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Higher order DNA structure in macronuclear chromatin of the hypotrichous ciliate *Oxytricha nova*

(DNA–protein interaction/multistranded DNA/chromosome structure)

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ABSTRACT **On lysis of macronuclei from the ciliated protozoan *Oxytricha* at 0.5–2 M NaCl, the DNA, which is normally found as discrete molecules ranging from 0.5 to 20 kilobases, appears in high molecular weight aggregates. Various treatments of the macronuclear lysate (i.e., nucleases, proteases, variation of salt, pH, and temperature) indicate that preservation of the aggregate structure depends on both nucleic acid–nucleic acid and nucleic acid–protein interactions. Purification of the DNA–protein complex after lysing the nuclei in 2 M NaCl shows that one major nuclear protein copurifies with the DNA. As shown by DNA–protein binding experiments, this protein has a high affinity for DNA; however, no evidence for sequence specificity of the protein binding was obtained. Chromatin reconstitution experiments suggest that the protein in itself is not sufficient for DNA aggregation in nuclei, but other factors, possibly the native chromatin structure, are required. Electron microscopy of the purified DNA–protein complex showed structures similar to those observed previously with *in vitro*-aggregated purified macronuclear DNA (14). A model is presented in which the terminal inverted repeat sequences found on all macronuclear DNA molecules interact with each other forming multistranded DNA complexes. The formation of these structures may be accelerated and stabilized by a protein *in vivo*.**

During macronuclear development in *Oxytricha nova* and other hypotrichous ciliates, macronuclear DNA becomes specifically fragmented into "gene-sized" DNA molecules (1–6). Each macronuclear DNA molecule carries the same terminal inverted repeat sequence (7), which is 5′-$C_4A_4C_4A_4C_4$ (8); similar terminal repeats have been found in other ciliates (8, 9). In addition, each macronuclear DNA molecule has a protruding 3′ single-stranded tail of the sequence 3′-$G_4T_4G_4T_4$ (8). (For a schematic drawing of one *Oxytricha* macronuclear DNA molecule, see Fig. 7A).

All macronuclear DNA fragments are associated with typical eukaryotic histones (10) and are organized into nucleosomes (11). When macronuclei are lysed in low salt-containing buffers (<50 mM NaCl), the chromatin occurs in short pieces, each corresponding to one macronuclear DNA molecule (12). However, electron microscopic studies of gently lysed macronuclei show only long continuous chromatin fibers that consist of closely spaced 300 Å superbeads, suggesting that the gene-sized chromatin fragments may be organized into higher order structures *in vivo* (ref. 13; K. G. Murti, personal communication). One possible mechanism of organizing gene-sized DNA molecules into higher order structure was suggested by the observation that purified macronuclear DNA can aggregate end-to-end *in vitro* by formation of multistranded DNA complexes at the ends of each molecule (14). In this paper, we present evidence that the gene-sized macronuclear DNA molecules form higher order structures *in vivo* and that DNA·DNA and DNA–protein interactions are involved in these associations.

MATERIALS AND METHODS

Oxytricha nova was grown on *Chlorogonium* and macronuclei were isolated as described (3). Macronuclei were lysed in 10 mM Tris·HCl, pH 7.0/10 mM EDTA containing 0.5–2 M NaCl, and the soluble chromatin was either used directly or purified by passing it through a Sepharose 4B (Pharmacia) column. Total macronuclear DNA was isolated as described by Elsevier *et al.* (4); the preparation of the terminal C_4A_4 probe will be described elsewhere.

DNA and DNA–protein complexes were analyzed on 1% agarose gels, and proteins were separated on 7.5–17.5% gradient $NaDodSO_4$/polyacrylamide gels (15). 5′ Labeling of nucleic acids was carried out as described by Maxam and Gilbert (16), 3′ labeling of nucleic acids was as described by Tu and Cohen (17), and nick-translation of DNA was done as described by Rigby *et al.* (18). Transfer of nucleic acids to diazobenzyloxymethyl paper was done by the technique of Alwine *et al.* (19). Transfer of proteins from $NaDodSO_4$/polyacrylamide gels to nitrocellulose filters and analysis of protein–DNA interactions were done essentially as described by Bowen *et al.* (ref. 20; see also Figs. 3–6). Protein concentration was determined by the technique of Lowry *et al.* (21). Electron microscopy of nucleic acids was carried out as described (6).

