Studies on the *Poky* Mutant of *Neurospora crassa*

**FINGERPRINT ANALYSIS OF MITOCHONDRIAL RIBOSOMAL RNA***

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Base sequence and methylation of mitochondrial ribosomal RNAs from wild type and *poky* strains of *Neurospora crassa* were compared to determine whether a mutational lesion exists in *poky* 19 S RNA. At the outset, new procedures were developed for the isolation of intact nucleic acids from *Neurospora* mitochondria based on the substitution of Ca**2**+ for Mg**2**+ in the isolation media to inhibit mitochondrial nuclease activity. Using these procedures, intact and highly purified **3**P-labeled ribosomal RNAs were extracted from purified mitochondrial ribosomal subunits of wild type and *poky* and compared using three complementary fingerprinting systems: two-dimensional electrophoresis of T1 plus phosphatase digests and homochromatography of T1 and pancreatic RNase digests. In supplementary experiments, **3**P-labeled wild type RNA was co-fingerprinted with **3**P-labeled *poky* and ratios of **3**P/**3**P radioactivity were determined in each fragment to detect possible differences in stoichiometry. In addition, levels and patterns of methylated nucleotides were compared using procedures based on in vitro labeling with [methyl-**3**H]methionine and [**3**P]orthophosphate. In all these experiments, no difference was detected between wild type and *poky* in base sequence or methylation of either 19 S or 25 S RNA. Levels of methylation of *Neurospora* mitochondrial ribosomal RNAs were extremely low (less than 0.1% of the nucleotides), and results based on fingerprint analysis and DEAE-cellulose chromatography of alkaline hydrolysates of the [**3**H]methyl-labeled RNA suggested that 25 S RNA contains two ribose methylations, while 19 S RNA contains no methylated nucleotides.

The *poky* (mi-1) mutant of *Neurospora crassa* was isolated by Mitchell and Mitchell in 1952 (2) as a slow growing variant with deficiencies in several cytochromes and other alterations in mitochondrial enzyme systems (2–6). The *poky* phenotype is presumed to result from a mutation in mitochondrial DNA since it shows cytoplasmic inheritance (2) and can be transmitted to wild type by micro-injection of purified mitochondria (7). Although the primary defect in *poky* is unknown, several lines of evidence center attention on the assembly of mitochondrial ribosomes. *Poky* mitochondria are deficient in small ribosomal subunits so that the mass ratio of small to large subunits is 1:10 rather than 1:2 as in wild type (8, 9). At the same time, many of the other phenotypic changes in *poky* (e.g. the cytochrome deficiency) can be accounted for by the diminished capacity for mitochondrial protein synthesis which results from the ribosome defect (4, 6, 10, 11). In recent studies on mt rRNA processing, Kuriyama and Luck (12) found that mature mt rRNAs (19 S and 25 S RNA) in *Neurospora* are produced by cleavage of a common high molecular weight precursor (32 S) RNA. In *poky* mitochondria, pulse-labeled 32 S RNA appears to accumulate and the 19 S portion is rapidly degraded after cleavage (13).

From genetic considerations, the most likely initial hypothesis was that *poky’s* defective ribosome assembly is due to a primary defect in mt rRNA. These are known gene products of mt DNA (14–16) and, since each mitochondrial genome contains one gene each for 19 S and 25 S RNA (12, 14, 16), mt rRNA mutations would not be obscured by redundancy as in nonmitochondrial systems. On the other hand, a mutation in a mitochondrial ribosomal protein was largely precluded from consideration since these were all thought to be synthesized extra-mitochondrially and presumably specified by nuclear genes (17). As a first approach, lesions in *poky’s* mt RNAs could be sought in fingerprint experiments if the difficulty of isolating intact and highly purified mt RNAs as starting material could be overcome. The present report is divided into two parts. The first describes a new method of isolating intact RNA from purified mt ribosomal subunits. The method is based simply on substitution of Ca**2**+ for Mg**2**+ in the buffers used to prepare mt ribosomes, the rationale being that Mg**2**+ activates *Neurospora* mitochondrial nucleases, while Ca**2**+ does not (18). The procedures were used to isolate mt RNA for fingerprinting...
experiments, which are the main focus of the report. Three complementary fingerprinting systems were employed and wild type and poky mt rRNAs were compared with respect to
primary structure and level and pattern of methylated nucleotides. In all these experiments, no significant difference was
found in poky. However, the experiments do emphasize a unique structural feature of mt rRNAs, their extremely low
level of methylation compared to other types of rRNAs.

