucleases used. In Fig. 3 *B* it can be seen that proteinase K treatment abolishes all label and that no labeled nucleotide fragments appear on these SDS gels.

When ^{125}I-labeled rDNA restriction fragments were eluted from agarose gels, they could be digested with DNase I and their proteins analyzed. Fig. 4 shows an autoradiograph of the Bam c complex, isolated as in Fig. 2, treated with DNase I and subjected to electrophoresis on a 15% SDS polyacrylamide gel. Track c shows proteins released by digestion of labeled DNA eluted from the agarose gel. Bands corresponding to proteins *I* (5,000–6,000 mol wt) and *II* (13,000 mol wt), as seen in Fig. 3, are visible. In this case, band *II* is resolved as two bands. Bands are also visible at 40,000–45,000 mol wt. These are not prominent when total rDNA is digested as in Fig. 3. They may represent aggregates of proteins or proteins and DNA. They may also represent proteins present in low numbers specifically at the rDNA termini. Label at the top of the gel is aggregated protein-DNA not digested by DNase I. Track *d* was obtained after digestion of labeled material that could not be eluted from the agarose gel Bam c band. Homogenized agarose was loaded directly onto the polyacrylamide gel. This labeled material is evidently protein-DNA bound to agarose and cannot be digested with DNase I. Comparison of Figs. 3 and 4 with Fig. 2 shows that proteins of low molecular weight (5,000 and 13,000) found on the rDNA molecule are located selectively at the termini.

Electron Microscope Visualization of Proteins Bound to rDNA

To see proteins bound to the rDNA in samples spread for electron microscopy, we used a procedure (10) that employs rabbit anti-DNP antibody to bind proteins previously derivatized using DNFB. This enhances the size of protein complexes on the DNA so that they can be recognized after shadowing. When intact rDNA is reacted with DNFB and antibody and spread as described in Materials and Methods, protein complexes are visualized as seen in Fig. 5. At the top of Fig. 5 is a representative rDNA molecule with complexes located within 3 kb of each end and another possible complex at ~8 kb. (For purposes of measurement all of these complexes were scored.) Of the full-length rDNA molecules measured, 92% had antibody complexes. Of these molecules, 61% had complexes in at least one terminal spacer and ~6% had complexes near both termini. At the bottom left of Fig. 5 are shown termini from six separate full-length rDNA molecules that possessed complexes. In each case a complex is located within 1–3 kb of the actual terminus; in only a small fraction of molecules were complexes seen directly at the terminus. We mapped the positions of complexes visualized on rDNA molecules treated with DNFB

FIGURE 3 SDS gel electrophoresis of ^{125}I-labeled proteins released from rDNA by nuclease treatment. (A) 15% Polyacrylamide gel showing bands released by treatment with DNase I. Each sample contained rDNA labeled and purified as described, 1,000 cpm, with (+) or without (−) treatment with DNase I. At the left of the gel, total *Physarum* histones were electrophoresed for comparison. The large band visible with Coomassie stain is BSA added to dialysis bags to prevent binding. (B) 12% Polyacrylamide gel showing results of treatment of labeled rDNA-protein complexes with DNase I (*DNase*), with or without subsequent treatment with proteinase K (*PK*), and staphylococcal nuclease (*SN*). Each sample contained rDNA labeled and purified as described, 1,000 cpm, and the indicated nuclease. Arrows adjacent to autoradiographs identify labeled bands released by nuclease treatment in the ranges of 5,000 daltons (*I*) and 13,000 daltons (*II*). Control sample rDNA (*cont*) received no enzyme treatment.

A.

DNase I

II→

I→

[125]I autorad

coomassie

B.