Enzymes used were Pronase, RNase A, RNase T1, RNase H, nuclease S1, DNase I, and exonuclease BAL-31.

RESULTS

Macronuclei were lysed in 10 mM Tris·HCl, pH 7.0/10 mM EDTA containing NaCl at 0.5–2 M. When these nuclear lysates were subjected to agarose gel electrophoresis, the typical low molecular weight macronuclear DNA banding pattern (Fig. 1, lanes 1 and 6) could not be seen; instead, the DNA appeared as a smear in the high molecular weight region of the gel (lanes 2–5). This pattern occurred with DNA at 100 μg/ml to 2 mg/ml and therefore was independent of chromatin concentration. To determine the stability of these high molecular weight structures under physiological ionic conditions, nuclei were lysed in 2 M NaCl and the DNA, along with any DNA-bound proteins, was separated from dissociated material by passing the lysate through a Sepharose 4B column. After this purification step, the DNA was extensively dialyzed against 150 mM NaCl/10 mM Tris·HCl, pH 7.0/10 mM EDTA and analyzed on agarose

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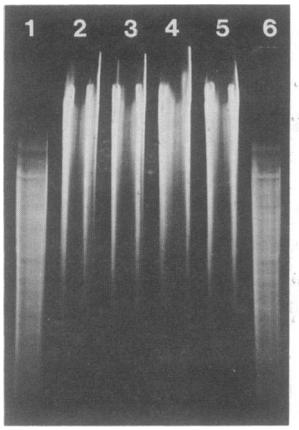

FIG. 1. Agarose gel electrophoresis of macronuclear DNA and macronuclear lysates. Lanes: 1 and 6, macronuclear DNA; 2–5, macronuclei lysed with 0.5, 0.9, 1.2, and 2 M NaCl, respectively. To exclude the effect of salt on the mobility of the different preparations, all samples were made 2 M NaCl prior to electrophoresis. DNA was separated on 1% agarose gels in 40 mM Tris acetate, pH 7.8/20 mM NaOAc/20 mM EDTA.

gels. As shown in Fig. 2*B*, the high molecular weight complex is preserved under physiological ionic conditions.

To define the molecular species involved in forming the high molecular weight structures, the nuclear lysate was heated, treated with $NaDodSO_4$, or treated with a number of enzymes (Table 1). Treatment with RNase A and T1, RNase H, or the single-strand-specific nuclease S1 did not destroy the high molecular weight DNA aggregates. High temperature treatment, however, did disaggregate the high molecular weight structure. The temperature necessary for disaggregation is dependent on the salt concentration in the nuclear lysate. Thus, at 0.5 M NaCl, heating for 10 min to 65°C was sufficient for disaggregation but, at 2 M NaCl, at least 10 min at 75°C was necessary to obtain the low molecular weight DNA banding pattern (Fig. 2*A*, lane 2). Further experiments showed that disaggregation of macronuclear DNA can also be achieved by treatment with 1% $NaDodSO_4$ (Fig. 2*B*, lane 4), dialysis against low salt (10 mM Tris·HCl, pH 7.0; data not shown; ref. 12), treatment with Pronase (Fig. 2*A*, lane 3, and 2*B*, lane 5), or washing nuclei at high pH (pH 9.5, see Fig. 6). The facts that disaggregation can be achieved by high temperature and that the temperature needed is dependent on the salt concentration used suggest that a nucleic acid–nucleic acid interaction is involved in the preservation of a higher order structure of DNA in chromatin. Further indications for a nucleic acid interaction between macronuclear gene-sized DNA molecules are the observations that disaggregation occurs when nuclei are lysed in very low salt (12) or when nuclei are washed at high pH prior to lysis (see Fig. 6). However, the fact that disaggregation can also be achieved by $NaDodSO_4$ or Pronase treatment indicates that proteins are required to maintain the high molecular weight aggregate.

