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Organization and expression of the mitochondrial genome of plants I. The genes for wheat mitochondrial ribosomal and transfer RNA: evidence for an unusual arrangement*

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ABSTRACT

We show here that mitochondrial-specific ribosomal and transfer RNAs of wheat (*Triticum vulgare* Vill. [*Triticum aestivum* L.] var. Thatcher) are encoded by the mitochondrial DNA (mtDNA). Individual wheat mitochondrial rRNA species (26S, 18S, 5S) each hybridized with several mtDNA fragments in a particular restriction digest (*Eco* RI, *Xho* I, or *Sal* I). In each case, the DNA fragments to which 18S and 5S rRNAs hybridized were the same, but different from those to which 26S rRNA hybridized. From these results, we conclude that the structural genes for wheat mitochondrial 18S and 5S rRNAs are closely linked, but are physically distant from the genes for wheat mitochondrial 26S rRNA. This arrangement of rRNA genes is clearly different from that in prokaryotes and chloroplasts, where 23S, 16S and 5S rRNA genes are closely linked, even though wheat mitochondrial 18S rRNA has previously been shown to be prokaryotic in nature. The mixed population of wheat mitochondrial 4S RNAs (tRNAs) hybridized with many large restriction fragments, indicating that the tRNA genes are broadly distributed throughout the mitochondrial genome, with some apparent clustering in regions containing 18S and 5S rRNA genes.

INTRODUCTION

Mitochondria contain distinctive species of ribosomal and transfer RNA which form part of an organelle-specific translation system [1,2]. In all systems examined to date, the rRNAs and most of the tRNAs are coded for by mitochondrial-specific DNA (mtDNA) [3-5]. In a number of recent studies, DNA/RNA hybridization techniques have been used to locate genes for ribosomal and transfer RNAs on physical maps of the mitochondrial genome. Such studies have revealed strikingly different patterns of organization of ribosomal and transfer RNA genes in different mtDNAs. In *Neurospora*, for example, there is clustering of genes for rRNAs and tRNAs in a segment representing about 27% of the mtDNA, with the genes for the large and small subunit rRNAs located about 5 kilobase pairs (kbp) apart [6-8]. In yeast, there is also pronounced clustering of tRNA genes, but in this case the rRNA genes are much farther

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(about 25 kbp) apart [9]. In contrast, rRNA genes are adjacent in animal mtDNA, while tRNA genes are scattered throughout the genome [10-12]. The significance of these varying patterns with respect to the evolution and expression of mtDNA is not yet known.

As in other eukaryotic systems, the mitochondria of higher plants contain species of tRNA [13-17] and high-molecular-weight rRNA [18-23] which can be distinguished by several criteria from their counterparts in the cytosol. A distinct 5S rRNA has also been identified in plant mitochondria [21,24]. Although it has been assumed that these plant mitochondrial RNAs are encoded by the organelle DNA, this has not yet been formally demonstrated, nor have there been any attempts to probe the organization of plant mitochondrial ribosomal and transfer RNA genes. As part of a systematic investigation of the organization and expression of the mitochondrial genome of wheat, we have used restriction endonuclease analysis coupled with DNA/RNA hybridization experiments in order to explore the genetic origin of wheat mitochondrial rRNAs (26S, 18S, and 5S) and tRNAs. Our results provide the first direct evidence that plant mitochondrial rRNAs and tRNAs are indeed transcribed from the mtDNA. Our data also suggest a novel arrangement of rRNA genes in plant mtDNA.

EXPERIMENTAL

(a) Isolation of Mitochondrial and Nuclear Fractions

Purified mitochondria were prepared from viable wheat embryos [25,26] (germinated in the dark, usually for 24 hr) by a modification of the method of Cunningham and Gray [22]. To minimize cytosol RNA contamination, a Mg^{2+}-free medium (0.44 M sucrose - 50 mM Tris·HCl (pH 8.0) - 3 mM EDTA - 1 mM 2-mercaptoethanol - 0.1% bovine serum albumin (fatty acid-free)) was used for the initial homogenization and in all subsequent differential and density gradient centrifugation steps. To reduce contamination by nuclear DNA (nDNA), additional low-speed centrifugations (1000-2000 x g, 6 min) of the initial filtered homogenate and the resuspended crude mitochondrial pellet were carried out. After centrifugation in discontinuous sucrose gradients (1.15 M, 1.30 M, 1.45 M steps), mitochondria were removed from the 1.30 M - 1.45 M interface, slowly diluted with 2 vol. homogenizing medium (lacking bovine serum albumin and mercaptoethanol), and recovered (18,000 x g, 20 min).

To prepare a nuclear fraction, germinating wheat embryos were homogenized as above, the brei squeezed through cheesecloth, and the filtrate passed through a layer of Miracloth (Calbiochem) and centrifuged (1000 x g, 6 min). The re-

sulting pellet was washed twice by resuspension in homogenizing medium followed by low-speed centrifugation.

(b) Isolation of Mitochondrial and Cytosol RNA

Purified mitochondria were lysed with Triton X-100, the lysate centrifuged, and RNA isolated from the supernatant by phenol extraction [27] below 5°, as described by Leaver and Harmey [24]. The final aqueous phase was saved and the phenol phases were then re-extracted in the same sequence with 0.1 M Tris·HCl (pH 9). Total mitochondrial RNA was precipitated from the final, combined aqueous phases with 2 vol. ethanol.