MATERIALS AND METHODS

Materials

The wild type strains were Em 5256A (FGSC #626), Lein 7A (FGSC #647; obtained from Fungal Genetics Stock Center, Humboldt State
University Foundation, Arcata, Calif.), Alohi 4A (FGSC #1259) and Lindegren a (the last two strains were obtained from Professor E. L.
Tatum, Rockefeller University). Auxotrophic strains considered "wild
type" for comparison with poky were wild-1 alb (3051-9-6a; obtained from E. L. T.) and me-3 KW-6 (derived from me-3 (Ref. 19)
as described in Refs. 13 and 20). The poky strain, poky (m-3), was that
used by Kuriyama and Luck (13). Procedures for maintaining strains and
preparing conidia have been described previously (21). The wild
type strains were maintained in vegetative growth on minimal medium
slants (22) and transferred monthly. The me-3 and poky strains were
stored as large stocks of lyophilized conidia from which fresh vegetative
cultures were started for each experiment. The pantothentic acid-
requiring strain was cultured on minimal medium supplemented with
10 mg of pantothenic acid/liter. For the methionine-requiring strains,
cultures were started for each experiment. The pantothenic acid-
requiring strain was cultured on minimal medium supplemented with
1.5 rnM L-methionine (carrier-free; New England Nuclear). Labeling with [methyl-3H]methionine was as described by
Lambowitz and Luck (20) except that the methionine supplement of
the medium was reduced to 0.375 m~ and the final specific activity was
found in poky. However, the experiments do emphasize a unique structural feature of mt rRNAs, their extremely low
level of methylation compared to other types of rRNAs.

Growth of Mycelia and Purification of Mitochondria

Mycelia were grown in liquid cultures as described previously (21,
22). For small mitochondrial isolations, mycelia were harvested in mid to late
log phase, 14-hour cultures for wild type strains and 24-hour cultures
for poky. Mycelia were disrupted by grinding with sand and mitochondria
were isolated using the flotation gradient method (24). One
flotation gradient accommodated mitochondria from 2 to 5 g wet weight
of cells (material from three to six culture flasks).

Labeling with Radioactive Precursors

14C labeled RNA for routine gel analysis was obtained from cells
grown in modified Vogel's minimal medium of low phosphate concen-
tration (0.2 to 1 mg of KH,PO/m containing [14C]phosphoric acid (carrier-free; New England Nuclear) at a final specific activity of 0.1 to
1 mCi/mmol. To obtain very high specific activity 14C-labeled RNA for
fingerprinting, the phosphate concentration of the medium was
reduced to 0.1 mg of KH,PO, and [14C]phosphoric acid was added to
a final specific activity of approximately 20 mCi/mmol. This specific
activity gave about 103 dpm/ug of mt RNA. The same procedures were
used for labeling with [32P]phosphoric acid (carrier-free, New
England Nuclear). Labeling with [methyl-3H]methionine was as described by
Lambowitz and Luck (20) except that the methionine supplement of
the medium was reduced to 0.375 m and the final specific activity was
approximately 250 mCi/mmol, equivalent to 1500 dpm/ug of mt RNA.
In control experiments, the lower methionine concentration was found to
inhibit mitochondrial nucleic acid synthesis. The methionine fraction from
each migration gradient was diluted with 3 to 4 volumes of 500 mM KC1/50
mm CaCl,25 mm Tris-HCl, pH 7.5/5 mm diithiothreitol (RSA Corp.,
N. Y.) and sedimented in a Beckman type 4 rotor (25,000 rpm, 1
min, 3'). The resulting pellet was resuspended in 3.8 ml of 500 K/50
mm CaCl, (diithiothreitol) and lysed by addition of 0.2 ml of 20%
Nonidet P-40. To separate mt rRNAs from membrane contaminants,
the lysate was layered over a 1.85 m sucrose cushion containing 500 K/25
mm CaCl,25 mm Tris-HCl (500 mM KC1/25 mm CaCl,25 mm Tris-HCl,
ph 7.5/5 mm diithiothreitol) and centrifuged in an IEC A321 rotor
(55,000 rpm, 17 hours, 37'). After centrifugation, the lysate and the top
of the sucrose layer were carefully withdrawn with a Pasteur pipette
and the sides of the tube were washed three times with glass-distilled
water to remove any contaminating nucleases. Then, the remainder of
the 1.85 m sucrose layer was removed and the transparent lipid-layered
pellet was resuspended quickly with about 1 ml of ice-cold glass-distilled
water. The ribosomal pellet was used to prepare RNA in either of two
ways.

