Volume 11 Number 6 1983 Nucleic Acids Research

Analysis of four tobacco mitochondrial DNA size classes

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Received 28 January 1983; Accepted 11 February 1983

ABSTRACT

Supercoiled mtDNAs were isolated from tobacco suspension culture cells and three of the smallest size classes (10.1, 20.2 and 30.3 kb) were characterized through denaturation, heteroduplex and restriction mapping. The 20.2 kb molecule was found to be a head-to-tail dimer of the 10.1 or X size class, while the 30.3 kb size class was found to contain two kinds of molecules, a head-to-tail trimer of X (X_3) and a second molecule, ABC. X and ABC had a 118 ±35 bp region of homology, and both size classes shared a degree of homology with at least one other size class. Restriction maps of both the X and ABC molecules are presented and the possible origin and role of the many plant mtDNA size classes are discussed.

INTRODUCTION

Although plant mitochondrial genomes are not as well characterized as those in animals or fungi, the available evidence suggests that plants have evolved a distinctive mitochondrial genome organization. In contrast to the human and yeast mt genomes that are found as a single size class, plant mt genomes are found as a heterogeneous population of covalently-closed circles ranging in size from less than 0.5 μm to more than 30 μm in length. Analysis of the complexity of plant mtDNAs by means of restriction digests or reassociation kinetics yields estimates ranging from 160 kb in Brassicus oleracea (1) to about 2500 kb in muskmelon (2). These values are 2 to 30 times larger than yeast mtDNA (78 kb) and about 10 to 156 times larger than human mtDNA (16 kb). Plant mt rRNAs are also larger (18S and 26S) (3) than yeast (15S and 21S) (4) or animal (12S and 16S) (5) mt rRNAs. In addition, plant mitochondria have 5S RNAs that have not been found in any other mitochondrial systems (6; 7).

Plant mitochondrial genomes appear to be significantly more complex and physically heterogeneous than other mitochondrial genomes. Furthermore, they function in a genetic environment of greater complexity than that found in animal or fungal cells. In addition to the nucleus, plant mitochondria share

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the cell with chloroplasts that have their own genomes. In fact, there has been a suggestion that the chloroplast genome codes for some of the ribosomal proteins found in mitochondrial ribosomes (8). Although the general framework of plant mitochondrial genomes is understood, the details of the organization have been, until recently, unknown. The major difficulty has been the inability to isolate significant quantities of intact supercoiled plant mtDNA. Comparisons of the complexity of mitochondrial genomes with the size of the largest mtDNA molecule found in plant mitochondria have led Quetier and Vedel (9) to conclude that different sequences must be present on different size classes. Spruill et al. (10), using cloned Bam HI restriction fragments, found that the sum of unique fragments exceeded the size of the largest mtDNA size class and also concluded that different mtDNA size classes represented discrete chromosomes. However, it is essential to isolate and study individual mtDNA size classes in order to investigate how plant mitochondrial genomes are organized among the heterogeneous range of size classes, to map the genome and to study the regulation of plant mitochondrial genomes.

Recently we reported the successful isolation and separation of the different size classes of several different plants grown as suspension culture cells (11; 12). Our initial results suggested that there was homology among at least some of the different size classes of tobacco, bean and corn since the lowest size classes of each species hybridized to many, but not all, of the other size classes of that species. Comparison of restriction digests of three of the smallest tobacco size classes (10.1, 20.2 and 30.3 kb) showed that the 20.2 kb molecule was a dimer of the 10.1 kb mtDNA and that the 30.3 kb molecule had restriction fragments identical in size to those found in the 10.1 kb mtDNA in addition to a number of unique bands. In this paper, we report the results from the first stage of our detailed analysis of the tobacco mitochondrial genome (approximate MW 250 kb) in which we have cloned and characterized three of the approximately 15 size classes that have been observed, i.e. the 10.1, 20.2 and 30.3 kb molecules.

MATERIALS AND METHODS

Chemicals and Enzymes: Chemicals and buffers were obtained from regular commercial sources. Ligase, DNA polymerase I, restriction enzymes, etc. were purchased from Bethesda Research Laboratories and New England Biochemicals. DNAase came from Worthington and isotopes were purchased from New England Nuclear.

