S1 and S2, the linear mitochondrial DNAs present in a male sterile line of maize, possess terminally attached proteins

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ABSTRACT

    S1 and S2 are short linear mitochondrial DNA molecules found in a particular male sterile cytoplasm of maize. We show here that these DNA molecules and two other related linear DNA species found in maize mitochondria, have proteins attached, probably covalently, to their 5' ends. This is the first demonstration of such a linear DNA-terminal protein association in higher eukaryotes. Such proteins may be involved in priming replication of these DNAs.

INTRODUCTION

    Each of the four different maternally inherited cytoplasms of U.S.A. corn belt maize has a characteristic array of low molecular weight double stranded mitochondrial DNA (mtDNA) molecules (1). The mtDNA of N, male fertile, cytoplasm contains linear molecules of 2.35 kb and supercoiled plasmids of 1.94 kb. T, cytoplasmically male sterile (cms), mtDNA has the same molecules except that the linear DNA has suffered a deletion of about 200 bp. C cms mtDNA has the same complement as N plus two additional circular DNAs of 1.57 and 1.42 kb. S cms also possesses the same molecules as N in addition to two linear DNAs of about 6.2 and 5.2 kb, termed S1 and S2 respectively (1,2). All these DNA molecules are observed as discrete bands when subjected to electrophoresis on 1.5% agarose gels (1).

    The linear structure of S1, S2 and the 2.35 kb molecules was inferred from two observations. When these DNA species were isolated from agarose gels and submitted to electron microscopy, no circular molecules were observed. Also, S1 nuclease treatment of a mtDNA preparation did not alter the electrophoretic mobility of these DNA species. These results were in contrast to the behavior, in parallel experiments, of the supercoiled 1.94 kb mtDNA species common to all maize cytoplasms (1).

    S1 and S2 probably arose by an excision and rearrangement of sequences from the high molecular weight mitochondrial genome, because closely related

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sequences are present in the high molecular weight DNA of N cytoplasm (4,5,6).
However, the homologous sequences are not full-length contiguous copies of S1
and S2. S1 and S2 are present in approximately a five-fold molar excess
compared to high molecular weight mtDNA sequences in S cytoplasm mitochondria
(4). They possess terminal inverted repeats of about 200 bp (7) and in S cms
plants which have spontaneously reverted to fertility (8), it has been
suggested that they have transposed into the mitochondrial genome (9),
although an alternative explanation, involving sequence rearrangement, is
possible (10). They are the only discrete bands observed when crude mtDNA
lysates are electrophoresed in agarose gels (11), suggesting that they are
resistant to endogenous nucleases.

The presence of linear DNA species in maize mitochondria prompted us to
investigate the nature of their DNA termini in order to understand how the
molecules are protected from nuclease degradation *in vivo* and the mode of DNA
replication used.

We report that the linear molecules have a protein bound at the 5'
termini. This protein may protect against degradation and be involved in the
replication of the DNA molecules.

MATERIALS AND METHODS
Maize genotypes
The genotypes used in this study were B37 carrying S, N and T cytoplasms,
B37 x H84 carrying S, W64A carrying N and T, WF 9 carrying C, C0192 x WJ
carrying CA, J, B (all members of the S group of cytoplasms; 12,13,14), LF
(member of the N group; 13,14), and HA (member of the T group; 12,13,14)
cytoplasm.
Isolation of mitochondrial DNA
MtDNA was isolated from 4-day old dark grown coleoptiles as previously
described (14). This procedure involved lysing intact mitochondria in 50 mM
Tris-HCl pH 8.0, 10 mM EDTA, 2% sarkosyl and 0.012% autodigested pronase or
proteinase K at 37°C for 1 h, prior to several phenol-chloroform (1:1)
extractions. In experiments designed to retain any covalently attached DNA-
protein complexes, mitochondria were isolated by the same method (14) and
lysed in a solution in which the pronase or proteinase K component had been
replaced with phenylmethylsulphonyl fluoride. The lysate was made 8M with
respect to guanidinium chloride and reincubated at 37°C for 1 h. Cesium
chloride (to 2.7 M) and buffer was added to reduce the guanidinium chloride
concentration to 4M and the DNA-protein complexes were purified by equilibrium

centrifugation. Gradients were fractionated, and an aliquot of each fraction was treated with proteinase K prior to electrophoresis in order to locate the position of the various DNA molecules.