To prepare 26S and 18S rRNAs, mitochondrial ribosomes were first isolated by making the clarified Triton lysate 0.3% in Brij 58 and centrifuging (50Ti rotor, 45,000 rpm, 90 min). The pellet was resuspended in 300 mM KCl - 3 mM $MgCl_2$ - 10 mM Tris·HCl (pH 7.5) [18], clarified (27,000 x g, 10 min), and the resulting supernatant layered over sucrose density gradients [28] containing the same buffer. After centrifugation (SW 25.1 rotor, 20,000 rpm, 16 hr), the gradients were fractionated and RNA isolated from the well-separated ribosomal subunit peaks by cold phenol extraction. The RNAs were then further purified by electrophoresis in 2.4% polyacrylamide gels in the cold (4°), as described by Leaver [29]; the bands corresponding to the intact 26S and 18S rRNAs were located by ultraviolet shadowing [30] and recovered by electrophoretic elution.

To prepare 5S and 4S RNAs, total mitochondrial RNA in aqueous solution was made 1 M in NaCl and held at 0° for 18 hr. After removal of precipitated high-molecular-weight rRNA by centrifugation, the NaCl-soluble fraction was recovered by ethanol precipitation and electrophoresed in 10% polyacrylamide gels; the 5S and 4S bands were located [30] and recovered.

Total cytosol RNA was prepared by cold phenol extraction of the upper 2/3 of the initial post-mitochondrial supernatant of a centrifuged wheat embryo homogenate, as described above for total mitochondrial RNA.

(c) Isolation of Mitochondrial and Nuclear DNA

When mitochondrial fractions were to be used for the preparation of mtDNA, residual nDNA was eliminated by DNase treatment [31] of the crude, washed mitochondrial pellet, just prior to sucrose density gradient centrifugation. The procedure of Kolodner and Tewari [31] was used to isolate DNA from purified mitochondrial and nuclear fractions. After recovery from CsCl-ethidium bromide density gradients, DNA preparations were freed of dye and salt by passage through Sephadex G-25 and were recovered by ethanol precipitation. In analytical CsCl gradients, wheat mtDNA and nDNA gave unimodal bands

with buoyant densities (relative to 1.731 g cm^{-3} for marker *M. luteus* DNA) of 1.705 and 1.701 g cm^{-3}, respectively, in agreement with literature values [32,33].

(d) Labeling of RNA with ^{32}P *in vitro*

(i) 5' End-Labeling [34,35]

Purified RNAs (5 µg in 10 µl H_2O) were partially hydrolyzed by incubation with 10 µl 100 mM Tris·HCl (pH 9.5) at 85° (4S and 5S) or 95° (26S and 18S) for 5 min. After this, 60 µl 10 mM Tris·HCl (pH 7.4) - 1 mM spermidine - 0.1 mM EDTA were added and the mixture heated to 50° for 3 min. Then, to label 5' ends, the following were added: 100 pmoles [γ-^{32}P]ATP, prepared as described by Walseth and Johnson [36], to a specific activity > 5000 Ci/mmole; 10 µl 0.5 M Tris·HCl (pH 9.5) - 0.1 M $MgCl_2$ - 0.05 M dithiothreitol; and several units of T4 polynucleotide kinase (Boehringer Mannheim), for a final volume of 100 µl. This mixture was incubated at 37° for 30 min, and the reaction then stopped by the addition of 200 µl 2 M ammonium acetate, 50 µg carrier RNA, and 750 µl ethanol. Repeated ethanol precipitations at -70° removed unreacted [γ-^{32}P]ATP until approximately 75% of the radioactivity was insoluble in 5% trichloroacetic acid. The specific activity of the 5' end-labeled RNA was > 10^6 cpm/ug.

(ii) 3' End-Labeling

Mitochondrial 5S rRNA was also 3' end-labeled by reaction with T4 RNA ligase (P-L Biochemicals) and [5'-^{32}P]pCp (prepared as in ref. 36), as described by Peattie [37]. After several ethanol precipitations, the labeled RNA was lyophilized, dissolved in buffer, heated at 50° for 3 min, and then electrophoresed under denaturing conditions in a 10% polyacrylamide gel [37]. Intact 5S [^{32}P]RNA was recovered by electrophoretic elution.

(e) Hydrolysis of DNA by Restriction Endonucleases and Separation of Fragments

Samples of DNA (3 µg) were digested to completion at 37° with *Eco* RI (Miles Laboratories), *Xho* I, *Sal* I, *Xba* I, *Msp* I, *Hin*c II (New England Biolabs), or *Hpa* II (Bethesda Research Laboratories), using conditions specified by the suppliers. The resulting DNA fragments were separated in horizontal slab gels of 1% agarose containing 50 mM Tris·HCl (pH 8.0) - 2 mM EDTA - 20 mM CH_3COONa - 18 mM NaCl. The DNA bands were visualized by staining the gels with ethidium bromide (1 µg/ml) and photographing under ultraviolet light.

(f) DNA/RNA Hybridization

After denaturation, the DNA fragments were transferred from the gel to nitrocellulose filters (Millipore, HAWP) using the standard Southern procedure

[38], modified to allow overnight elution from slab gels. Filters were rinsed, dried, and baked under vacuum at 80° for 2 hr. For hybridization experiments, DNA filters were incubated (18-22 hr, 42°) with [^{32}P]RNA in 0.6 M NaCl - 0.06 M sodium citrate (= 4 x SSC)/100% deionized formamide (1/1, v/v) in heat-sealed polyethylene bags. About 40-400 ng [^{32}P]RNA in 0.3-0.6 ml hybridization medium was used for each filter strip containing 3 μg fragmented DNA. Filters were washed once in the hybridization medium at 42°, twice in 4 x SSC at 42°, and twice in 4 x SSC at room temperature, before being dried and subjected to autoradiography using Kodak X-Omat R film and intensifying screens at -70° [39].