Plant Material: Nicotiana tabacum, variety Wisconsin 38, suspension culture

cells were grown in Linsmaier and Skoog (13) liquid medium containing indole-acetic acid (3.0 mg/liter) and N^6-dimethylallyl adenine (0.3 mg/liter).

mtDNA: Mitochondria (mt) and mtDNA were isolated as described (14). Super-coiled mtDNAs were subjected to electrophoresis in 0.4% agarose gels with continuous buffer circulation (40mM Tris base/20mM sodium acetate/2mM Na_2 EDTA, pH 8.0) at 2 V/cm for 48 hr. Specific size classes were extracted from gels by using the freeze technique of Thuring et al. (15). Restricted mtDNA samples were subjected to electrophoresis in 1 to 2% gels.

Nick Translation and Southern Hybridizations: Isolated mtDNA was nick translated using the Bethesda Research Laboratories nick-translation kit, and the products were extracted with phenol and precipitated with ethanol. The specific activities of the samples were 10^7-10^8 dpm/μg of template DNA. Filter hybridizations were done according to the procedure of Jeffreys and Flavell (16).

Cloning: Supercoiled mtDNA extracted from agarose gels as described above was digested with Sal I or Pst I and ligated with pBR322 that had been similarly restricted. The ligated samples were used to transform E. coli K12 and plated out on the appropriate agar plates (either with ampicillin or tetracycline). Clones were picked and plated on either ampicillin or tetracycline plates. Positive clones were grown up and the plasmid DNA isolated according to the protocol of Davis, Botstein and Roth (17).

Electron Microscopy: Supercoiled DNA molecules were nicked once with DNAase I in the presence of ethidium bromide according to Greenfield et al. (18). Heteroduplex preparation and DNA spreading were essentially as described by Davis et al. (19). The heteroduplex mixture was diluted and spread in a less denaturing condition (lower formamide concentration) to detect base pairing between short complementary sequences.

The set of isodenaturing spreading conditions described by Davis and Hyman (20) was used for partial denaturation studies. The electrolyte concentration was held constant and the denaturing power of the solvent was controlled by various concentrations of formamide in the spreading solutions and in the hypophase as specified in the figure legends. The length standards for double-stranded and single-stranded DNAs were SV40 (5224 bp) and ØX174 (5370 bp), respectively. Length measurements were made with a Numonics electronic graphic calculator. Electron micrographs were obtained with a JEM 100 CX electron microscope from JOEL Company.

RESULTS

The presence of several enzymes (Sal I, Xba I, Xho I, Pst I and Bgl I) that made only a single cut greatly facilitated cloning the 10.2 kb (X) tobacco mtDNA size class (Figure 1). Using the Sal I site we were able to clone the entire X size class into the Sal I site of pBR322 as a single fragment. We also cloned X into the Pst I site of pBR322 with equal success. No heterogeneity was observed in the X size class nor was any loss or rearrangement of the X insert observed among the more than 40 clones we analyzed.

Earlier studies of the 20.2 kb mtDNA (11) had shown that it was a head-to-tail dimer of X. The situation with the 30.3 kb molecule was, however, more complex. None of the restriction enzymes we used gave a single cut in the 30.3 kb size class. We therefore decided to clone the Sal I fragments into pBR322. A Sal I digest of the 30.3 kb size class gave three bands on an agarose gel in an approximate ratio of 1:4:1. We characterized 45 positive clones and found four distinct fragments, one of which was identical to the X size class, one designated B which had almost the same molecular weight as the X size class, and two others we designated A and C.

Heteroduplex analysis of the four fragments revealed a small region of homology between X and A. This region was approximately 118 ±35 bp (N=10) long (see Figure 2). Examination of the individual denatured Sal I fragments in the EM revealed the existence of a short inverted repeat sequence near one end of the C fragment (Figure 3a). The sequences in the short inverted repeat were mapped at 270 ±42 bp (N=20) and 1760 ±80 bp (N=20) from the Sal I site. Upon renaturation of the denatured C fragments, structures as shown in Figure