Electrophoresis of DNA

Electrophoresis of mtDNA molecules in 1% or 1.5% agarose gels was as described previously (14).

DNA modification reactions

DNA was 5' end labelled with γ-^{32}P-ATP (Amersham) using polynucleotide kinase (Boehringer) and alkaline phosphatase (BRL) (15). Cordycepin 5' triphosphate and terminal deoxynucleotidyl transferase (New England Nuclear) were used to ^{32}P label the 3' ends (16). Linearized pBR322 was used as a positive control in both the above reactions. Xba I, Eco RI and Hae III were purchased from BRL. Exonuclease III and lambda α-exonuclease were gifts from Dr. W. Holloman (Dept. Immunology and Medical Microbiology, University of Florida). ^{3}H labelled P22 DNA was used as a positive control for both exonuclease reactions.

RESULTS

Mitochondria from a S cms line were isolated and lysed in the absence of pronase or proteinase K. The mtDNA was then purified by banding in a cesium chloride equilibrium gradient containing 4M guanidinium chloride. The gradient was fractionated and each fraction was dialysed against 10 mM Tris-HCl pH 7.5, 1 mM EDTA and divided into two equivalent samples. One sample from each fraction was incubated at 37°C for one hour with 500 μg. ml^{-1} proteinase K. Each pair of samples was then fractionated by electrophoresis in adjacent lanes (Fig. 1). S1, S2 and n (the 2.35 kb linear DNA) only appear as discrete bands in the gel after proteinase K treatment. In the absence of proteinase K treatment, these DNAs either do not fully enter the gel or else their migration is retarded. We attribute this effect to exclusion by the gel matrix of a DNA-protein complex. The levels of S1 and S2 visible in the untreated samples of fractions 10 to 13 are due to non-specific protolysis during the mtDNA preparation. The mobility in the gel of the high molecular weight mtDNA band is not markedly affected by proteinase K treatment, and therefore provides an internal control. Fractionation of the cesium chloride-guanidinium chloride gradient showed that the high molecular weight mtDNA banded tightly in a bell-shaped distribution (Fig. 1, fractions 9-13). In contrast, the distribution of all the linear molecules was skewed towards the upper, less dense part of the gradient. The molecules behaved similarly when
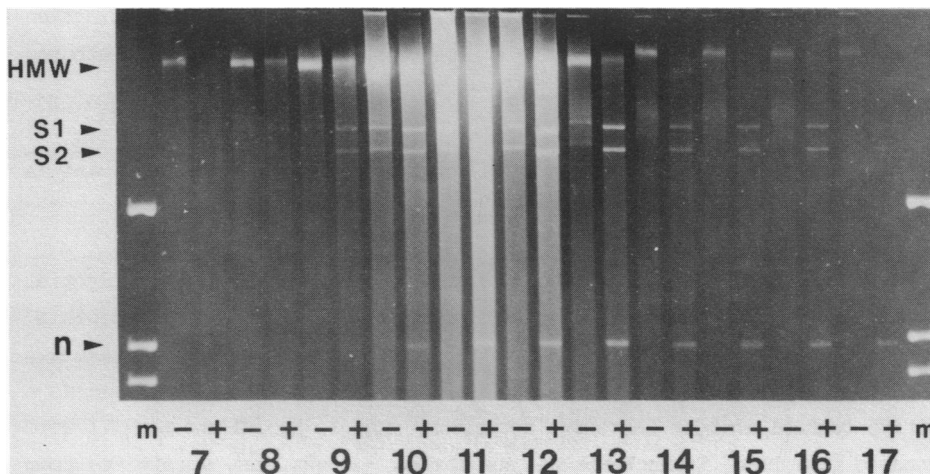
Figure 1. Electrophoretic analysis in 1.5% agarose of cesium chloride-guanidinium chloride gradient fractions of S cms mtDNA. Only the area around the center of the gradient, where the mtDNA banded, is shown, i.e. fractions 7 to 17. The fraction at the bottom of the gradient was designated 1 and the fraction at the top 26. Each aliquot was either not treated (-) or treated (+) with proteinase K (0.05% w/v), 37°C, 1 hour prior to electrophoresis. m represents size marker lanes of Hae III digested λ DNA. Vertical labelling indicates DNA molecules of interest (HMW represents high molecular weight mtDNA).