+SN +DNase+PK +DNase cont

←II

←I

[125]I autorad

A   B   C   D   E   C   D

— 43.0

— 25.7

— 18.4

— 14.3
— 12.3
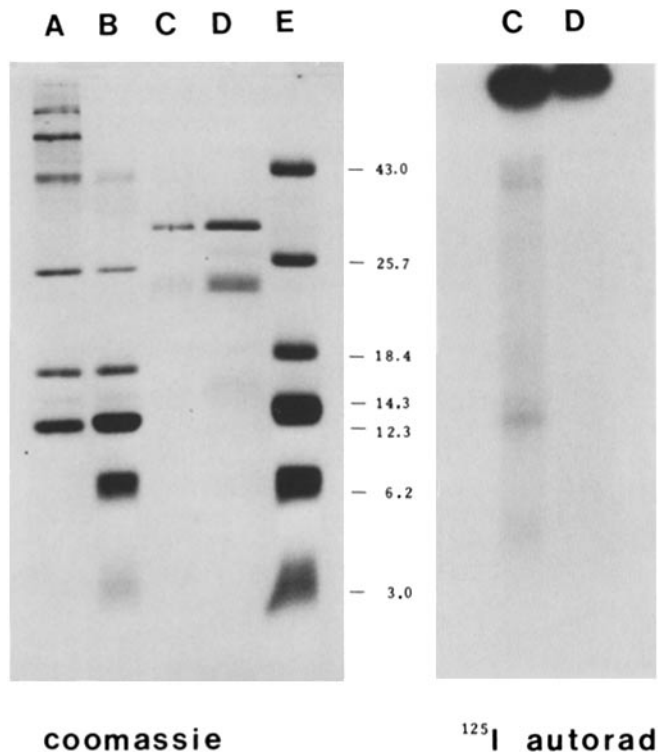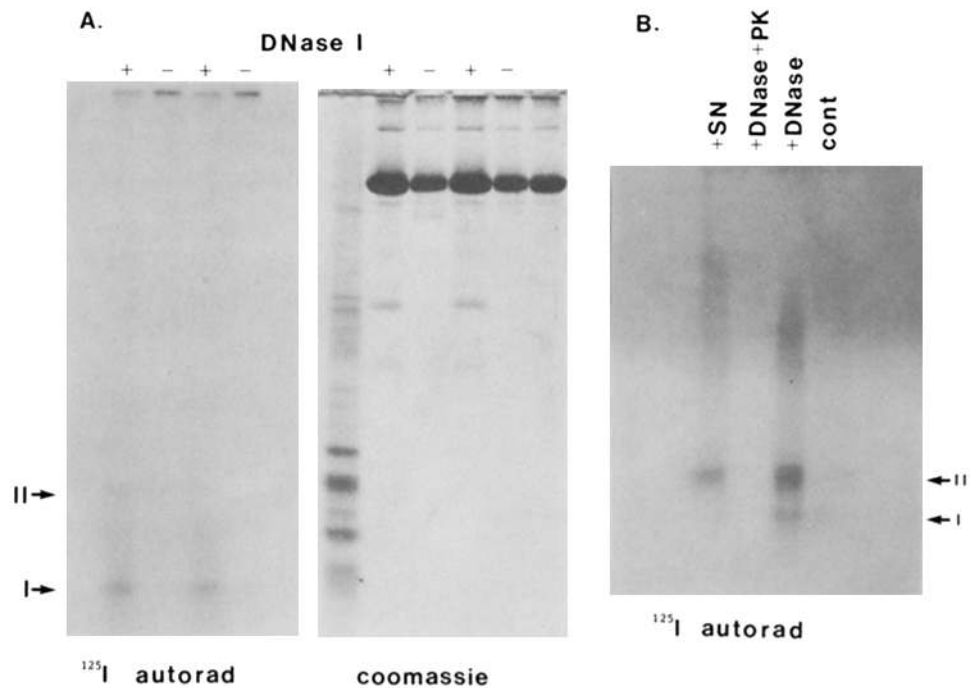
— 6.2

— 3.0

coomassie   [125]I autorad

FIGURE 4 Release of labeled protein from the Bam HI rDNA terminal restriction fragment by DNase I. The terminal rDNA Bam c fragment was isolated by electoelution after digestion of rDNA by

and antibody as shown in the histogram at the bottom right of Fig. 5. As a control we mapped positions of complexes on the same number of rDNA molecules treated with antibody but not with DNFB. This control is adequate to demonstrate that antibody does not selectively associate with single-strand regions on the DNA. It is clear that proteins recognized by the anti-DNP antibody are preferentially located within 1-2 kb of the actual rDNA termini. Complexes seen on other parts of the rDNA molecule may represent proteins bound to rDNA, but their distribution does not differ significantly from that of complexes seen without DNFB. Comparison with the map at the top of the histogram shows that the region of protein binding corresponds approximately to that region known to possess multiple inverted repeats and selectively-located single-strand gaps. The location of bound protein relative to the