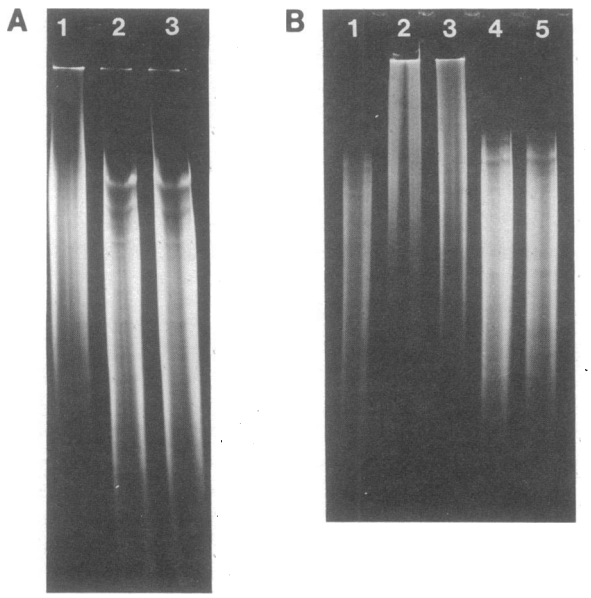

FIG. 2. Effects of different treatments on nuclear lysates. (*A*) Effect of temperature and Pronase on the high molecular weight complex. Macronuclei were lysed in 2 M NaCl/10 mM Tris·HCl, pH 7.0/10 mM EDTA (lane 1), treated for 10 min at 75°C (lane 2), or digested for 30 min with Pronase at 1 mg/ml (lane 3). (*B*) Macronuclei were lysed as described in *A*, and the lysate was passed through a Sepharose 4B column equilibrated with the same buffer. Lanes: 1, macronuclear DNA; 2, nuclear lysate after purification over Sepharose column; 3, purified lysate was dialyzed against 150 mM NaCl/10 mM Tris·HCl, pH 7.0; 4, 1% $NaDodSO_4$ was added to the sample shown in lane 3; 5, the same sample was digested with Pronase at 1 mg/ml for 30 min.

Table 1. Effects of different treatments on the nuclear lysates

Treatment	Time	Effect
Heat (65–75°C)	10 min	Disaggregation; temperature needed depends on salt concentration (Fig. 2*A*)
$NaDodSO_4$ (1%)	—	Disaggregation (Fig. 2*B*)
Pronase (1 mg/ml)	30 min	Disaggregation (Fig. 2 *A* and *B*)
RNase A (200 μg/ml) and 3000 units of RNase T1	60 min	No effect on aggregation
RNase H (500 units; A_{260}, 20)	60 min	No effect on aggregation
Nuclease S1 (10^5 units; A_{260}, 20)	60 min	No effect on aggregation
Exonuclease BAL-31 (12.5 units; A_{260}, 20)	60 min	No effect on aggregation
High pH*	—	Disaggregation (Fig. 6)
Low ionic strength†	—	Disaggregation (12)

* Nuclei were extracted twice with 150 mM NaCl/50 mM Tris·HCl, pH 9.5.

† Nuclei were lysed without salt.

To identify the proteins still bound to macronuclear DNA at 2 M NaCl, we lysed macronuclei at this salt concentration and purified the DNA–protein complex by passing it through a Sepharose 4B column. The purified DNA–protein complex was either used to determine the DNA/protein ratio or analyzed on $NaDodSO_4$/polyacrylamide gels; for comparison, total macronuclear proteins were subjected to electrophoresis on the same gel. As shown in Fig. 3*A* (lanes 1 and 3), a major nuclear protein (M_r, ≈50,000) copurifies with the DNA. This protein also comprises a major band in total macronuclear protein (Fig. 3*A*, lane 2). Determination of the DNA/protein ratio showed 4–6 μg of protein per 100 μg of DNA. Since the average molecular weight of *Oxytricha* macronuclear DNA is $\approx 1.5 \times 10^6$ and the approximate molecular weight of the protein is 50,000, one or two protein molecules should be present per macronuclear DNA molecule. To provide further evidence that the M_r 50,000 protein represents a DNA-binding protein, total macronuclear proteins were transferred from $NaDodSO_4$/polyacrylamide gels to nitrocellulose filters and incubated with either ^{32}P-labeled macronuclear DNA or ^{32}P-labeled terminal repeat sequence (C_4A_4 probe; unpublished). Macronuclear DNA binds to many proteins, including strong binding to the M_r 50,000 protein (Fig. 3*B*, lane 1), while the C_4A_4 probe binds somewhat more strongly to the M_r 50,000 protein than to other proteins. DNA binding to the M_r 50,000 protein can still be detected after extensive washing of the filters, indicating that it has a high affinity for DNA.

