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# Mapping of rRNA genes in an inverted repeat in Nicotiana tabacum chloroplast DNA

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## ABSTRACT

Nicotiana tabacum chloroplast DNA contains two copies each of 16S and 23S rRNA genes. These genes are located in an inverted order as determined from restriction fragment mapping and Southern hybridization to restriction fragments. The position of these genes on the N. tabacum chloroplast DNA molecule has been established relative to a complete map of SalI and SmaI restriction enzyme cleavage sites.

## INTRODUCTION

Recently, there has been intensive research to determine the structural properties of chloroplast DNA from higher plants and algae. Several common structural features have been observed. Kolodner and Tewari (1) found that chloroplast DNA from spinach, oats, corn, peas and lettuce exists as supercoiled circular molecules of 130-150 kilobase pairs (kbp). Circular chloroplast DNA from the algae Chlamydomonas reinhardii and Euglena gracilis has been observed (2,3) which is consistent with the circular map of uniparental genes of Chlamydomonas (4).

Electron microscopic studies have revealed the presence of two copies of a large sequence which are in inverted order in the chloroplast DNA of maize, spinach, oats, and lettuce (5). Restriction endonuclease mapping has verified the presence of these inverted repeats in spinach and maize chloroplast DNA (6,7). A similar repeated sequence has also been found in Chlamydomonas chloroplast DNA (2). Southern hybridization analysis of chloroplast DNA restriction fragments from maize, spinach and Chlamydomonas

 

has shown that a chloroplast rRNA cistron maps in each of these repeated sequences (2,6,7) and that they are also in inverted order. However, not all chloroplast DNA's possess this inverted repeat. For instance, chloroplast DNA of Euglena has three copies of the rRNA cistrons arranged in tandem (8), and pea chloroplast DNA has the characteristic two copies of rRNA genes which, however, seem to be arranged in tandem (5).

Chloroplast DNA of tobacco (Nicotiana tabacum) was shown (9) to have a kinetic complexity of about 150 kbp and can be isolated as large circular molecules (10-12). Two copies of genes for 16S and 23S chloroplast rRNA have been found on tobacco chloroplast DNA (10,11,13). We have mapped restriction enzyme sites on tobacco chloroplast DNA to facilitate establishing the location of specific genes which might be present on this DNA (10,11 and Jurgenson and Bourque, in preparation). We find the size of tobacco chloroplast DNA to be 146 kbp, based on measurements of the size and stoichiometry of restriction fragments and based on contour length measurements (11 and Jurgenson and Bourque, in preparation).

This paper shows that the genes for tobacco chloroplast rRNA are arranged in an inverted orientation as are the repeated sequences in which they exist. These conclusions result primarily from two dimensional gel electrophoretic analysis of double restriction enzyme digests of chloroplast DNA. These analyses establish the restriction map order, relative to SalI fragments, of several XhoI and KpnI fragments which contain the repeated sequences.

## METHODS

Chloroplast DNA was prepared by a modification of the methods described by Wong and Wildman (14). NaI was used in place of CsCl in the step gradients used for isopycnic purification of the DNA. Chloroplast DNA was digested with restriction enzymes, the fragments separated on agarose gels and