RESULTS AND DISCUSSION

(a) Restriction Patterns of Wheat Mitochondrial DNA are Unexpectedly Complex

When purified wheat mtDNA was hydrolyzed with the restriction endonucleases *Eco* RI, *Xho* I, or *Sal* I, a discrete and reproducible fragment pattern was generated by each enzyme (Fig. 1A). These restriction patterns were invariant for different preparations of mtDNA, were not altered by increasing enzyme concentration and/or digestion time, and were the same whether the mtDNA was isolated from dormant or germinating (24-72 hr) embryos. In contrast, and as expected from its much greater complexity, wheat nDNA had an *Eco* RI restriction pattern composed of a continuous rather than discrete spectrum of DNA fragments (Fig. 1A), although some distinct bands were detectable among the products. As discussed elsewhere (L. Bonen and M.W. Gray, in preparation), wheat nDNA appears to be highly resistant to digestion with either *Xho* I or *Sal* I.

Compared to the restriction patterns of chloroplast and other mitochondrial DNAs, the restriction patterns of higher plant mtDNA appear to be unexpectedly complex [40,41]. In this regard, our results with wheat mtDNA confirm the previous observations of Quetier and Vedel [40]. Not only is there a large number of bands (ranging from about 30 to more than 50, depending on the enzyme), but some of the bands are clearly present in fractional amounts, whereas others appear to be multiples. This complexity is most readily seen in densitometer tracings of the gel patterns (Fig. 2). Disregarding possible multiplicities of some bands, the sum of the estimated molecular weights of the DNA fragments in our restriction digests ranged from about 160 x 10^6 (*Eco* RI) to as much as 230 x 10^6 (*Xho* I), although these estimates are complicated by considerable uncertainty in the calculated molecular weights of the larger DNA fragments (see Table 1).

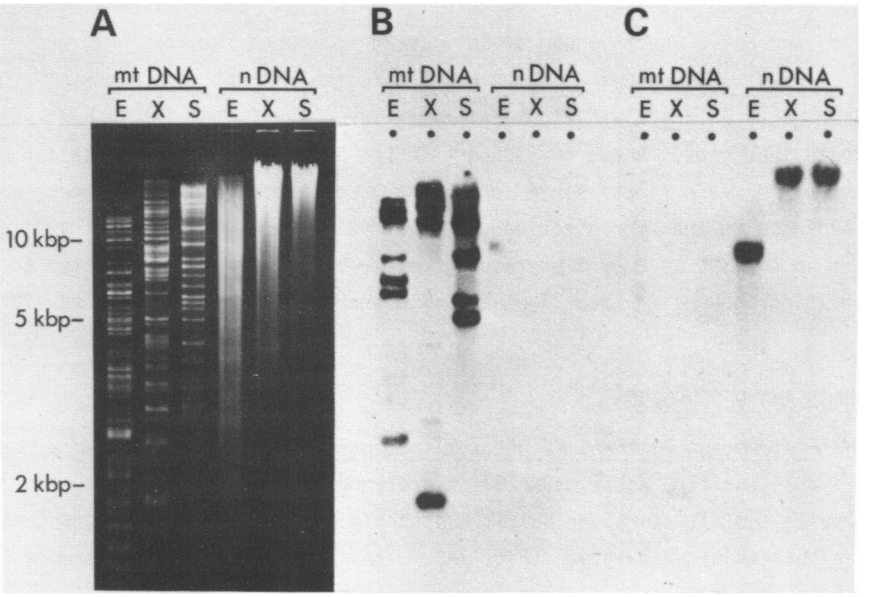

Figure 1. (A), Ultraviolet photograph showing the resolution of restriction endonuclease hydrolysis products of wheat mtDNA and nDNA, after electrophoresis in a 1% agarose gel. DNA fragments were visualized by staining with ethidium bromide and were then transferred to nitrocellulose filters. E, *Eco* RI, X, *Xho* I; S, *Sal* I. (B), Autoradiogram of nitrocellulose-bound restriction fragments after hybridization with ^{32}P-labeled wheat mitochondrial total RNA. (C), Autoradiogram of nitrocellulose-bound restriction fragments after hybridization with ^{32}P-labeled wheat cytosol total RNA.

Studies of plant mtDNA have so far provided no uniform picture of the size of the mitochondrial genome in different higher plants [*cf.* 41]. However, the *minimum* molecular weight estimates obtained from analysis of restriction digests are appreciably higher than the *maximum* values based on electron microscopy (60-120 x 10^6; ref. 42-43) or renaturation kinetics (70-140 x 10^6; ref. 43-45). Thus, the situation with plant mtDNA, where complex restriction patterns may reflect a heterogeneous population of molecules [40,41], contrasts strikingly with that of chloroplast DNA, whose simpler restriction patterns are compatible with the presence of a homogeneous population of molecules.

(b) Wheat Mitochondrial DNA Contains the Genes for Mitochondrial-Specific RNAs

The total RNA populations from mitochondrial and post-mitochondrial (=

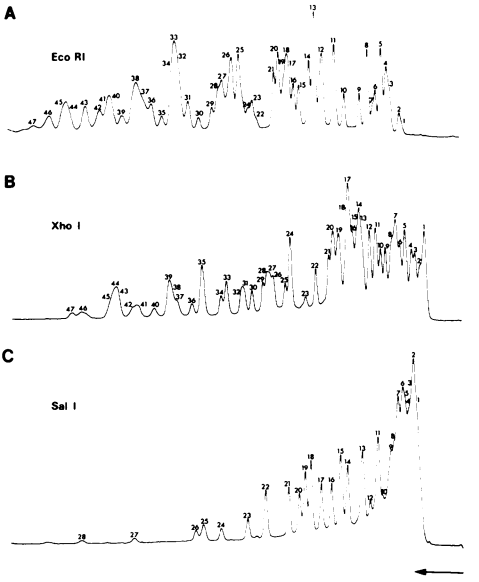

Figure 2. Densitometer tracings illustrating the restriction patterns obtained when wheat mtDNA was hydrolyzed with *Eco* RI (A), *Xho* I (B), or *Sal* I (C). The negatives of the ultraviolet photographs (Fig. 1A) were scanned with a microdensitometer (Joyce, Loebl & Co. Ltd.). Fragments which could be identified as discrete bands on positive prints (Fig. 1A or longer runs) have been numbered individually in order of decreasing size. The direction of electrophoresis (arrow) corresponds to migration from top to bottom in Fig. 1A.