Method A—radioactive RNA was prepared directly from the pellet by sedimen-
through SDS-sucrose gradients. The ribosomal pellet was
dissolved in a buffer containing 1% SDS/600 mm NaCl/25 mm Tris-HCl,
ph 7.4/0.5 mM EDTA and heated to 35'c for 3 min. Aliquots of the
resulting solution (0.3 to 0.4 ml containing 1 to 3 x 106 units) were then
layered over linear 6 to 23% sucrose gradients made up in the same
buffer but with 0.5% SDS and centrifuged in an IEC SB283 rotor
(35,000 rpm, 8 hours, 24'). The gradients were fractionated by
monitoring absorbance at 254 nm with an ISCO density gradient
fractionator. Separated 19 S and 25 S RNAs were collected and
precipitated twice by addition of 2 volumes of ethanol and storage at
-20'. Yields were 5 to 10 kg of 19 S RNA per culture flask with
respectively larger quantities of 25 S RNA.

Method B—The ribosomal pellet was used to prepare ribosomal
subunits from which RNAs were extracted with phenol/propane/SDS.
The ribosomal pellet was dissolved in 500 mM KC1/25 mm MgCl,25 mm
Tris-HCl, pH 7.5 and incubated with 1 m pmuromycin (adjusted
to pH 7.6 with KOH) for 15 min at 20' to obtain ribosomal subunits
(25). Aliquots of the solution (0.3 to 0.4 ml containing 2 to 4 x 106 units)
were then layered over linear 5 to 30% sucrose gradients in 500 K/25
mm MgCl, Tris and centrifuged in a rotor (40,000 rpm, 4
hours, 3'). Separated large and small ribosomal subunits were
precipitated by addition of 2 volumes of ethanol, with yeast tRNA
(Sigma) sometimes added to ensure quantitative precipitation. RNAs
were extracted using a modification of the phenol/propane/SDS
method of Suzuki and Brown (26). The subunits were resuspended in
2.0 ml of a buffer containing 0.10 M NaCl; 1% SDS; 2 mM EDTA; 25 mM
tris-HCl, pH 7.5; and 10 kg of polyvinyl sulfate, and then
deproteinized by incubation with pronase (100 kg per ml; Calbiochem)
for 20 min at 35' (the pronase had been preincubated for 1 hour at
35'). The RNAs were then purified by two cycles of extraction with
phenol/chloroform/isoamyl alcohol (0.5/0.5/0.02 volumes per 1 vol-
ume aqueous phase) at room temperature. RNAs were precipitated
twice with ethanol before fingerprinting. Yields were approximately 2
kg of 19 S RNA per culture flask.

RNA Extraction Using SDS-diethylpyrocarbonate

In several experiments, RNA was prepared for gel analysis by
extraction of whole mitochondria or mitochondrial lysates using the
SDS-diethylpyrocarbonate method (24, 27). Generally, yeast tRNA
was added as carrier so that the final RNA concentration was
approximately 1 x 106 unit/ml.

Gel Electrophoresis

Electrophoretic analysis of RNAs was carried out essentially as
described by Pescock and Dingman (28) on gels (6 mm thick) containing
2.4% acrylamide/0.8% agarose in 30 mM NaH,P04/3 mM Tris-HCl,
ph 7.9. The gels were run at 140 V (3') until the bromphenol blue marker had migrated two thirds of
the way through the gels. After electrophoresis, gels were stained with
Stains-all (Eastman-Kodak, Co., Rochester, New York) and cut
into 1-mm slices which were then placed into scintillation vials. The slices were incubated in 1 ml of 2 M NH₄OH overnight at 55° to hydrolyze the RNAs. The NH₄OH was then evaporated leaving approximately 0.8 ml of H₂O which was eluted with 10 ml of xylene-based scintillation fluid (29) containing butyl-PBD (5 g/liter; New England Nuclear) and Triton X 114 (30%, v/v). Samples were counted using a Beckmann LS250 scintillation counter.