Bam HI and electrophoresis of resulting fragments on an agarose gel as in Fig. 2. Track C: electroeluted Bam c was ethanol precipitated, redissolved in 50 μl of digestion buffer, and treated with 5 U of DNase I for 2 h at 37°C. The digestion was stopped by adding 3 × SDS sample buffer, and electrophoresis was on a 15% polyacrylamide gel. Track D: labeled Bam c protein remaining bound to the agarose gel after electroelution was treated with DNase I. The agarose containing the Bam c band was homogenized, dialyzed against the DNase I digestion buffer, digested, and stopped as described. The reaction mixture was then loaded directly into the gel sample well. Markers (tracks A, B, and E) are ovalbumin (43,000), α-chymotrypsinogen (25,700), β-lactoglobulin (18,400), lysozyme (14,300), cytochrome c (12,300), bovine trypsin inhibitor (6,200), and insulin (3,000). The prominent Coomassie blue–stained bands in tracks C and D are the result of added DNase I.
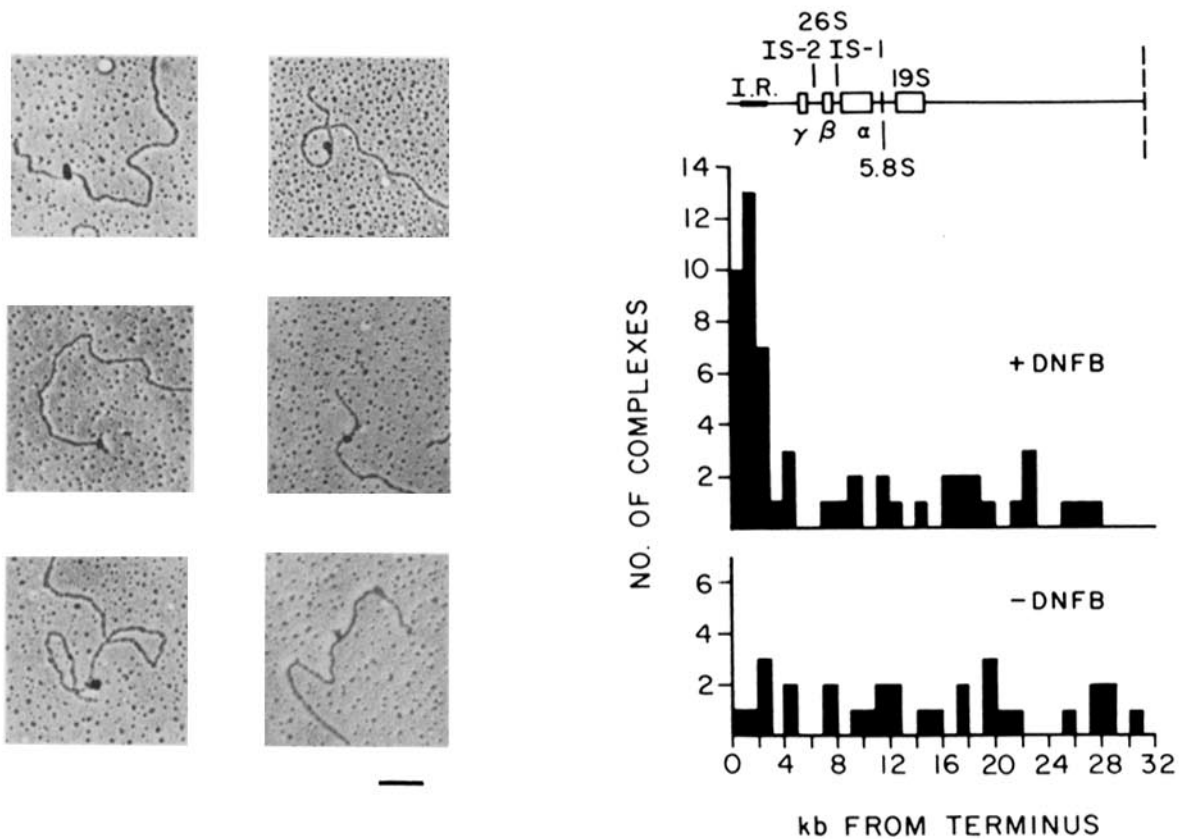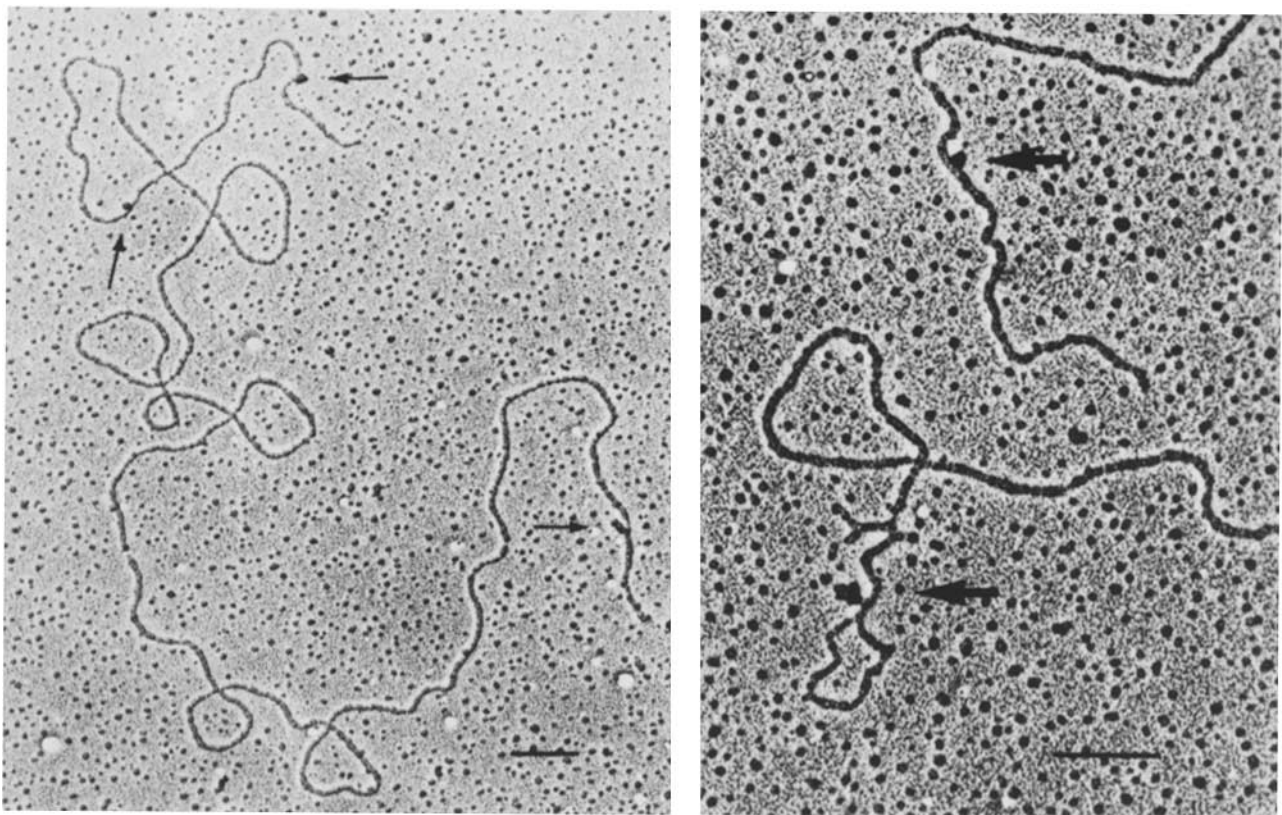
FIGURE 5   Electron microscope visualization of rDNA-protein complexes after treatment with DNFB and rabbit anti-DNP antibody. The rDNA-protein complexes were extracted and purified by CsCl gradient centrifugation. Reaction with DNFB followed by complexing with antibody and the *S. aureus* protein A were as described in Materials and Methods. DNA was spread from a solution containing 50% formamide onto a 20% formamide hypophase and shadowed with Pt-Pd as previously described (4, 5). Lengths were determined using as standards rRNA R-loops spread simultaneously. Each terminus shown is from a full-length (55–65 kb) rDNA molecule. Bars indicate 1 kb. Arrows indicate electron-dense structures scored as rDNA-protein complexes. Of full-length rDNA molecules measured, 72% possessed at least one antibody complex. For the histogram at bottom, the locations of complexes on 25 experimental rDNA molecules and 25 control rDNA molecules (without DNFB) were mapped. The high-magnification micrograph at upper right shows one end of a molecule with a circularized terminus and a protein complex located distal to a region of rDNA secondary structure.