cytosol) fractions of wheat embryo homogenates are similar in that each contains as the major species the large (26S) and small (18S) ribosomal subunit RNAs, as well as 5S and 4S RNAs. The mitochondrial 26S and 18S rRNAs can be distinguished from their cytosol counterparts by their modified nucleoside contents [22], T_1 oligonucleotide fingerprints [21], and, in the case of the 18S rRNAs, their T_1 oligonucleotide catalogues [23]. By the latter two criteria, wheat mitochondrial and cytosol 5S rRNAs are also distinct species [21]. The physicochemical properties of wheat mitochondrial 4S RNA (tRNA) have not yet been examined in detail.

When wheat mitochondrial total [^{32}P]RNA was annealed to separated and immobilized restriction fragments of wheat mtDNA and nDNA, the mitochondrial RNA was found to hybridize almost exclusively with mtDNA (Fig. 1B). About 5-6 major bands appeared on the autoradiograms for each of the enzyme digests. Thus, as in other eukaryotes, *wheat mitochondrial-specific RNAs are transcribed from wheat mtDNA*. In the converse experiment, wheat cytosol total

[^{32}P]RNA was found to hybridize with nDNA rather than mtDNA (Fig. 1c). In this case, radioactivity was confined to one narrowly-defined region of the autoradiograms, corresponding in the case of *Eco* RI digests to (a) DNA fragment(s) about 10 kbp in size, but at the position of non-restricted nDNA in the case of *Xho* I and *Sal* I digests. These labeled regions contain the genes for cytosol 26S and 18S rRNAs (unpublished results), which are detectable because of their high copy number in the wheat nuclear genome [46].

A slight degree of hybridization of nDNA with mitochondrial [^{32}P]RNA is apparent in Fig. 1B, whereas Fig. 1C shows a low level of hybridization of mtDNA with cytosol [^{32}P]RNA. From the positions of the labeled bands, it seems likely that this "cross hybridization" can be attributed to slight contamination of the mitochondrial and cytosol RNA probes with each other. Thus, we conclude that there is no extensive homology between wheat mitochondrial RNA species and their cytosol counterparts. There was no evidence from these hybridization experiments of contamination of nDNA with mtDNA, or *vice versa.*

(c) The Genes for Wheat Mitochondrial 26S and 18S rRNAs are Far Apart

We next examined the hybridization characteristics of the separated and purified mitochondrial 26S and 18S rRNAs. As shown in Fig. 3, each [^{32}P]rRNA hybridized with several DNA fragments (ranging in molecular weight from about 1.2×10^6 to $> 10 \times 10^6$) in each restriction digest, and the resulting radioactive bands were not of equal intensity. With shorter electrophoretic runs, to ensure that small DNA fragments were retained on the gel, an additional band in the *Xho* I digest was labeled to a minor extent with 18S [^{32}P]RNA.

Of particular interest was the observation that in each restriction digest the 26S RNA hybridized to *different* DNA fragments than did the 18S RNA, as indicated by the autoradiograms (Fig. 3) and by densitometer tracings of these (Fig. 4). This same result was obtained in experiments using five additional restriction endonucleases (*Sma* I, *Xba* I, *Msp* I, *Hpa* II, *Hin*c II): in each case, the hybridization patterns for 26S and 18S RNAs were clearly different. Since it is highly unlikely that each of 8 different restriction endonucleases would cleave precisely in the spacer region between closely linked 26S and 18S rRNA genes, we conclude that *the genes for wheat mitochondrial 26S and 18S rRNAs are far apart on the wheat mitochondrial genome.*

It should be emphasized that for these experiments, it was imperative that the 26S and 18S rRNA probes not be contaminated with each other. For this reason, it was necessary to prepare the individual rRNAs from separated ribosomal subunits and to further purify the isolated RNAs by polyacrylamide gel electrophoresis. Occasionally, in spite of these precautions, careful