**Fingerprinting Procedures**

**Enzymatic Digestion**—Digestion of RNA with ribonuclease T₁ (Sanyko Co., Tokyo, Japan) and pancreatic ribonuclease A (PRNase; Sanger) was carried out using the conditions of Sanger et al. (30, 31). 10 to 20 µg of RNA (rRNA plus carrier tRNA) were digested using an enzyme to substrate ratio of 1:15 for 30 min at 37°. Digestion with ribonuclease T₁ plus bacterial alkaline phosphatase (electrophoretically purified; BAPF-Worthington) was carried out as described by Maed and Salim (32) with 10 to 20 µg of RNA and an enzyme to substrate ratio of 1:15 for ribonuclease T₁ and 1:5 for alkaline phosphatase. A freshly prepared phosphatase solution was used for each incubation.

**T₁ plus Phosphatase Fingerprints**—The first dimension was electrophoresis on cellulose acetate strips (3 x 55 cm; Schleicher and Schuell, Dassel, Germany) impregnated with pH 3.5 buffer containing urea (7 M ures/5% acetic acid, adjusted to pH 3.5 with pyridine). Standard pH 3.5 buffers (0.5% pyridine/5% acetic acid (v/v)) was used in the electrophoresis tanks (Savant Instruments). Electrophoresis was for approximately 1½ hours at 3000 volts. For the second dimension, the entire strip except for a small portion near the origin was transferred to DEAE paper (85 x 47 cm; Whatman DE81). Electrophoresis was in 7% formic acid at 1000 volts for 16 hours until just before the blue marker had reached the top of the paper.

**Homochromatography**—The first dimension was electrophoresis on cellulose acetate as described above except for a shorter time (about 45 min). The region between the blue and second pink markers was transferred to DEAE-cellulose thin layer plates (Polygram Cel 300 DEAE; 40 x 20 cm; Brinkmann). Chromatography was as described by Brownlee and Sanger (35) using Homomixture c.

**Autoradiography**—Film was RP Royal X-Omat (Kodak). Exposure times were 3 days to 1 week.

**Counting of Radioactivity in Spots**—Spots were marked on the fingerprints using a template autoradiogram, cut out, transferred to scintillation vials, and counted in toluene plus Omnifluor.

**Elution of Spots**—In the analysis of the fingerprints of [H]methyl-labeled RNA, because of the very low counting efficiency of [H] in DEAE-paper (3 to 10%), it was necessary to elute the labeled oligonucleotides to obtain sufficient [H] counts. After counting as above, scintillation fluid was decanted and the spots were eluted rinsed twice with 10 ml of toluene. The paper was then dried at 60° and extracted twice with 15 ml triethylamine carbonate (30%, v/v). Extracted controls were developed for RNA isolation as described in detail under "Materials and Methods." In both cases, the starting material was mitochondria purified on flotation gradients to eliminate cytoplasmic RNA contamination (24). The mitochondria were lysed with Nonidet in buffers containing 5 to 50 mM CaCl₂ in combination with low and high salt (10 and 500 mM KCl, respectively). The lyses were incubated for 1 hour at 3°, after which the RNAs were extracted and analyzed by gel electrophoresis as shown in Fig. 1A. The most striking observation was that mt RNAs (and mt DNA; uppermost light band) were recovered intact from both low and high salt buffers in the presence of 50 mM Ca²⁺. The relatively high Ca²⁺-concentration was presumably required to displace endogenous mitochondrial Mg²⁺ from enzyme active sites, although it is also possible that Ca²⁺ binding to RNA directly inhibits interaction with nucleases (cf. Ref. 40). In any event, as the Ca²⁺ concentration was decreased there was progressively more degradation of mt RNAs which was more pronounced in the low than in the high salt buffer. The latter finding presumably reflects the decreased binding of nucleases to RNA in high salt media and, on this basis, the buffer containing 50 mM KCl and 50 mM Ca²⁺ (500 K⁺/50 Ca²⁺/Tris/dithiothreitol; see "Materials and Methods") was selected for further development. In other experiments, rRNAs isolated in the presence of 50 mM Ca²⁺ were found to contain no "hidden" breaks as judged by electrophoretic profiles after the RNA had been preheated to 60° in Sarkosyl-EDTA gel buffer. The advantage of Ca²⁺-containing buffers is strikingly illustrated in Fig. 1B by the integrity of mt RNAs, which can be extracted from ribosomal pellets prepared by sedimentation of mitochondrial lysates through a cushion of 1.85 M sucrose. For a shorter time (about 45 min).