rebanded on a second gradient.

Similar results were obtained when this experiment was conducted with mtDNA isolated from N, T (Fig. 2), and C (data not shown) cytoplasm lines. Figure 2 shows that the migration of high molecular weight DNA and the 1.94 kb plasmid is unaffected by proteinase K, indicating that these species are not tightly associated with protein. However, both the linear DNA species n and t migrate in the gel as discrete bands only after treatment by proteinase K. The distribution of both species in the gradients is skewed towards the less dense fractions.

Heating the DNA preparations to 70°C and treatments with sodium lauryl sulphate, 8M guanidinium chloride, phenol, chloroform, β-mercaptoethanol and dithiothreitol did not make the linear DNA species enter the gel.

Optical density measurements of the linear DNAs also indicate that they are associated with protein. A cesium chloride-guanidinium chloride gradient fraction enriched in S1, S2 and n (e.g. fraction 16 in Fig. 1), after removal of the salts, has a 260/280 nm value of 1.47. Treatment of the same fraction with proteinase K at 37°C for one hour followed by three phenol-chloroform
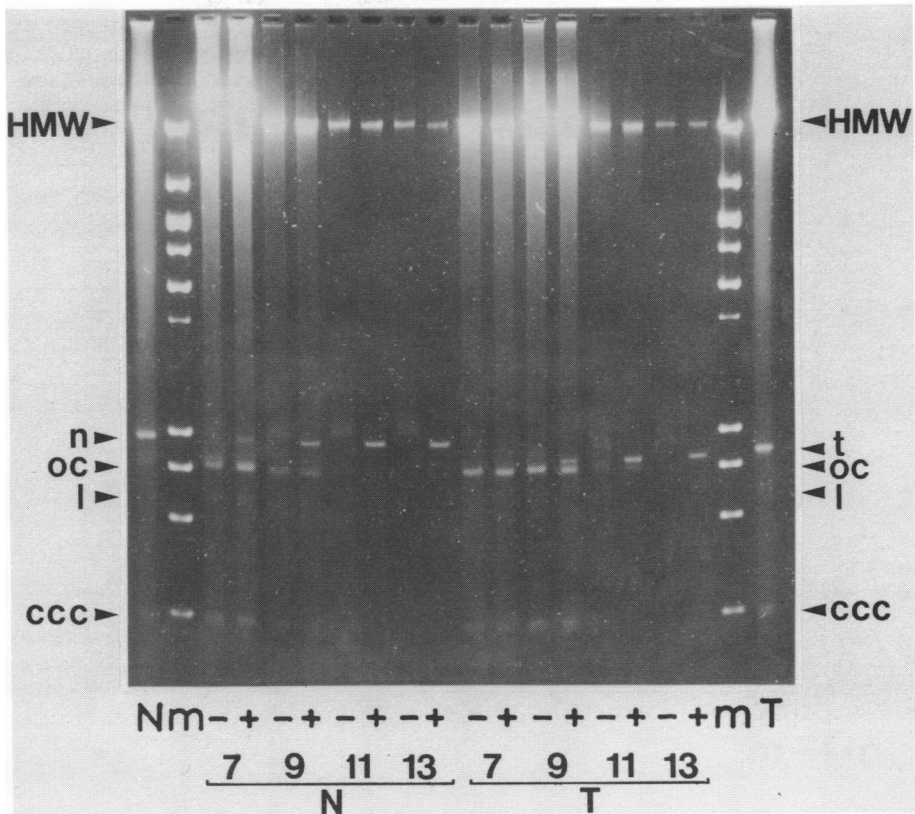
Figure 2. Electrophoretic analysis in 1.5% agarose of cesium chloride-guanidinium chloride gradient fractions of mtDNA isolated from seedlings with N and T cytoplasms. Only alternate fractions around the center of each gradient, where the mtDNA banded, are shown. The fraction at the bottom of the gradient was designated 1, and the fraction at the top 22. Labelling key as in Figure 1 except that the size marker lanes (m) are a mixture of Eco RI and Hae III digests of λ DNA. Lanes N and T represent total mtDNA from N and T mitochondria respectively which were lysed in the presence of pronase and not subjected to cesium chloride-guanidinium chloride gradient centrifugation. ccc, oc and 1 represent the position of the supercoiled, open-circular and linear conformations respectively of the common 1.94 kb plasmid.