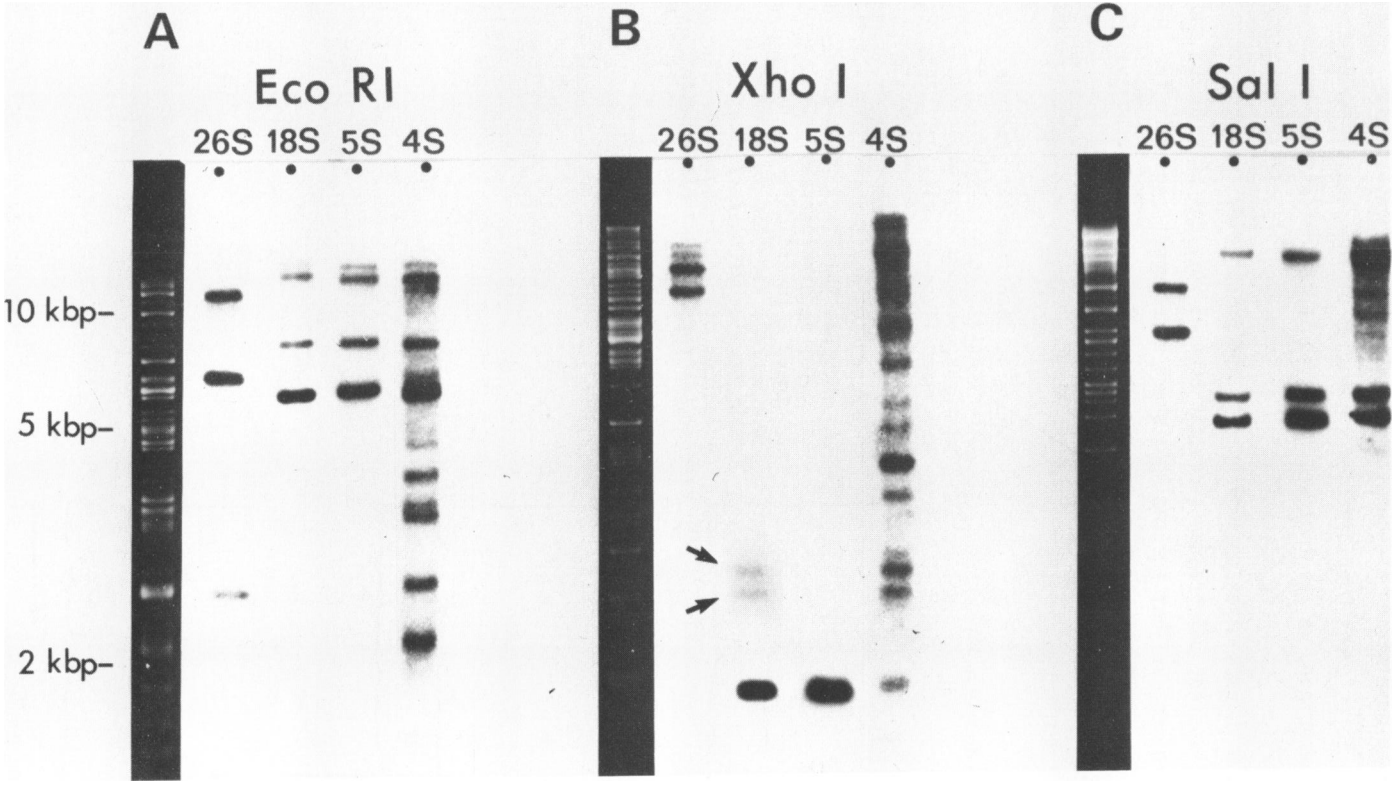

Figure 3. Autoradiograms showing the hybridization of ^{32}P-labeled wheat mitochondrial 26S, 18S, 5S, and 4S RNAs to nitrocellulose-bound fragments of wheat mtDNA hydrolyzed with *Eco* RI (A), *Xho* I (B), or *Sal* I (C). Ultraviolet photographs of the ethidium bromide-stained DNA fragments are shown to the left of each set of autoradiograms. The arrows in (B) indicate the positions of *Xho* I fragments X36 and X37 (see text).

examination of autoradiograms and densitometer tracings did reveal some evidence of possible "cross hybridization". In view of the sensitivity of the hybridization technique, we are inclined to attribute this occasional very weak "cross hybridization" to trace contamination of even highly-purified 26S and 18S RNA probes with each other, rather than to the possibility that the particular "cross-labeled" DNA fragments contain unequal portions of both 26S and 18S structural genes, or that the two RNAs share some common sequences [*cf.* 47]. Nevertheless, we intend to confirm our conclusion by fine structure analysis of cloned mtDNA fragments containing the rRNA genes.

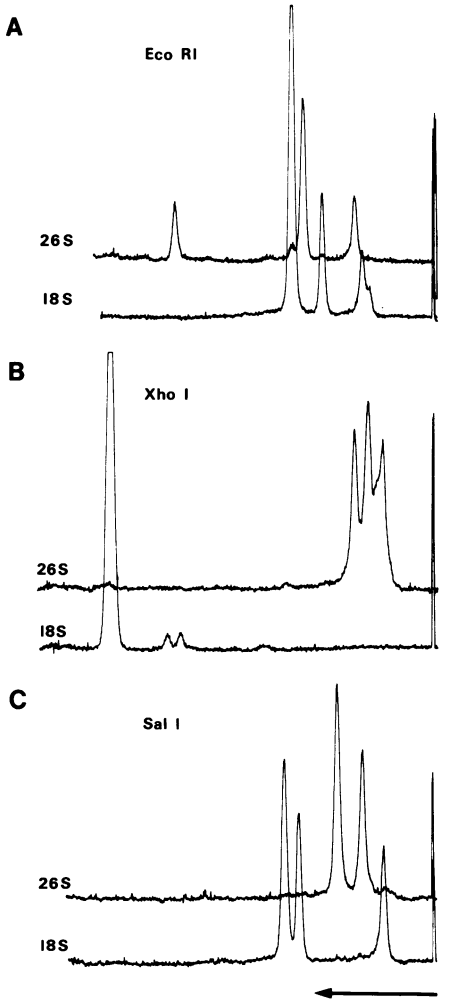

Figure 4. Densitometer tracings illustrating the hybridization patterns obtained when separated and immobilized restriction fragments of wheat mtDNA were hybridized with purified wheat mitochondrial 26S and 18S [^{32}P]rRNAs. (A), *Eco* RI; (B) *Xho* I; (C), *Sal* I. Following autoradiography, the developed X-ray films (Fig. 3) were scanned directly in a Joyce, Loebl microdensitometer. The direction of electrophoresis (arrow) corresponds to migration from top to bottom in Fig. 3.

(d) The Genes for Wheat Mitochondrial 18S and 5S rRNAs are Closely Linked

Since a distinct mitochondrial 5S RNA has so far been identified only in higher plants [21,24], we were particularly interested in determining the genetic origin of wheat mitochondrial 5S rRNA. For these experiments, we found it necessary to isolate 5S RNA from the 1 M NaCl-soluble fraction of total mitochondrial RNA rather than from the large subunit of mitoribosomes, since in the latter case the 5S RNA proved to be contaminated with fragments of mitochondrial 26S RNA.