Since the ends of the DNA molecules might be involved in

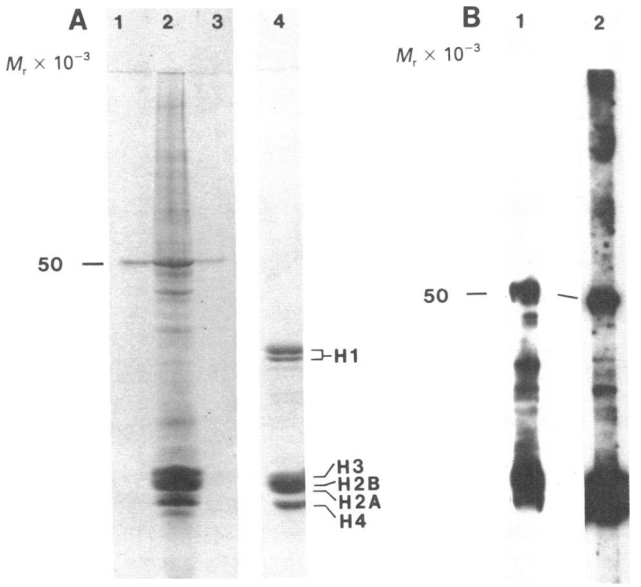

FIG. 3. (*A*) Gradient (7.5–17.5%) $NaDodSO_4$/polyacrylamide gel electrophoresis of total macronuclear proteins and proteins copurified with the DNA after lysing nuclei in 2 M NaCl. Lanes: 2, total macronuclear proteins were prepared for electrophoresis by boiling purified macronuclei in $NaDodSO_4$ mixture (15) for 5 min; 1 and 3, purification of the proteins still bound to DNA at 2 M NaCl was achieved by passing the nuclear lysate over a Sepharose 4B column, and the flow through was dialyzed against distilled water, freeze dried, suspended in $NaDodSO_4$ mixture, and boiled for 5 min; 4, calf thymus histones extracted from calf thymus nuclei with 0.2 M H_2SO_4. (*B*) Total macronuclear proteins were separated on 7.5–17.5% gradient $NaDodSO_4$/acrylamide gels and transferred to nitrocellulose filters. Filters were incubated in Denhardt's medium/100 mM NaCl/2 mM EDTA/0.1 mM dithiothreitol/10 mM Tris·HCl, pH 7.0, containing either ^{32}P-labeled total macronuclear DNA or ^{32}P-labeled C_4A_4 probe. After incubation, filters were washed at least five times in the incubation medium but without DNA probe. Lanes: 1, protein filters incubated with total macronuclear DNA; 2, protein filters incubated with the C_4A_4 probe. Bovine serum albumin, cytochrome *c*, and calf thymus histones were used as molecular weight standards.

the preservation of the high molecular weight DNA–protein complex, we tested whether these ends are somehow protected in chromatin against exonuclease activity. We digested purified macronuclear DNA and macronuclei with exonuclease BAL-31 for various times. After digestion, purified DNA was incubated in 2 M NaCl for 36 hr and then subjected to electrophoresis on a 1% agarose gel to determine whether it was still able to aggregate. Nuclei were extracted with 2 M NaCl after the digestion, and the nuclear lysate was also analyzed on an agarose gel. As shown in Fig. 4, purified DNA was not able to aggregate after digestion for 5–15 min, a time that would be sufficient to remove 30–60 bases under our digestion conditions. Thus, the sequence at the macronuclear DNA ends (i.e., 5′-$C_4A_4C_4A_4C_4$) plays an essential role in DNA aggregation. However, no or very little effect on the high molecular weight DNA–protein complex was observed after 60 min of digestion, showing that the ends of the gene-sized DNA molecules in chromatin are somehow protected against exonuclease activity (Fig. 4).