the DNA transferred to nitrocellulose by the Southern procedure as modified by Wahl, et al. (15). Chloroplast rRNA was isolated from ribosomes (16), repurified on sucrose gradients, and labeled in vitro with $^{125}I$ to a specific activity of 3 x $10^6$ dpm/ug (17,18). Hybridization mixtures contained, per ml, 2 x $10^6$ cpm $^{125}I$ in either 16S or 23S rRNA. If radioactive 23S rRNA was used, a 10-fold excess of 16S rRNA was added to compete with possible contaminating, labeled 16S rRNA. If $^{125}I$-16S rRNA was used, a similar excess of 23S rRNA was added. In addition, 25 ug of phenol-extracted E. coli tRNA, 0.2% SDS, 2mM KI, 1 mM EDTA, 25mM Tris Ph 8.0, 4xSSC (standard saline-citrate) and 50% formamide were included in the hybridization solution. Hybridization was in a sealed polyethylene bag for 14 hr at 47°C. The filters were then washed twice for 30 min in the hybridization mixture lacking RNA at 47°C and then washed for 30 min in 2xSSC. Filters were then treated for 1 hr at 23°C with 25 ug/ml RNAse A in 2xSSC, and finally washed in 2xSSC by stirring for 2 to 4 hr or until no radioactivity could be detected on the filters with a survey monitor. Autoradiographic exposures were from 2 to 10 days at -80°C with a Dupont lightning-plus fluorescence screen on each side of the filter and with a sheet of Kodak RP Royal X-Omat film adjacent to the filter. The two dimensional agarose gel system used to resolve products of double restriction enzyme digestion were made as previously described (19). Molecular weights of chloroplast DNA restriction fragments were estimated relative to EcoRI digestion fragments of lambda cI857 S7 and $T5^+$ DNA.

## RESULTS

The data in Figure 1 establish the presence of two copies of 16S and 23S rRNA genes on tobacco chloroplast DNA. The two largest XbaI fragments (25.6 and 18.0 kbp) hybridize to 23S chloroplast rRNA, as do SalI fragments of 25.6 and 18.0 kbp. The XbaI and SalI fragments of equal size are not identical. Cleavage with SalI reduces the size of each of the two large XbaI

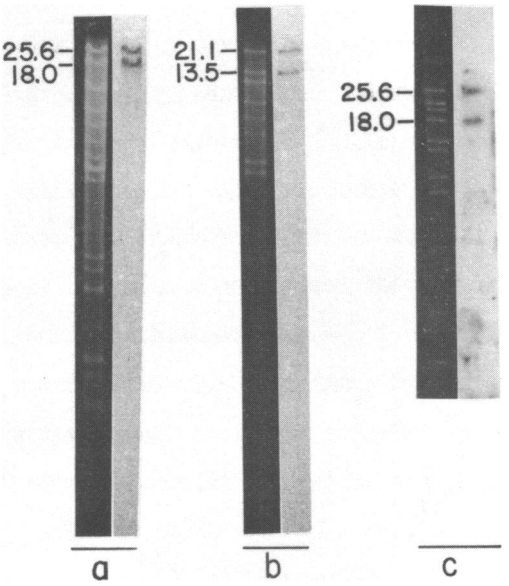


Figure 1: Restriction enzyme digests of N. tabacum chloroplast DNA with XbaI (a), XbaI and SalI (b), SalI (c) and their hybridization to 23S chloroplast rRNA. Gels in (a) and (b) were 0.8% agarose; gel in (c) was 0.6% agarose. Photograph of ethidium bromide-stained gels are on right and radioautographs are on left in each case.

fragments by 4.5 kbp (Figure 1), and the resulting double digestion products also hybridize to 23S rRNA. Results obtained using 16S chloroplast rRNA as labeled hybridization probe are identical to those described for 23S rRNA. It can be concluded from these data that SalI does not cleave either of the two 16S or 23S rRNA genes. Two dimensional gel analysis (Figure 2) shows that the 25.6 and 18.0 kbp XbaI fragments contain extensive sequence homology, since their partial EcoRI digestion products are resolved into an almost identical series of lower molecular weight fragments by electrophoresis in the second dimension. Figure 3 shows the position of these XbaI fragments on a complete map of SmaI and SalI fragments. The presence of 22.6 kbp repeated regions indicated in Figure 3 has been determined from molecular weight measurements and mapping of SmaI, XbaI and SalI fragments (11 and Jurgenson and Bourque, in