**RESULTS**

**Preparation of Intact mt rRNAs**—When the work was initiated, procedures for isolating RNA from Neurospora mitochondria were inadequate for fingerprinting experiments, which absolutely require intact and highly purified RNA as starting material. In Neurospora, the nucleic acid released during mitochondrial lysis is resistant to the standard inhibitors (18, 34). As a result, RNA isolated after purification of mt ribosomes was found to consist largely of heterogeneous fragments, even under optimal conditions (34). It was possible to isolate intact nucleic acids by direct phenol or SDS extraction of whole mitochondria, but only in the presence of diethylpyrocarbonate for nuclelease inhibition (24, 35). These procedures do not permit isolation of ribonucleoprotein particles and since diethylpyrocarbonate has nucleotide-modifying properties (36, 37), the procedures are still unsuitable for fingerprinting experiments.

Faced with this situation, our first objective was to develop alternate procedures for isolating intact mt RNA. The methods devised are based on the simple expedient of substituting Ca²⁺ for Mg²⁺ in the isolation media, the rationale being that Mg²⁺ activates Neurospora mt nucleases, while Ca²⁺ does not (18). At the same time, Ca²⁺ can effectively replace Mg²⁺ in maintaining ribosome structure (38, 39). In initial experiments, to determine whether Ca²⁺-containing buffers could be used to isolate intact rRNA, mitochondria were lysed with Nonidet in buffers containing 5 to 50 mM CaCl₂ in combination with low and high salt (10 and 500 mM KCl, respectively). The lyses were incubated for 1 hour at 3°, after which the RNAs were extracted and analyzed by gel electrophoresis as shown in Fig. 1A. The most striking observation was that mt RNAs (and mt DNA; uppermost light band) were recovered intact from both low and high salt buffers in the presence of 50 mM Ca²⁺. Calcium and Mg²⁺-containing buffers were developed for RNA isolation as described in detail under "Materials and Methods." In both cases, the starting material was mitochondria purified on flotation gradients to eliminate cytoplasmic RNA contamination (24). The mitochondria were lysed with Nonidet in buffers containing 500 mM KCl and 50 mM Ca²⁺ (500 K⁺/50 Ca²⁺/Tris/dithiothreitol; see "Materials and Methods") was selected for further development. In other experiments, rRNAs isolated in the presence of 50 mM Ca²⁺ were found to contain no "hidden" breaks as judged by electrophoretic profiles after the RNA had been preheated to 60° in Sarkosyl-EDTA gel buffer. The advantage of Ca²⁺-containing buffers is strikingly illustrated in Fig. 1B by the integrity of mt RNAs, which can be extracted from ribosomal pellets prepared by sedimentation of mitochondrial lysates through a cushion of 1.85 M sucrose. For a shorter time (about 45 min).
Mitochondrial rRNAs in Neurospora

Fig. 1. A, gel electrophoresis of RNAs from mitochondrial lysates incubated in Ca\(^{2+}\)-containing buffers. Mitochondrial preparations, isolated from wild type strain Em 5565A using flotation gradients, were divided into several parts. Each of the final mitochondrial pellets was resuspended at a density of less than 2 mg of protein/ml in 2.0 ml of buffers containing 10 or 500 mM KCl, 5 to 50 mM CaCl\(_2\), 25 mM Tris-HCl, pH 7.5, and 5 mM dithiothreitol as indicated in the figure. Then, the mitochondria were lysed by addition of 1% Nonidet P-40, the lysates were incubated for 1 hour at 3\(^{\circ}\), and the RNAs were extracted using the SDS-diethylpyrocarbonate method. Yeast tRNA was added as carrier during the extraction. The extracted RNA was precipitated twice with ethanol and aliquots were taken for electrophoretic analysis. The direction of electrophoresis is from top to bottom and the heavily stained material near the bottom of the gels is 4 S RNA. B, gel behavior in high salt sucrose gradients made up in either Ca\(^{2+}\)- or Mg\(^{2+}\)-containing buffers (data not shown), indicating that Ca\(^{2+}\) can effectively maintain the structure of Neurospora mt ribosomes, at least in the presence of high salt.