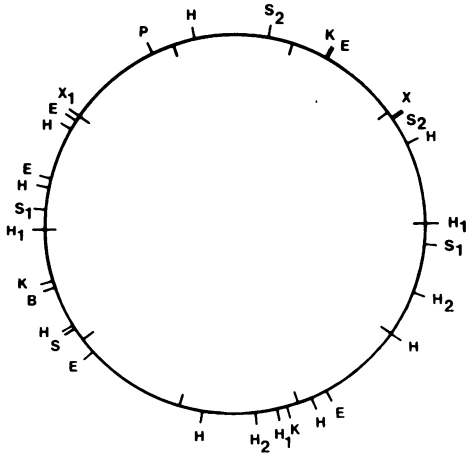

Fig. 1. A composite restriction map of X (10.1 kb size class). The internal divisions are 1 kb apart. The restriction enzymes used were: H=Hha I; H_1=Hind III; H_2=Hpa I; K=Kpn I; S=Sal I; S_1=Sac I; S_2=Sma I; X_1=Xba I; X=Xho I; E=Eco RI; B=Bgl I and P=Pst I.

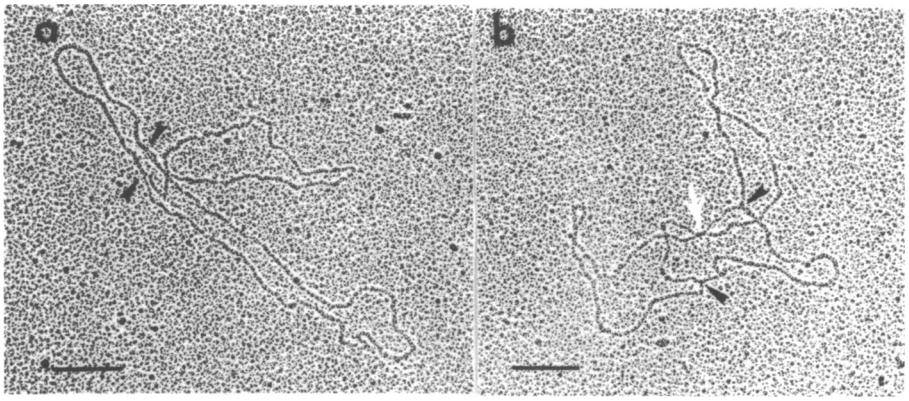

Fig. 2. Electron micrographs of heteroduplex molecules between pBR322-A and pBR322-X. The perfect duplex regions between the two arrowheads are pBR322: a) spread from 40% to 10% formamide; b) spread from 30% to 5% formamide. The white arrow points to the short homologous sequence between A and X which is 118 ±35 bp in length and is located 520 ±40 bp and 1580 ±90 bp from the Sal I site on X and A, respectively. The fact that the pairing between the homologous sequence observed in 2b is not stable under the spreading condition of 2a suggests the possibility of incomplete homologous pairing within this region. The bar represents 1 kb.

3b were frequently observed. In these structures, the pairing of the short inverted repeat prevented the complete renaturation of two single-stranded molecules as described by Broker et al. (21). The size of this short inverted repeat was estimated to be 70 ±28 bp (N=20).

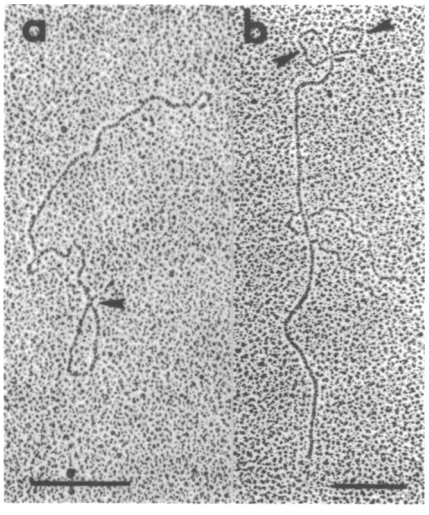

Fig. 3. Electron micrographs of (a) a single-stranded C fragment with an arrow pointing to the short inverted repeat, (b) a "double-stranded" C fragment which was sequentially denatured and renatured. Two single-stranded loops are marked with arrows. Both micrographs are prepared from 40% to 10% formamide spreads. The short inverted repeat is 70 ±28 bp (N=20). The bars represent the length of 1 kb double-stranded DNA.