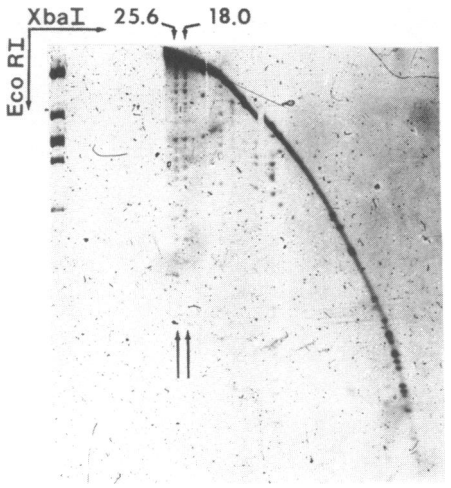


Figure 2: Two dimensional gel separation of XbaI and EcoRI double digests of chloroplast DNA. Arrows indicate the 25.6 and 18.0 kbp XbaI fragments separated in the first dimension which are partially digested with EcoRI and whose double digest fragments are resolved by electrophoresis in the second dimension. An EcoRI digest of lambda DNA was run on the left of the slab for reference. The diagonal ethidum bromide stained streak is nuclear DNA which was present in this chloroplast DNA preparation. XbaI fragments which are not cut by EcoRI are seen along this streak. Discrete fragments appear as spots on two dimensional gels (see also Figure 5).

preparation. SalI fragments A and D (25.6 and 18.0 kbp) contain the rRNA genes and are adjacent (data not shown). The positioning of the SmaI and SalI fragments on the map in Figure 3 suggests that the repeated sequences and the rRNA genes are in inverted order. Figure 6 shows details of the region of the map containing the rRNA as deduced from the data presented on this paper.

Hybridization of 16S and 23S rRNA to chloroplast DNA fragments generated by XhoI, KpnI and double digests in various combinations with these enzymes and with SalI, strongly supports the proposed inverted orientation of the chloroplast rRNA genes (Figure 3). Southern hybridization of 23S and 16S rRNA to KpnI digestion fragments shows (Figure 4a) that both 16S and 23S rRNA hybridizes to two KpnI fragments (30.2 and 25.3 kbp). 16S rRNA hybridizes equally to these bands but 23S rRNA hybridizes more strongly to the 25.3 kbp

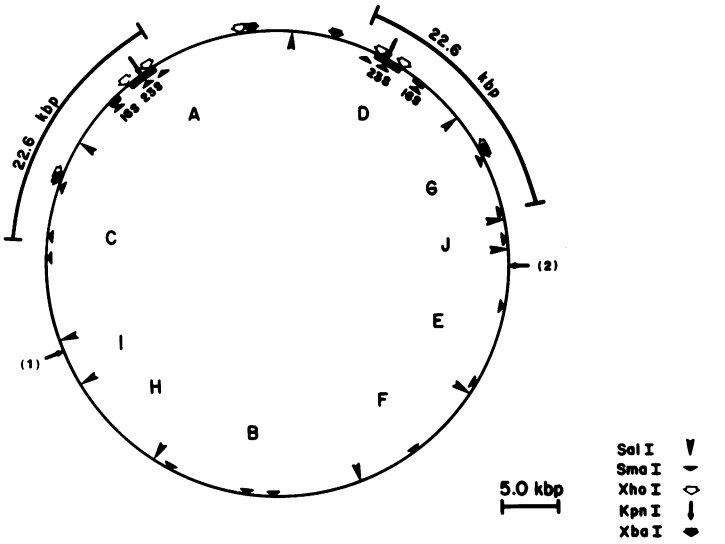


Figure 3: Restriction map of chloroplast DNA showing the location of SmaI enzymes and with SalI, strongly supports the proposed inverted orientation of and SalI sites. Location of the XbaI, XhoI and KpnI sites which generate fragments containing portions of 16S and 23S rRNA genes are indicated. SalI fragments are indicated by capital letters (A-J) in order of decreasing size. The position of the inverted repeat region is shown by brackets.