We have considered the possibility that the 3′ single-stranded protruding ends of the macronuclear DNA molecules may be filled in with a low molecular weight nucleic acid or nucleic acid–protein complex forming bridges between molecules, resulting in linear aggregates. To identify low molecular weight nucleic acids that may fill in the 3′ overhang, nuclei were lysed in 2 M NaCl and the DNA–protein complex was purified on Sepharose columns. This complex was then treated with

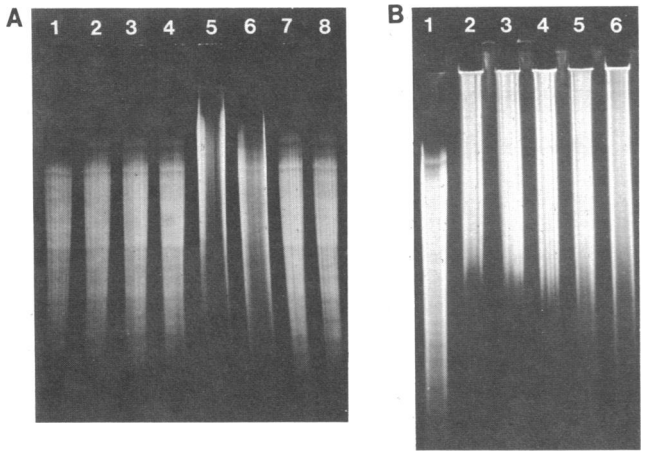

FIG. 4. Effect of exonuclease BAL-31 on DNA aggregation and on the high molecular weight DNA–protein complex. Twenty micrograms of purified DNA or macronuclei (A_{260}, 0.4) was digested with 0.25 unit of BAL-31 in 150 mM NaCl/12 mM $CaCl_2$/12 mM $MgCl_2$/1 mM EDTA/20 mM Tris·HCl, pH 8.0, for 5–60 min. After digestion, an aliquot of the DNA was made 2 M in NaCl and allowed to aggregate for 36 hr at room temperature. Ability to aggregate was analyzed on 1% agarose gels. (*A*) Lanes: 1–4, purified DNA was digested for 0, 5, 15, and 30 min, respectively, with BAL-31; 5–8, aliquots of the DNA were incubated for 36 hr in 2 M NaCl after digestion for 0, 5, 15, and 30 min, respectively. (*B*) Lanes: 1, macronuclear DNA; 2–6, macronuclei were digested with BAL-31 for 0, 5, 15, 30, and 60 min, respectively, and lysed in 2 M NaCl, and the lysates were analyzed on 1% agarose gels.

Pronase and the mixture was extracted with phenol several times. Nucleic acids were 5′ labeled and analyzed on 10% and 20% acrylamide gels. A number of low molecular weight bands were found; however, the banding pattern was not consistent in different DNA preparations. Furthermore, when these bands were transferred to diazobenzyloxymethyl paper and hybridized with the C_4A_4 probe, no hybridization could be detected. Therefore, we assume that the low molecular weight nucleic acids observed in these preparations are either degradation products copurified with the DNA–protein complex or created during the purification and labeling procedure.

Evidence that the nucleic acid interaction in chromatin may be similar to the *in vitro* aggregation observed with purified macronuclear DNA (14) was obtained from electron microscopic examination of the nuclear lysates. When macronuclear DNA was allowed to aggregate *in vitro*, end-to-end joining of several gene-sized DNA molecules to form V-like ("necks") junction structures was seen in the electron micrographs. As outlined before (14), these structures are not expected if the DNA aggregates via complementary single-stranded ends. In the present study, similar structures were detected by electron microscopy of the DNA–protein complex. Since the high molecular weight DNA–protein aggregate was obtained by lysing macronuclei in 2 M NaCl followed by purification on Sepharose 4B, no chromatin structure can be seen but long aggregates of end-to-end joined gene-sized DNA molecules are present. More than 90% of the DNA molecules were found in these aggregates and the number of joined DNA molecules in one aggregate varied from 3 to >15. This variable number may be due to partial disintegration of DNA–protein complexes during preparation. The M_r 50,000 protein apparently involved in linkage of the DNA molecules is too small to be detected by the electron microscope techniques used (Fig. 5).

Several attempts to reconstitute the high molecular weight DNA–protein complex from disaggregated material were made. When the purified DNA–protein complex was dissociated in low salt or by heat treatment and then dialyzed against 150 mM

NaCl/10 mM Tris·HCl, pH 7.0/10 mM EDTA, the high molecular weight aggregates were not reformed, suggesting that in addition to the protein other factors must be required for the reconstitution. We therefore took advantage of the fact that, after extracting macronuclei several times with 150 mM NaCl/50 mM Tris·HCl, pH 9.5, the high molecular weight DNA–protein aggregate is no longer observable (Fig. 6*A*) although, as shown by gel electrophoresis of nuclear proteins, the M_r 50,000 protein is still present in the nuclei. Dialysis of chromatin treated as described above against 150 mM NaCl/50 mM Tris·HCl, pH 7.0, results in rapid reconstitution of the high molecular weight DNA–protein complex (Fig. 6*B*).