inverted repeat region can be seen in the few micrographs obtained in which a terminus possessed both a protein complex and a foldback sequence, the latter possibly because of partial denaturation with formamide during spreading. An electron micrograph with these features is seen at the top right of Fig. 5. In this case the protein complex is located ~200–500 bp distal to the inverted repeat region.

DISCUSSION

Our results indicate that protein is very tightly bound to the *Physarum* rDNA molecule in a region 1–2 kb from the termini. This location corresponds approximately to a region previously shown to contain multiply repeated inverted repeat sequences of ~100 bp and specific nicks or gaps (5). Nick translation at these discontinuities begins with the sequence CCCTA (5). An unusual chromatin subunit structure containing a repeated sequence at the termini of *Tetrahymena* rDNA has been reported, suggesting the presence of nonhistone proteins on rDNA termini in that organism (11). We have not yet determined the precise positions of the *Physarum* rDNA proteins with respect to inverted repeat sequences and single-strand gaps. Based on specific activity of labeling with ^{125}I Bolton-Hunter reagent, it can be estimated that 1–5% of rDNA molecules possess protein selectively bound tightly to termini. This could reflect inefficiency of labeling but could also indicate that protein is bound as part of a transient enzymatic process and is not constantly associated with all termini.

When either rDNA or isolated rDNA termini are digested with DNase I, discrete protein bands of 5,000 (*I*) and 13,000 (*II*) mol wt are detected, the latter most likely two bands (Figs. 3 and 4). Digestion of terminal restriction fragments also releases protein bands of 40,000–45,000 mol wt (Fig. 4). The structural relationship of these protein bands is not known. They may be subunits of a larger protein-DNA complex or they may be structurally and functionally distinct. It is notable that staphylococcal nuclease efficiently releases band *II* but not band *I* from rDNA (Fig. 3). This suggests that band *II* may be a dimer of band *I* connected by a short DNA segment resistant to staphylococcal nuclease. Proteins of the same molecular weights are detected on rDNA whether or not nucleoli are treated with phenol immediately after isolation. Thus proteolysis is probably not a factor in these experiments although this cannot be ruled out entirely. In Fig. 2, significant protein label is observed on the large Bam a rDNA fragment, which consists primarily of the rRNA transcription unit. This label could be attributable to the presence of undigested rDNA, but it may also represent protein attached to sequences on or near the transcription unit. Electron micrographs have not thus far revealed any selective location for such protein (Fig. 5). Specific activities of iodination alone do not allow calculation of the number of proteins per rDNA molecule, but in some electron micrographs we could see more than one complex per terminus, as seen at the bottom right of the six photographs of rDNA ends in Fig. 5, suggesting multiple binding sites for proteins. The nature of the chemical bond between protein and rDNA has yet to be determined. It has been observed that proteins linked to termini of herpes simplex virus DNA segments are apparently not covalently bound even though they are SDS resistant (12). In the present case, resistance to SDS, GuCl, CsCl, formamide, and urea suggests a covalent linkage, and lability to NaOH would be consistent with a phosphoester linkage although other bonds are possible.

In adenovirus a single bound protein is linked directly to the 5′ terminus of each strand and may serve as a primer for initiation of replication (13). This protein is present on a high percentage of adenovirus molecules and is attached through a phosphoserine linkage. It is unlikely that rDNA bound protein in *Physarum* serves an identical primer function because rDNA replication initiates in the center of the molecule and proceeds toward the ends (6). The terminal protein of *Physarum* may, however, be involved in rDNA replication because some unknown priming mechanism must be involved in replicating 3′ ends of the linear molecule. It is conceivable that covalent attachment of proteins occurs during formation of single-strand discontinuities. (Eucaryotic DNA type I topoisomerases may function via covalent DNA intermediates [14].) If such nicks are made in terminal repeats, a replication mechanism can be envisioned in which DNA synthesis and strand displacement occur at a repeat followed by circularization of the terminus, ligation, and strand scission to form a completed 3′ end. Such a mechanism has been proposed for mitochondrial DNA in *Tetrahymena* (15). The involvement of terminal protein in this way is consistent with the electron micrograph in Fig. 5 showing a circularized terminus attached near a protein complex, a frequently observed configuration.

Terminal protein may also be involved in integration of the rDNA with chromosomal DNA components. Little is known about any such process in *Physarum*, but the suggestion is based on observations that the terminal inverted repeat sequences are reiterated in chromosomal DNA (5). Terminal protein could organize a transient chromosomal association of rDNA resulting in equitable distribution of ribosomal genes during mitosis or meiosis.

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