As shown in Fig. 3, the mitochondrial 5S RNA hybridized to mtDNA; further, it hybridized to the *same* DNA fragments as 18S RNA, but not to those fragments with which 26S RNA hybridized. The results shown in Fig. 3, obtained using an intact 3' end-labeled probe, were confirmed in separate experiments with 5' end-labeled 5S RNA. From this, we conclude that *the genes for wheat mitochondrial 18S and 5S RNAs are closely linked, whereas the genes for the mitochondrial 5S and 26S RNAs are far apart.* It should be noted that the mitochondrial 5S RNA consistently hybridized to all of the DNA fragments containing the genes for mitochondrial 18S RNA *except* bands X36 and X37 in the *Xho* I digest (Fig. 3B, arrows). This might be explained by the presence of a cleavage site within 18S structural genes, which would generate DNA fragments containing that terminal portion of an 18S gene which was not physically linked to a 5S gene.

(e) Genes for Transfer RNAs Appear to be Both Scattered and Clustered in the Wheat Mitochondrial Genome

The mixed population of wheat mitochondrial 4S RNAs (tRNAs) hybridized with many large DNA fragments (at least a dozen in each restriction digest) (Fig. 4). Several additional small molecular weight DNA fragments, retained in shorter electrophoretic runs, were also labeled. The multiplicity of labeled bands suggests that *the genes for mitochondrial tRNA are widely distributed throughout the wheat mitochondrial genome. Nevertheless, some clustering of tRNA genes is also indicated,* since certain bands (e.g. S19 and S21 in the *Sal* I digest) were much more intensely labeled than others. In both the *Eco* RI and *Sal* I digests, fragments hybridizing with 18S RNA also hybridized strongly with 4S RNA. However, the bulk of this latter hybridization could not have been due to contamination of the 4S RNA probe with degradation products of 18S RNA, since in the *Xho* I digest the 4S [^{32}P]RNA probe only weakly labeled the area comprising fragments X43 - X45, with which almost all of the 18S RNA hybridization occurred.

(f) An Unusual Arrangement of Ribosomal RNA Genes in the Wheat Mitochondrial Genome

Table 1 summarizes the hybridization patterns observed for each of the wheat mitochondrial rRNAs in the three restriction digests (*Eco* RI, *Xho* I, *Sal* I) analyzed in detail in the present study. Enumeration of DNA fragments containing rRNA genes was based both on the data presented here and on results

Table 1 Size of mtDNA Fragments Carrying rRNA Genes

Endonuclease	RNA Probe	Fragment No.	M.W. (x 10^{-6})	Chain Length (kbp)[a]
Eco RI	26S	5	7.6	11.3
		12	4.3	6.4
		32-34[b]	1.7-1.8	2.6
	18S	2	*9.8*	*14.7*
		3	*8.4*	*12.6*
		10	5.2	7.7
		14	3.9	5.8
Xho I	26S	4	*12.5*	*18.7*
		5	*10.6*	*15.8*
		7	*9.0*	*13.4*
		10	7.2	10.7
	18S[c]	36	1.9	2.8
		37	1.7	2.6
		43-45[b]	1.1-1.2	1.7-1.8
Sal I	26S	11	7.3	10.9
		14	5.2	7.8
	18S	5-6[b]	*10.9-11.5*	*16.3-17.6*
		19	3.7	5.5
		21	3.3	5.0

Restriction fragments to which mitochondrial 26S and 18S rRNAs hybridized are numbered according to the densitometer tracings shown in Fig. 2. Molecular weights were estimated from a standard curve constructed using the known molecular weights of *Eco* RI restriction fragments of λ DNA [59]. Due to the extreme deviation from linearity in the curve above a molecular weight of about 8 x 10^6, the estimated sizes of the largest restriction fragments (numbers in italics) can only be considered approximate.

[a] Assuming one kbp corresponds to a molecular weight of 670.

[b] Because several DNA fragments co-migrate in these regions, it is unclear which one(s) are hybridizing to rRNA.

[c] An additional fragment (molecular weight about 0.6 x 10^6, estimated by linear extrapolation of the standard curve) was weakly labeled by 18S [^{32}P]RNA in short electrophoretic runs of *Xho* I digests.

from longer electrophoretic runs, in which the larger DNA fragments were better separated. Nevertheless, where a number of bands were closely spaced (e.g. bands S5 - S8) or not separated at all (e.g. bands X43 - X45), a precise correlation between autoradiogram and stained gel could not be made; in these cases, DNA fragments containing rRNA genes are identified tentatively.

Previous work in this laboratory [23,48] has demonstrated that wheat mitochondrial 18S rRNA is clearly prokaryotic in nature, showing significantly more sequence homology with prokaryotic and chloroplast 16S rRNAs than could have been expected to arise by chance. However, the arrangement of ribosomal genes in the wheat mitochondrial genome (18S and 5S close together but both distant from 26S) is quite different from the arrangement in prokaryotes and chloroplasts, where 23S and 5S genes are closely linked [49-52] and where 23S and 16S genes, although separated by a spacer region of up to 2 kbp [e.g. 50], are nevertheless part of the same transcriptional unit [53,54]. In animal mitochondria, the genes for the large and small subunit rRNAs are adjacent [10-12], but in some other mitochondrial systems, notably *Neurospora* [6-8], yeast [9], and *Tetrahymena* [55], the genes for the large and small subunit rRNAs are far apart [> 5 kbp]. However, in none of these mitochondrial systems has a 5S rRNA molecule been described. It is clear, therefore, that not only is there considerable variation in the physico-chemical properties of mitoribosomes and their constituent RNAs [1,2], but that this variation is reflected in quite different arrangements (and presumably modes of transcription and regulation) of rRNA genes in the different mitochondrial genomes that have been studied to date.