DISCUSSION

Our results show that the DNA from *Oxytricha* macronuclei lysed in high salt occurs as a high molecular weight aggregate. Since *in vitro* aggregation of purified macronuclear DNA is a very slow process (14), we conclude that the structures observed are not due to aggregation of DNA after cell lysis. The results do not necessarily prove that these structures are the *in vivo* configuration of DNA in macronuclear chromatin, although experiments at physiological ionic strength show that they are stable under physiological ionic conditions. Most of our experiments were carried out in 2 M NaCl, since under these conditions most of the nuclear proteins are dissociated from the DNA and the number of variables is kept to a minimum.

The experiments testing the stability of these high molecular weight DNA aggregates suggest that both nucleic acid–nucleic acid and protein–nucleic acid interactions are required for preservation of these structures. In fact, it was shown that, after lysing the nuclei in 2 M NaCl and purification of the DNA–protein complex, one major nuclear protein copurifies with the DNA. One to two molecules of this protein appeared to be present per macronuclear DNA molecule. This number is as expected for a protein involved in linkage of the gene-sized DNA fragments into larger units. Our DNA binding experiments to nuclear proteins provide further evidence that this protein has a high affinity to DNA. However, the preliminary reconstitution experiments suggest that this protein in itself is not sufficient for DNA aggregation. Other factors, possibly the native chromatin structure, may be necessary to ensure the correct action of this protein. Although the M_r 50,000 protein alone may not be sufficient for DNA aggregation, it certainly accelerates formation of the high molecular weight complex in chromatin to a great extent. The observation that the ends of macronuclear DNA molecules are protected against exonuclease activity suggests that they are associated with a protein, perhaps the M_r 50,000 protein.

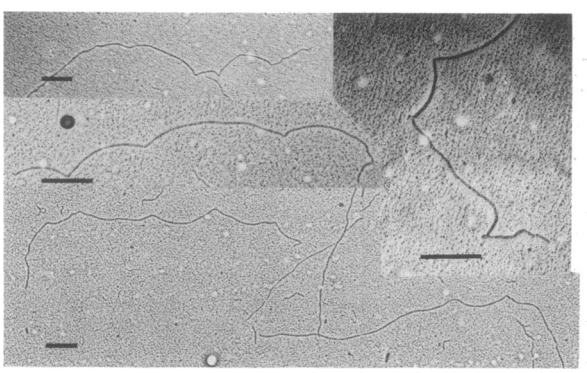

FIG. 5. Electron micrographs of nuclear lysates. Macronuclei were lysed in 2 M NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0, and the DNA–protein complex was purified on a Sepharose 4B column. Electron microscopy was carried out as described (10). Bar = 0.2 μm.

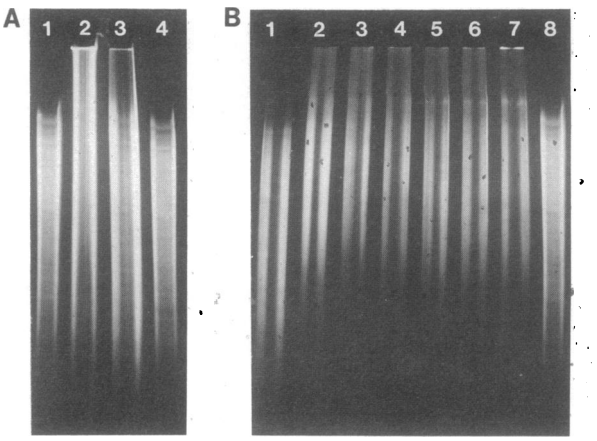

FIG. 6. (*A*) Effect of high pH on the high molecular weight DNA–protein complex. Lanes: 1, macronuclear DNA; 2, macronuclei were lysed in 2 M NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0; 3, macronuclei were extracted twice with 150 mM NaCl/10 mM EDTA/50 mM Tris·HCl, pH 9.5, washed once with 150 mM NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0, and extracted with 2 M NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0; 4, macronuclear DNA. (*B*) Reconstitution of the high molecular weight complex in chromatin. Nuclei were extracted twice with 150 mM NaCl/10 mM EDTA/50 mM Tris·HCl, pH 9.5, and then dialyzed against 150 mM NaCl/10 mM EDTA/50 mM Tris·HCl, pH 7.0. After various times, the chromatin was pelleted by centrifugation, washed once with 150 mM NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0, and extracted with 2 M NaCl/10 mM EDTA/10 mM Tris·HCl, pH 7.0, and analyzed on a 1% agarose gel. Lanes: 1–8, 0, 1, 2, 4, 6, 8, and 24 hr of dialysis, respectively.