fragment, suggesting that more of the 23S rRNA gene is present in the 25.3 kbp fragment. Hybridization of 23S rRNA occurs to XhoI fragments of 14.9, 9.7 and 3.3 kbp (Figure 4b). Double digestion of the XhoI fragments with SalI cuts the 14.9 kbp fragment to give a 9.0 kbp fragment which hybridizes to 23S rRNA (Figure 4c). Double digestion of XhoI fragments with KpnI (Figure 4e) cleaves the 3.3 kbp XhoI fragment to a 0.5 kbp fragment and one of 2.7 kbp which still hybridizes to 23S rRNA. The 0.5 kbp Kpn-XhoI fragment has run off the gel shown in Figure 4e. We have not detected hybridization to the 0.5 kbp fragment. 16S rRNA hybridizes to a single XhoI fragment of 11.2 kbp (present in two copies) which is cleaved by SalI to yield two copies of a fragment (7.5 kbp) which still hybridize to 16S rRNA (Figure 4c). KpnI does not cleave the 11.2 kbp XhoI fragment (Figure 4e). When chloroplast DNA is digested with

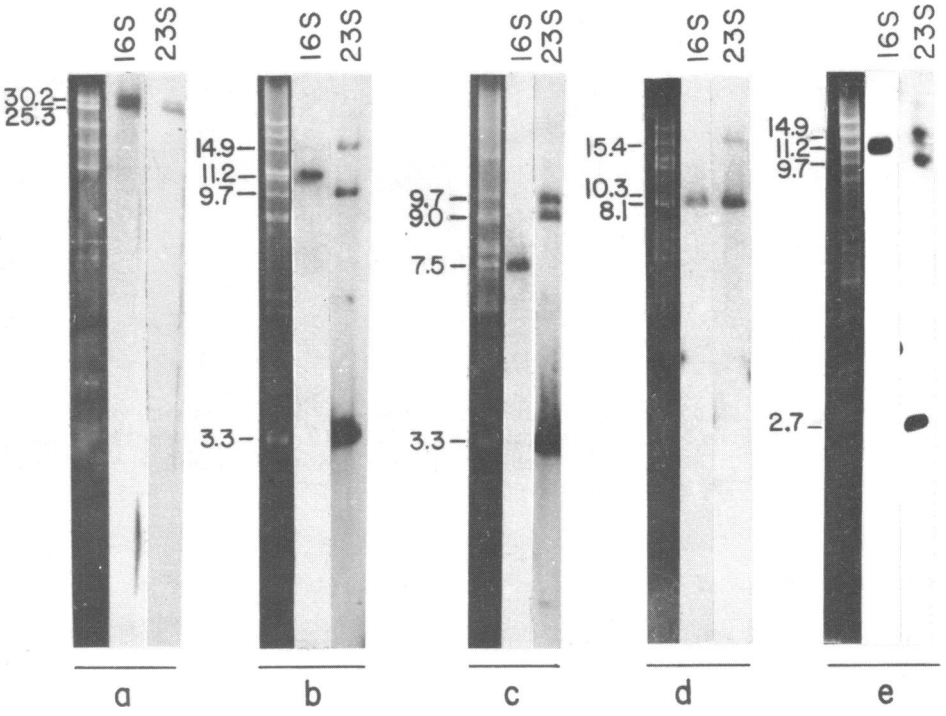


Figure 4: Chloroplast DNA digestion with KpnI (a), XhoI (b), XhoI and SalI (c), KpnI and SalI (d), KpnI and XhoI (e). Hybridization to 16S and 23S rRNAs are shown for each digest. Photographs of ethidium bromide-stained gels are on the left in each group.

KpnI and SalI, (Figure 4d) 23S rRNA hybridizes to fragments of 15.4, 10.3, and 8.1 kbp, whereas 16S hybridizes only to an 8.1 kbp fragment (present in two copies) of tobacco chloroplast DNA.