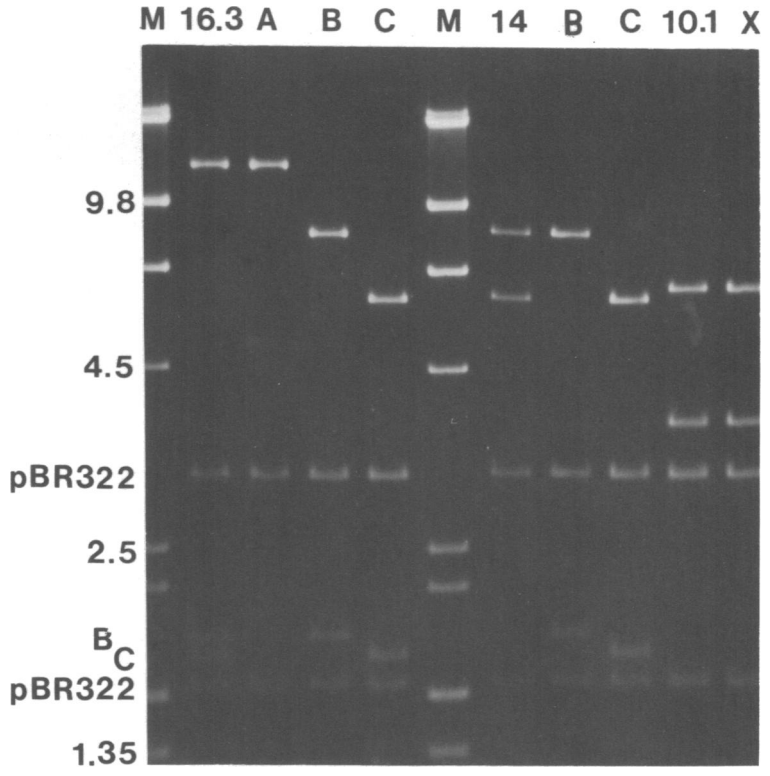

Fig. 4. A double-restriction digest (Sal 1 and Pst 1) of the three Pst I clones that have inserts of approximately 16.3, 14 and 10 kb, compared with double digests of the A, B, C and X Sal clones all in pBR322. The lanes marked with M are a mixture of a Hind III digest of λDNA and a Hae III digest of ØX174.

It was clear from the above analysis that the 30.3 kb size class was heterogeneous since we had four Sal I fragments with a minimal total molecular weight of 40 kb, 10 kb larger than the size of this molecule as measured in the EM. Digestion of X, A, B and C, with a variety of restriction endonucleases, revealed that Pst I did not cut the A fragment but did cut X, B and C once each. Digestion of the 30.3 kb size class with Pst I gave three fragments with approximate molecular weights of 16.3 kb, 14 kb and 10.1 kb. Comparison of double digests (Sal I and Pst I) of the X, A, B and C Sal I clones with the 16.3, 14 and 10.1 kb Pst I clones (Fig. 4) showed that the 16.3 kb Pst I clone was made up of the 12.9 kb A fragment, a 1.8 kb fragment from B and a 1.6 kb fragment from C (see arrows). The 14 kb Pst I fragment consisted of an 8.2 kb fragment from B and a 5.8 kb fragment from C. The 10.1