The complexity of the wheat mtDNA hybridization patterns (each rRNA hybridizing with several DNA fragments in each restriction digest) may have its origin in one or more of the following:

(1) *Physical heterogeneity in the mtDNA population,* as postulated by Quetier and Vedel [40] to explain the complex restriction patterns of plant mtDNA. Different physical arrangements of the same gene within different mtDNA molecules could give rise to a variety of restriction fragments containing a particular rRNA coding sequence. The relative proportions of these fragments would reflect the relative proportions, in the total population, of the mtDNA molecules containing them. Our finding of a multiplicity of bands, labeled unequally by a given [^{32}P]rRNA probe in each restriction digest of wheat mtDNA, is consistent with this model.

(2) *Heterogeneity of methylation at potential restriction sites* [40]. If the same restriction site were either methylated or non-methylated in differ-

ent (but otherwise physically homogeneous) mtDNA molecules, failure of a restriction endonuclease to cleave the methylated site could generate some of the larger mtDNA fragments which contain rRNA genes. Such partial cleavage would be consistent with the complexity of both the restriction and hybridization patterns. As documented elsewhere (L. Bonen and M.W. Gray, in preparation), this possibility seems less likely as a result of experiments using the isoschizomers *Hpa* II and *Msp* I, which can distinguish whether or not the same restriction site is methylated.

(3) *Multiple copies of each of the rRNA structural genes.* If each gene copy had different flanking sequences, then such multiple units might be found in different fragments after cleavage of the mtDNA with a given restriction endonuclease. The gene frequency for each of the wheat mitochondrial rRNAs is not yet known, since the interpretation of quantitative hybridization experiments is limited by lack of agreement concerning the genome size of plant mtDNA [41]. In other mitochondrial systems, only a single copy of each rRNA gene is present per genome equivalent [3,4], although *Tetrahymena* mtDNA does contain two copies (in an inverted repeat arrangement) of the gene for the large subunit rRNA [55]. Chloroplast DNA, which is similar in size and complexity to plant mtDNA, contains two or three complete sets of rRNA genes; these are either in inverted [50-52] or tandem [56] repeats within the DNA, so that the multiple rRNA genes may end up in multiple copies of the *same* DNA fragment(s) after restriction endonuclease cleavage, rather than in different fragments.

(4) *Cleavage within rRNA structural genes.* This is difficult to reconcile with the observation that in certain of the digests, several very large restriction fragments were labeled by a given [^{32}P]rRNA species. This was the case, for example, in the *Xho* I digest probed with mitochondrial 26S rRNA (Fig. 3B), where there were four labeled fragments 10-15 times the size of an rRNA structural gene--each fragment approaching or even exceeding the size of the entire animal mitochondrial genome! Admittedly, the presence of long intervening sequences within rRNA structural genes [6-8,57] might be expected to increase the probability of a gene being distributed between two or more restriction fragments, as, for example, in the case of the ovalbumin gene [58]. However, the possibility of internal cleavage is not compatible with the fact that both 18S and 5S rRNAs hybridized to *all of the same* fragments in *Eco* RI and *Sal* I digests of wheat mtDNA (Fig. 3A and 3C).

In the absence of a physical map of wheat mtDNA (and a unique map may be impossible to construct, if the mtDNA population is indeed heterogeneous),

more detailed analysis of the wheat mitochondrial genome will have to rely heavily on fine structure mapping of the individual restriction fragments, and determination of relationships among fragments in different restriction digests. Towards this end, we are undertaking the cloning and nucleotide sequence analysis of restriction fragments containing the genes for wheat mitochondrial rRNAs and tRNAs.

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REFERENCES

1 Borst, P. (1972) *Annu. Rev. Biochem. 41*, 333-376.
2 Avadhani, N.G., Lewis, F.S. and Rutman, R.J. (1975) *Sub-Cell. Biochem. 4*, 93-145.
3 Borst, P. and Grivell, L.A. (1973) *Biochimie 55*, 801-804.
4 Borst, P. (1977) *Trends in Biochemical Sciences 2*, 31-34.
5 Linnane, A.W. and Nagley, P. (1978) *Arch. Biochem. Biophys. 187*, 277-289.
6 Hahn, U., Lazarus, C.M., Lünsdorf, H. and Küntzel, H. (1979) *Cell 17*, 191-200.
7 de Vries, H., de Jonge, J.C., Bakker, H., Meurs, H. and Kroon, A. (1979) *Nucleic Acids Res. 6*, 1791-1803.
8 Heckman, J.E. and RajBhandary, U.L. (1979) *Cell 17*, 583-595.
9 Borst, P. and Grivell, L.A. (1978) *Cell 15*, 705-723.
10 Angerer, L., Davidson, N., Murphy, W., Lynch, D. and Attardi, G. (1976) *Cell 9*, 81-90.
11 Dawid, I.B., Klukas, C.K., Ohi, S., Ramirez, J.L. and Upholt, W.B. (1976) in *The Genetic Function of Mitochondrial DNA* (Saccone, C. and Kroon, A.M., eds), pp. 3-13, North-Holland Press, New York.
12 Saccone, C., Pepe, G., Bakker, H. and Kroon, A. (1977) in *Mitochondria 1977: Genetics and Biogenesis of Mitochondria* (Bandlow, W., Schweyen, R.J., Wolf, K. and Kaudewitz, F., eds), pp. 303-315, de Gruyter, New York.
13 Meng, R.L. and Vanderhoef, L.N. (1972) *Plant Physiol. 50*, 298-302.
14 Guderian, R.H., Pulliam, R.L. and Gordon, M.P. (1972) *Biochim. Biophys. Acta 262*, 50-65.