Further examination of Sal digestion products of KpnI fragments on two dimensional gels (Figure 5) reveals that the 30.2 kbp KpnI fragment is cleaved into products of 20.6 kbp, 8.1 kbp and a smaller fragment. The 25.3 KpnI fragment is cleaved by SalI into four major products of (15.4, 11.8, 10.3 and 8.1 kbp) and also two smaller fragments which migrated off the gel in Figure 5. This data reveals that the 25.3 kbp KpnI band must consist of two

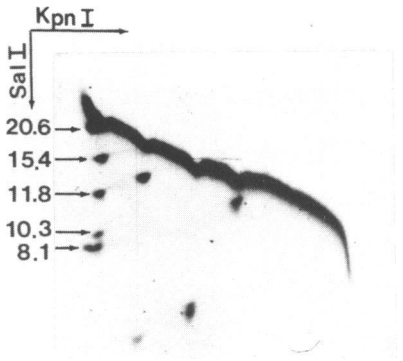


Figure 5: Separation of SalI digestion products of KpnI fragments on a two dimensional agarose gel. Arrows point to 8.1, 10.6, 11.8, and 15.4 kbp fragments discussed in the text. Ethidim bromide stained diagonally oriented streak is nuclear DNA which was present in this chloroplast DNA sample.

different fragments of the same size, since the sum of the sizes of the six digestion products equals 50 kbp. Furthermore, our results (Figure 4 and 5) indicate that there are four KpnI and SalI fragments which have sequence homology to 23S rRNA: an 8.1 kbp KpnI - SalI fragment derived from the 30.2 kbp KpnI fragment and the 15.4, 10.3, and 8.1 kbp KpnI - SalI fragments produced by SalI cleavage of the 25.3 kbp KpnI fragments.

## DISCUSSION

In summary, several observations have been made which confirm the presence of two copies each of 16S and 23S chloroplast rRNA genes on tobacco chloroplast DNA. The relative intensity of ethidium bromide fluorescence of the 3.3 and 11.2 kbp XhoI fragments (Figure 4) indicate that they are both present as two copies per genome. Since they contain sequences homologous to 23S and 16S rRNA, respectively, the presence of two copies of each of these genes are indicated. This conclusion is further supported by the observation of the two other XhoI fragments (14.9 and 9.7 kbp) which also contain part of the 23S rRNA genes.

From the KpnI and SalI double digestion data it is clear that KpnI cleaves each 23S gene at a point 8.1 kbp from a SalI site. Since the 16S rRNA genes are located on the 8.1 kbp KpnI and SalI fragments, it can be concluded that the 16s rRNA genes must be located between the SalI sites and the 23S rRNA genes. Most of the sequences for the 23S rRNA genes are also on this fragment as shown in Figure 4d. From hybridization to XhoI fragments, we conclude (1) that the KpnI site located within the 23S rRNA gene is between the XhoI sites which give the 3.3 kbp fragment (Figure 6), and (2) this KpnI site is 0.5 kbp from the XhoI site distal to the 16S rRNA gene(s) (Figure 4e). This is compatable with our observations that most of the 23S rRNA gene is between the KpnI site and the 16S rRNA gene. However, the relative hybridization to the 14.9 and 9.7 kbp XhoI fragments is curious, relative to the KpnI hybridization data. It appears that 23S rRNA hybridizes to chloroplast DNA over a distance which is 0.5 kbp larger than the mature 23S rRNA. This data suggests that a 0.5 kbp segment of DNA which does not hybridize to 23S rRNA may be located between the KpnI and XhoI sites in question. This region is indicated in Figure 6. Further work is necessary to conclusively demonstrate the presence and precise location of this presumptive intron.