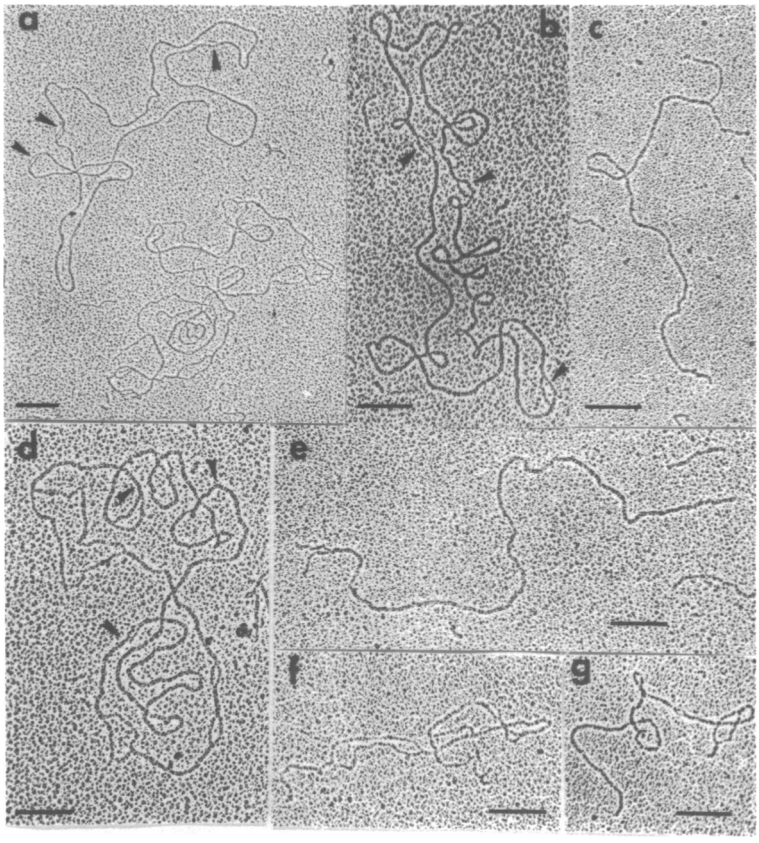

Fig. 5. Electron micrographs show the denaturation profiles of (a) a mixture of X_3 and ABC, the three symmetrical denaturation loops in X_3 are marked with arrows. ABC is extensively denatured under these conditions. The spreading solution contained 0.1M Tris, 0.01M Na_2EDTA, pH 8.5, 80% formamide. The hypophase contained 0.01M Tris, 1mM Na_2EDTA, pH 8.5, 50% formamide. (b) and (c), X_3 and X, respectively, spread under stronger denaturing conditions using 85% formamide in the spreading solution and 55% formamide in the hypophase. (d), (e), (f) and (g) show the denaturation profiles of ABC, A, B and C, respectively, using moderate denaturation conditions, i.e., spreading from 70% to 40% formamide. The arrows in (d) point to the approximate positions of Sal I sites on ABC. The bar represents 1 kb.

kb Pst clone was identical to X. The 10.1 kb or X fragment was never found linked with any other molecule. We therefore concluded that there were probably two classes of 30.3 kb molecules, one a trimer of X (X_3) and the other composed of the three Sal I fragments ABC.

Analysis of the partial denaturation profiles of the 30.3 kb molecules in the EM also revealed the existence of two types of molecules. One class of

molecules was more resistant to formamide denaturation. Under the strong denaturation conditions described in the legend of Figure 5, three equally spaced denaturation loops (approximately 10 kb apart) were observed in this type of molecule (Fig. 5a, b). X had a single denatured region under the same conditions while A, B and C were almost totally denatured. We concluded that the 30.3 kb size class with three equally spaced loops was a trimer of X. The second class of molecules was extensively denatured under the strong denaturation conditions (Fig. 5a). When moderate denaturation conditions were used, the second type of molecule gave an asymmetrical denaturation profile (Fig. 5d). When fragments A, B and C were spread under the same denaturation conditions (Fig. 5e, f and g), characteristic denaturation loops of the entire molecule were detected in each individual fragment. Therefore, we concluded that the second type of molecule contained one copy each of the A, B and C Sal I fragments. The relative numbers of X_3 and ABC molecules were approximately equal in the sample we examined.

A restriction map of the ABC molecule was constructed through the use of the overlapping Pst I and Sal I clones, as well as partial and double digests of the various cloned fragments. A composite restriction map is shown in Figure 6.

The absence of extensive homology between X and ABC and the presence of a trimer of X suggested that much of the hybridization observed when X was hybridized to a Southern blot of total tobacco supercoiled mtDNA (11) was due to X hybridizing to multimers of itself. In order to see if X or ABC shared homology with any other size classes, we nick-translated X, A, B and C and

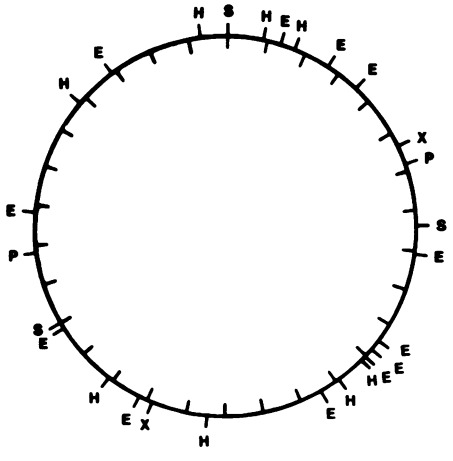

Fig. 6. A composite restriction map of the ABC size class. The internal divisions are approximately 1 kb apart. The restriction enzymes used were H=Hind III, E=Eco RI, S=Sal I, P=Pst I and X=Xba I.