extractions changed the value to 1.8. Total S cms mtDNA preparations which have been isolated in the presence of pronase and extracted three times with phenol and chloroform routinely exhibit a 260/280 nm value of about 1.95.

To determine if the protein is attached at or near the termini of the linear DNAs, a cesium chloride-guanidinium chloride gradient fraction enriched in S1, S2 and n DNAs was, after dialysis, treated with the restriction
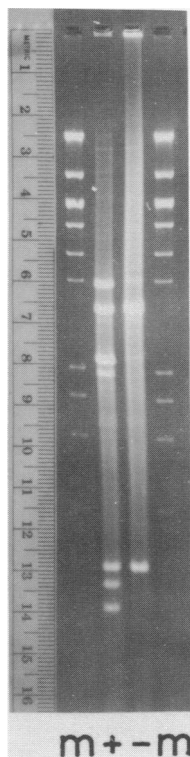
Figure 3. Electrophoretic analysis in 1.5% agarose of an XbaI digest of a cesium chloride-guanidinium chloride gradient fraction enriched in S1, S2 and n DNAs. Size markers (m) are as in Figure 2. Treatment was with (+) or without (-) proteinase K.

endonuclease Xba I. This enzyme has two cleavage sites in S1 producing terminal fragments of 2447 and 717 bp and an internal fragment of 3016 bp. It also has two cleavage sites in S2 producing terminal fragments of 3333 and 879 bp and an internal fragment of 963 bp (7). There is no Xba I cleavage site in n DNA. After completion of the restriction digest the sample was divided into two parts. One was treated with proteinase K, the other was not. Both were then subjected to agarose gel electrophoresis (Fig. 3). In the sample not treated with proteinase K, only two major bands are visible. These correspond to the internal Xba I fragments of S1 and S2, i.e. 3016 and 963 bp respectively. The terminal fragments of both S1 and S2 and the entire n DNA did not migrate into the gel unless the sample was treated with proteinase K. This indicates a specific association of protein at or near the ends of the linear DNAs. The minor bands showing fluorescence in Figure 3 are Xba I fragments derived from the relatively low concentration of high molecular weight mtDNA in the gradient fraction.