Several experiments were carried out to investigate the nature of the nucleic acid–nucleic acid interaction involved in preservation of the DNA–protein complex. Treatment with RNase A and T1, RNase H, or nuclease S1 did not destroy the aggregate, even when all enzymes were used in large excess.

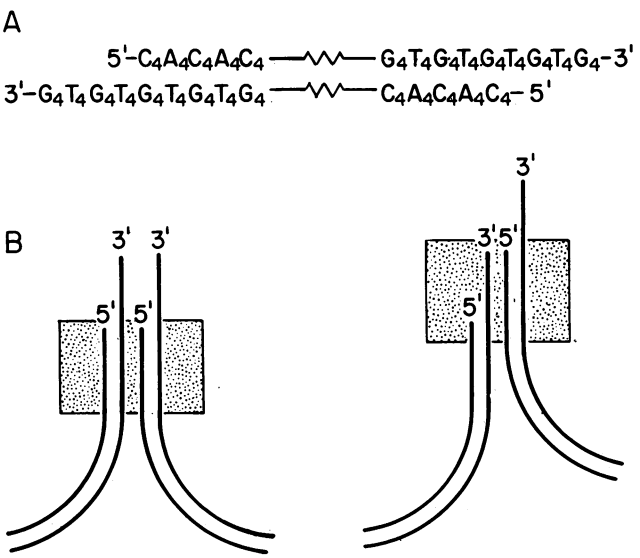

FIG. 7. (*A*) Suggested structure for gene-sized macronuclear DNA molecules showing inverted terminal inverted repeats and the 3′ single-stranded tail. (*B*) Suggested models for linkage of gene-sized DNA molecules into larger units. (*Left*) Terminal inverted repeat sequences form tetrastranded DNA complexes that are stabilized by a protein. (*Right*) Since the 3′ single-stranded tail is a repeat of the terminal repeat sequence, formation of triple-stranded DNA complexes is possible; this structure would also be stabilized by a protein.

Although this result could be explained by protection of the sequences involved, we were unable to identify any specific low molecular weight nucleic acids in our DNA preparations. Therefore, it appears unlikely that a nucleic acid species forms a bridge between macronuclear DNA molecules. These facts make it more likely that the C_4A_4 sequences at ends, but not the single-strand tails of the macronuclear DNA molecules, interact with each other. This suggestion is also supported by the appearance of the DNA–protein complex in the electron microscope. Structures similar to those observed with *in vitro* aggregated DNA (14) can be seen. We therefore believe that the two mechanisms are somehow related. It is also of interest that our electron micrographs resemble the structure of two DNA molecules with homologous ends held together by recA protein (22). From the data discussed here, we propose a model for the linkage of DNA molecules in the chromatin of macronuclei (Fig. 7). In the model, terminal inverted repeat sequences interact with each other, forming multistranded DNA complexes (Fig. 7*B Left*). It has been shown (14) that such an interaction is possible and that the 3′ single-stranded tail does not seem to be necessary for this process. Space filling models demonstrate that such multistranded structures are reasonable (23). Since additional hydrogen bonds would be formed between the two double helices, formation of such structures would be salt dependent and could be reversed by either low salt, high temperature, or high pH. The same arguments hold for the model presented in Fig. 7*B Right*. Since the 3′ single-stranded tail is an extension of the terminal inverted repeat, formation of a triple-stranded DNA region would be possible. Structures like this have been described in synthetic polymers (24) and are known to be stable when associated with recA protein (see refs. 22, 25). Since the formation of three- and four-stranded structures is very slow and such structures would be very unstable at physiological ionic strength, such formation could be accelerated and stabilized by a protein that does not have to be sequence specific but must be at least to some extent structure specific.

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