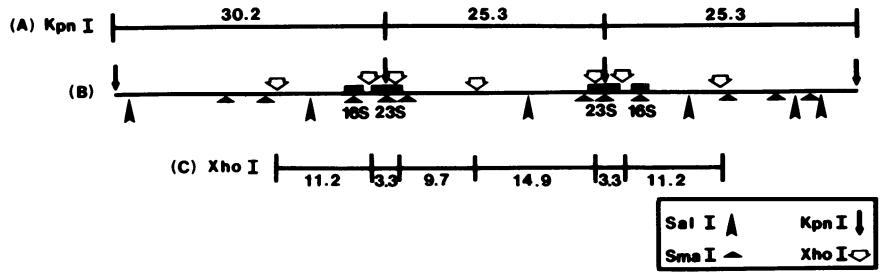


Figure 6: Details of the rRNA gene-containing region between the marks (1) and (2) on the map shown in Figure 3. All fragment lengths are in kbp. KpnI sites are mapped in (A), XhoI sites are mapped in (C), and their locations relative to the SalI, SmaI map are indicated in (B).

Utilizing the data presented here we have mapped on the N. tabacum chloroplast chromosome those KpnI and XhoI fragments that hybridize to the chloroplast 16S and 23S rRNA genes (Figure 3). Since the 25.6 and 18.3 kbp SalI fragments each contain a copy of 16S and 23S rRNA genes (Figure 1) and since these fragments are adjacent (Figure 3 and in preparation), an inverted order is the only arrangement of these genes compatable with the data (Figure 4). The KpnI-SalI and XhoI-SalI double digestion products demonstrate that the 16S rRNA genes are closer to a SalI site than the 23S rRNA genes. There is also a large (25.6 kbp) KpnI fragment with a SalI site that contains part of each 23S rRNA gene and which does not contain either 16S rRNA gene. This could only be true if the arrangement of these genes is in a inverted orientation. The 9.7 kbp and 14.7 kbp XhoI fragments and their cleavage patterns (Figure 4) by SalI and KpnI, respectively, support the location of this 25.6 kbp Kpn 1 fragment on the restriction map (Figure 3).

We have tried to fit the data presented in this paper to other possible arrangements for the rRNA genes. If, for example the 18.6 kbp SalI fragment D (Figure 3) were inverted to arrange the rRNA genes in tandem this might agree with the KpnI data. However, this tandem arrangement is not compatable with the XhoI hybridization data, particularly that for 16S rRNA. Inversion of the arrangements of both SalI fragments A and D, or just fragment A, relative to that shown in Figure 3 is incompatable with all of our data.

Recent studies with DNA gyrase (20,21) and DNA molecules containing both inverted and tandem repeats substantiate arguments (22) that an inverted orientation would withstand possible inactivation by intramolecular recombination better than similar molecules which have repeated sequences in tandem. For these reasons it is possible that genes which exist in inverted, repeated sequences would be stabilized by arrangements in which two identical copies of each gene are also present in inverted order. Reasons for the presence of two copies of a sequence on chloroplast DNA which collectively

represent one third of the available coding capacity are, at best, speculative with present knowledge. However, the reported absence of an inverted repeat in at least one higher plant, peas, (5) argues that the utility of inverted repeat sequences may be compensated by other mechanisms as well.

If the genes present in the inverted sequence are stabilized and present in a higher copy number than the rest of the genome, it is tempting to suggest that the rRNA genes and others which may be present are necessary for active maintenance of protein synthesis in the chloroplast. For example, inverted repeat sequences in spinach chloroplast DNA contain genes for rRNA and several tRNA species (23). Perhaps synthesis of chloroplast ribosomes is facilitated by transcription of multiple copies of these genes, thus allowing the RNA and/or ribosomal protein synthetic rates to be increased to levels above those which might be achieved by transcription of single copy genes. Since, in E. coli, ribosomal protein genes are located close to rRNA and tRNA genes (24), it is possible that genes for ribosomal proteins might also exist in the inverted repeat region of tobacco chloroplast DNA. Given that the average size of a chloroplast ribosomal protein is about 20,000 daltons (Capel and Bourque, in preparation), a large number of these genes could be present in the repeat sequences. Identification of those ribosomal protein genes (and other protein genes) which might be present in the repeat sequence and those encoded in the rest of the chloroplast DNA molecule or in nuclear DNA may help elucidate the nature and mechanisms of interactions that must occur with nuclear genes to maintain and regulate chloroplast structure and function.

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