Exonuclease III, which has a 3' to 5' exonuclease activity, and lambda α-

exonuclease, which has a 5' to 3' exonuclease activity, were used to determine if protein was attached at each end of the linear DNA strands (Fig. 4). A cesium chloride–guanidinium chloride gradient fraction enriched in S1, S2 and n DNAs was, after dialysis, divided into three parts. One part was treated with exonuclease III, one with λ-exonuclease and the other part was untreated. They were then incubated with proteinase K prior to agarose gel electrophoresis. The expected bands corresponding to S1, S2 and n DNAs are observed in the control sample not treated with exonucleases (Fig. 4, lane 4). Exonuclease III completely digested all three linear DNAs (lane 5) whereas they were resistant to λ-exonuclease digestion (lane 6). Lanes 7, 8 and 9 represent a repeat of the experiments in lanes 4, 5 and 6 respectively except that the proteinase K step prior to electrophoresis was omitted. As expected, no discrete bands are seen in any of the three lanes. However, a smear of fluorescence is visible in the untreated (lane 7) and λ-exonuclease treated
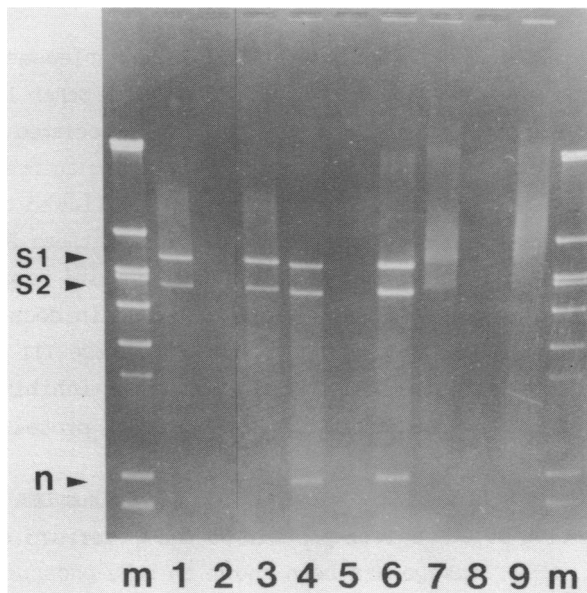

Figure 4. Electrophoretic analysis in 1% agarose of exonuclease treatments. Samples in lanes 1, 4 and 7 were untreated, lanes 2, 5 and 8 have been treated with exonuclease III and lanes 3, 6 and 9 have been treated with λ-exonuclease. DNA samples used were: proteinase K treated S1 and S2 DNAs purified from other mtDNAs on a linear sucrose gradient prior to exonuclease treatment (lanes 1, 2, 3); a cesium chloride–guanidinium chloride gradient fraction treated with the exonucleases then with proteinase K (lanes 4, 5, 6); and the same gradient fraction treated with the exonucleases but not the proteinase K (lanes 7, 8, 9). Labelling as in Figure 2.

(lane 9) samples indicating that a small quantity of DNA-protein complex had migrated in the gel but not to the position corresponding to protein-free DNA. This smear of fluorescence is completely absent in the exonuclease III treated samples (lane 8). Sl and S2 DNAs isolated from proteinase K treated mitochondria and separated from other mtDNAs on a linear sucrose gradient (4) were either untreated (lane 1), treated with exonuclease III (lane 2) or λ-exonuclease (lane 3). The two linear DNAs are visible in lane 1, completely absent in lane 2 and visible in lane 3, indicating that even after proteinase K treatment the 5' ends of the DNAs are still protected by peptide residues.

These results have been verified by end labelling experiments. We were able to ^{32}P label the 3' ends (16) of Sl and S2 whether or not the DNAs had been treated with proteinase K. However we were unable to label the 5' termini of Sl and S2 with γ-^{32}P-ATP and T4 polynucleotide kinase (15), despite labelling λ-EcoRI termini included as an internal control (Fig. 5).

DISCUSSION

Using proteases, exonucleases, restriction endonucleases and DNA end-labelling techniques, we have shown that Sl, S2 and two other linear DNAs (n and t) found in maize mitochondria have a 5' terminal associated protein. The resistance of the DNA-protein complexes to many denaturing agents suggests the linkage may be covalent. Proteinase K treatment, followed by phenol and chloroform extractions, does not leave the 5' termini of these DNAs available for end-labelling (Fig. 5), presumably because the termini remain linked to amino acid(s). The presence of the 5' attached protein does not however sterically hinder the 3' ends of the DNA, since exonuclease III digestion and 3' terminal labelling with terminal transferase is not inhibited. Current experiments are aimed at characterizing the terminal protein by in vitro labelling with ^{125}I.