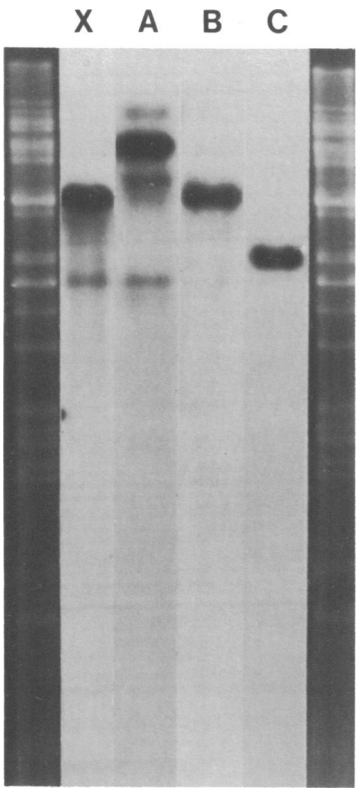

Fig. 7. Hybridization of nick-translated X, A, B and C to a Sal I digest of total tobacco mtDNA that had been electrophoresed in a 0.7% agarose gel. The two outside lanes show the ethidium bromide stained pattern of the total Sal I digest. The short region of homology between X and A is not seen under the stringent hybridization and wash conditions used in this experiment.

hybridized them to a Sal I digest of total tobacco mtDNA. Hybridization to any fragments other than X, A, B and C would indicate that other size classes shared some homology with one or both of these size classes. The results (Fig. 7) showed that B and C hybridize only to themselves or at least to fragments identical in molecular weight to B and C. X hybridized to itself and cross-hybridized slightly to one other fragment, while A hybridized to itself and three other fragments. Thus, both X and ABC shared slight homology with at least one other size class. Since both X and A appeared to hybridize to one of the same fragments, there may be a larger size class that shares some homology with both X and ABC.

We had a newer W38 N. tabacum cell line that had a totally different mtDNA size class pattern in which there did not appear to be a 10 kb size class (Fig. 8a). However, despite the size class differences, the two lines had almost identical mtDNA restriction digest patterns (Fig. 8b). If one hybridized X to a Sal I digest of the new line, one observed significant

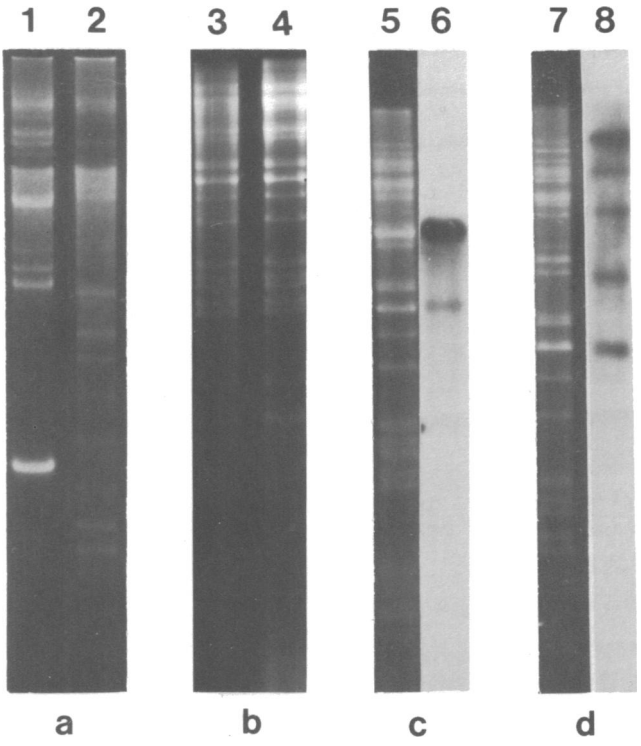

Fig. 8. (a) Supercoiled mtDNA from our old (lane 1) and our new (lane 2) lines of N. tabacum run on a 0.7% agarose gel. (b) Sal I restriction digest of total mtDNA from the old (lane 3) and new (lane 4) tobacco lines electrophoresed on a 1% agarose gel. Hybridization of the X size class to (c) (lane 6) a total Sal I restriction digest of the old line shown in lane 5. Hybridization of the X size class to (d) (lane 8) a total Sal I restriction digest of the new line electrophoresed on different 1% gels shown in lane 7.