15 Guillemaut, P., Steinmetz, A., Burkard, G. and Weil, J.H. (1975) *Biochim. Biophys. Acta 378,* 64-72.
16 Guillemaut, P. and Weil, J.H. (1975) *Biochim. Biophys. Acta 407,* 240-248.
17 Jeannin, G., Burkard, G. and Weil, J.H. (1975) *Biochim. Biophys. Acta 442,* 24-31.
18 Leaver, C.J. and Harmey, M.A. (1973) *Biochem. Soc. Symp. 38,* 175-193.
19 Pring, D.R. (1974) *Plant Physiol. 53,* 677-683.
20 Pring, D.R. and Thornbury, D.W. (1975) *Biochim. Biophys. Acta 383,* 140-146.
21 Cunningham, R.S., Bonen, L., Doolittle, W.F. and Gray, M.W. (1976) *FEBS Lett. 69,* 116-122.
22 Cunningham, R.S. and Gray, M.W. (1977) *Biochim. Biophys. Acta 475,* 476-491.
23 Bonen, L., Cunningham, R.S., Gray, M.W. and Doolittle, W.F. (1977) *Nucleic Acids Res. 4,* 663-671.
24 Leaver, C.J. and Harmey, M.A. (1976) *Biochem. J. 157,* 275-277.
25 Johnston, F.B. and Stern, H. (1957) *Nature 179,* 160-161.
26 Marcus, A., Efron, D. and Weeks, D.P. (1974) *Methods in Enzymology 30,* 749-754.
27 Parish, J.H. and Kirby, K.S. (1966) *Biochim. Biophys. Acta 129,* 554-562.
28 Payne, P.I. and Loening, U.E. (1970) *Biochim. Biophys. Acta 224,* 128-135.
29 Leaver, C.J. (1973) *Biochem. J. 135,* 237-240.
30 Hassur, S.M. and Whitlock, H.W., Jr. (1974) *Anal. Biochem. 59,* 162-164.
31 Kolodner, R. and Tewari, K.K. (1975) *Biochim. Biophys. Acta 402,* 372-390.
32 Suyama, Y. and Bonner, W.D., Jr. (1966) *Plant Physiol. 41,* 383-388.
33 Wells, R. and Ingle, J. (1970) *Plant Physiol. 46,* 178-179.
34 Maizels, N. (1976) *Cell 9,* 431-438.
35 Maxam, A.M. and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. U.S. 74,* 560-564.
36 Walseth, T.F. and Johnson, R.A. (1979) *Biochim. Biophys. Acta 562,* 11-31.
37 Peattie, D.A. (1979) *Proc. Natl. Acad. Sci. U.S. 76,* 1760-1764.
38 Southern, E.M. (1975) *J. Mol. Biol. 98,* 503-517.
39 Swanstrom, R. and Shank, P.R. (1978) *Anal. Biochem. 86,* 184-192.
40 Quetier, F. and Vedel, F. (1977) *Nature 268,* 365-368.
41 Levings, C.S.,III and Pring, D.R. (1978) *Stadler Symp. 10,* 77-93.
42 Wolstenholme, D.R. and Gross, N.J. (1968) *Proc. Natl. Acad. Sci. U.S. 61,* 245-252.
43 Kolodner, R. and Tewari, K.K. (1972) *Proc. Natl. Acad. Sci. U.S. 69,* 1830-1834.
44 Vedel, F. and Quetier, F. (1974) *Biochim. Biophys. Acta 340,* 374-387.
45 Wells, R. and Birnstiel, M. (1969) *Biochem. J. 112,* 777-786.
46 Liang, G.H., Wang, A.S. and Phillips, R.L. (1977) *Can. J. Genet. Cytol. 19,* 425-435.
47 Scott, N.S. and Ingle, J. (1973) *Plant Physiol. 51,* 677-684.
48 Cunningham, R.S., Gray, M.W., Doolittle, W.F. and Bonen, L. (1977) in *Acides Nucléiques et Synthèse des Protéines Chez les Végétaux* (Bogorad, L. and Weil, J.H., eds), pp. 243-248, Editions du Centre National de la Recherche Scientifique, Paris.
49 Lund, E., Dahlberg, J.E., Lindahl, L., Jaskunas, S.R., Dennis, P.P. and Nomura, M. (1976) *Cell 7,* 165-177.
50 Bedbrook, J.R., Kolodner, R. and Bogorad, L. (1977) *Cell 11,* 739-749.
51 Whitfield, P.R., Hermann, R.G. and Bottomley, W. (1978) *Nucleic Acids Res. 5,* 1741-1751.
52 Rochaix, J.D. and Malnoe, P. (1978) *Cell 15,* 661-670.
53 Pace, N.R. (1973) *Bacteriol. Rev. 37,* 562-603.
54 Hartley, M.R. and Ellis, R.J. (1973) *Biochem. J. 134,* 249-262.

55 Goldbach, R.W., Borst, P., Bollen-de Boer, J.E. and Van Bruggen, E.F.J. (1978) *Biochim. Biophys. Acta 521,* 169-186.
56 Gray, P.W. and Hallick, R.B. (1978) *Biochemistry 17,* 284-289.
57 Bos, J.L., Heyting, C., Borst, P., Arnberg, A.C. and Van Bruggen, E.F.J. (1978) *Nature 275,* 336-338.
58 Breathnach, R., Mandel, J.L. and Chambon, P. (1977) *Nature 270,* 314-319.
59 Thomas, M. and Davis, R.W. (1975) *J. Mol. Biol. 91,* 315-328.