A similar DNA-protein complex described for adenovirus DNA is due to the covalent linkage of a 55,000 dalton protein to the 5' termini of the linear DNA strands (17). The linkage has been shown to be a phosphodiester bond between the β-OH of a serine residue and the 5'-OH of the terminal deoxycytidine residue in the adenovirus DNA (18). The protein is required for virus replication since it serves as a primer for DNA polymerase enabling the enzyme to initiate daughter strand synthesis at the 5' termini (17,18). It is tempting to hypothesize that the proteins attached to the linear maize mtDNAs could provide a similar priming function.

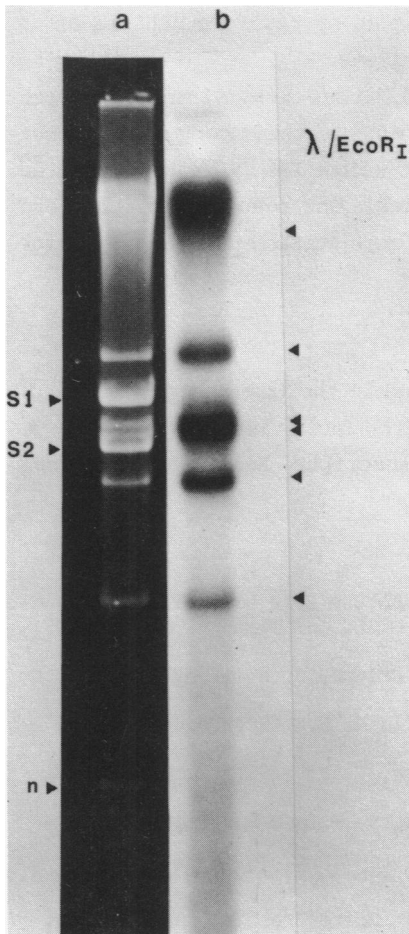Recently, we have identified, in maize mitochondria, replicative

Figure 5. 2 μg of S mtDNA and 1 μg of a λ EcoRI digest were each incubated with 0.8 units of calf alkaline phosphatase in 0.1 M Tris-HCl pH 8.0, 10 mM MgCl$_2$, 1 mM ZnCl$_2$. After phenol and chloroform extractions, and precipitation with ethanol, the samples were combined and incubated with 4 units T$_4$ polynucleotide kinase and 50 μCi γ-^{32}P-ATP (Specific activity 2000 Ci. mmol^{1}). One fifth of the sample was fractionated by electrophoresis in 1% agarose and stained with ethidium bromide (lane a). Lane b is an autoradiograph of the same gel. The unmarked arrows represent the position of the EcoRI fragments of λ. Other labelling as in Figures 1 and 2.

intermediates of S1 and S2 which also appear to have protein associations (10). Similar DNA-protein associations have been reported in Streptomyces spp. (19) and phage φ29 of Bacillus subtilis (20) in addition to adenovirus, but our study, we believe, is the first report of such a linkage in higher eukaryotes.

If the intact linear DNA species were to be cloned, for example, by homopolymeric tailing, we would expect the blocked 5' termini to be unavailable for ligation. However, such a procedure has been reported to give full-length clones (6). Such clones could result from phosphodiesterase activity during DNA isolation or in the bacterium causing hydrolysis of the peptide-DNA bond. Alternatively, the blocked residue might be eliminated by

nuclease action in the bacterium, and replaced by repair synthesis of an unmodified terminus, using the 3' end as a template.

The discovery of these plasmid-like mtDNA species, S1 and S2, which apparently replicate independently (on the basis of their copy number), but are able to recombine with the high molecular weight DNA (9,10) has suggested their use as vectors for plant transformation. The results reported here suggest that the DNA-protein complexes may be more suitable than naked DNA for such a role.

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