hybridization to five different bands, three of them larger than 10 kb and two smaller (Fig. 8d). Similar results had been obtained in hybridizations of the A fragment to the new line (unpublished results). Thus, although the restriction digests of the two lines looked identical, there appeared to have been rearrangements and/or recombinations in the new line with regard to the genetic information present on the X size class. Similar rearrangements have been recently reported by Sederoff et al. (22) in different lines of teosinte, a close relative of corn.

DISCUSSION

The physical heterogeneity of plant mitochondrial genomes reflects in

part a comparable genetic heterogeneity. The situation is more complex than this, however, since some of the physical heterogeneity can be attributed to oligomers of specific size classes while in other cases genetically distinct molecules have the same molecular weight. The work presented in this paper documents the existence of examples in both these categories. We have shown that in addition to a dimer of X (X_2) there is a trimer, X_3, which is unresolvable from the ABC size class on agarose gels. Although ABC and X_3 have approximately the same molecular weight, they share only about a 118 bp region of homology. The existence of both unique and related size classes has also been observed in the mtDNAs of bean and corn (11). Other than the 118 bp overlap, X_3 (or X) and ABC are genetically distinct from one another. Given the limited homology between X and other mtDNA fragments, it seems likely that some of the genetic information on X is found there exclusively. The same seems to be true for much of the genetic information on the ABC molecule, since neither the B nor C fragments appear to hybridize to any other restriction fragments. Fragment A does hybridize weakly to several other restriction fragments not found in the ABC molecule, however, so there is at least one other size class sharing some homology with the 30.3 kb ABC molecule.

The presence of discrete genetic elements raises the question of whether this mtDNA arrangement arose from, or is involved in, amplification of specific mitochondrial genes or whether it is simply an artifact of rearrangements and recombinational processes going on in plant mitochondria. As discussed previously (11), there are size classes found in bean and corn that appear to carry only fragments of genes. It seems more likely that such defective mtDNAs are the by-products of rearrangements of larger size classes possessing the complete genes, rather than the product of a specific amplification process. Also the existence of totally different size class populations among two cell lines of the same tobacco variety suggests that it is relatively unimportant how plant mt genomes are arranged physically as long as essential sequence information is present. However, it is still possible that plants do take advantage of their unique dispersed genome to amplify specific genes under certain conditions.

Regardless of the role played by the different size classes, one would like to know how such a genome arrangement evolved in plants. Single mitochondrial chromosomes seem to be the rule among nearly all other organisms under normal growth conditions. The distribution of a mitochondrial genome over a population of distinct size classes seems to be unique to plant mtDNAs. It may be that plants always have had large genomes and multiple size classes.

However, this seems unlikely since nearly all other organisms have relatively small mitochondrial genomes located on a single chromosome with the exception of the genome found in trypanosomes in which there is a maxicircle and a grid of interlocking non-coding minicircles (23). The alternative viewpoint is that plant mt genomes were once found as a single chromosome and as a result of a variety of recombinational events, amplifications, rearrangements, etc., the genome was distributed over many different size classes, some of which share sequences in common and others which are unique. This may have occurred to different degrees in different plants and a continuum may exist extending from plants which have retained a single unique size class to those in which the mt genome is found as a series of different molecules. In between there may be species which still retain the original size class along with other molecules carrying different parts of the genome. The absence of similar heterogeneous mitochondrial genome arrangements in animals or fungi would suggest that the process which produced the physical heterogeneity is unique to plants or at least occurs at a higher frequency in plants than in animal or fungal mt genomes. Ward et al. (2) have come to a similar conclusion in their effort to explain the unusually complex mt genomes in some members of the cucumber family.

ACKNOWLEDGMENTS

We wish to thank Drs. I. Sussex and J. Messing for laboratory space and Dr. D. Ward for helpful discussions and advice. This work was supported in part by U.S. Department of Agriculture Grant 80-0011, National Science Foundation Grant PCM-8110118, NIH training grant 5-T32 6 MO 7223, and NIH research grant GM31127-01.

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