Fig. 2A shows gel analyses of 19 S and 25 S RNAs isolated from wild type (me-3) and poky mitochondria using the two methods described above. All of the RNAs appear intact with relatively little visible contamination. These observations were confirmed by gel profiles as shown in Fig. 2B. The highest contamination was usually associated with poky 19 S RNA which was present initially in the smallest proportion (the gel pattern in Fig. 2B shows small peaks corresponding to mt DNA and 25 S RNA at slices 20 and 35, respectively). However, even in this case, the contamination amounts to less than 10% of the RNA, which is negligible for fingerprinting purposes. In the present work, all RNAs used for fingerprinting were isolated from purified ribosomal subunits according to Method b. This procedure diminishes the possibility of contamination by free RNAs and pronase treatment precludes variations in RNA-fingerprint patterns which might result from residual protein binding.

Fingerprinting: T\(_1\) plus Phosphatase Fingerprints—The major aim of the present work was to compare the primary structures of wild-type and poky mt rRNAs, primarily looking for a difference in poky 19 S RNA. To maximize the possibility of finding such a difference, three complementary fingerprinting systems were employed, each giving optimal resolution of a different set of sequences. Initial experiments centered on T\(_1\) plus phosphatase fingerprints. These give particularly good resolution of smaller fragments (up to 10 nucleotides) and were used to quantitatively compare the frequency of occurrence of individual sequences. As a supplemental approach, homochromatographic fingerprints were obtained for T\(_1\) and P-RNase digests in order to resolve larger fragments (those containing more than 10 nucleotides) which are poorly resolved by electrophoresis. Such large fragments are of particular interest because they may consist of unique nucleotide sequences characteristic of individual RNAs. The two types of enzymatic digestion are complementary, T\(_1\) cutting after G residues and P-RNase after pyrimidines.

Figs. 3 to 6 show T\(_1\) plus phosphatase fingerprints of 19 S and 25 S RNAs along with corresponding oligonucleotide maps (Figs. 4 and 6) which establish a standard geometry and some of the data are presented in a miniprint format immediately following this paper. Figs. 4 and 6 and Tables I through IV will be found on pp. 3094-3096. For the convenience of those who prefer to obtain the data in the form of 13 pages of full size photocopies, they are available as JBC Document Number 75M-1292. Orders should specify the title, authors, and reference to this paper, the JBC Document Number, and the number of copies desired. Orders should be addressed to the Journal of Biological Chemistry, 9650 Rockville Pike,
numbering system. Fingerprints were obtained for poky and three different wild type strains: Lein 7A, Abbott 4A, and Lindegren a. The primary comparison was between poky and Lein 7A, since poky was derived from Lein 7A and presumably has closely isogenic mtDNA. The other wild types were used to assess the extent of random variation in mt rRNA sequences and the possible influence of nuclear genes on the fingerprint patterns. It should be noted that the poky strain, poky f+ me-3, has a different nuclear background than the original (13).

Analysis of the T1 plus phosphatase fingerprints is facilitated by the fact that the fragments are arranged in a strict geometry determined by their composition (as described in Refs. 30 and 31 and summarized in the Legend of Fig. 3). Overall, the fingerprint patterns appear similar for poky and the various wild types, and this conclusion is supported by two types of more detailed comparison. The first focuses on longer fragments which generally consist of unique sequences, whose presence or absence may be scored by qualitative examination. In Neurospora T1 plus phosphatase fingerprints, a number of 5 to 12 nucleotide-containing fragments were identified as probable unique sequences on the basis of their position in the fingerprints and their proportion of the total radioactivity (Tables I and II; see supplementary material). These include for 19 S RNA; spots 23 to 27, 32 to 38, 40, 42 to 45, 49, 51, 57, 59, 65, 74, 80, and 84, and for 25 S RNA, spots 35 to 38, 47, 54, 58, 60, 62, and 73. No difference was apparent in the unique sequences resolved in wild type and poky in either 19 S or 25 S RNA.

The remaining spots in the T1 plus phosphatase fingerprints consist of redundant sequences or overlaps, so differences would appear only as differences in intensity. In order to determine whether such differences exist and also as a basis for future sequence analysis of 19 S and 25 S RNA, the proportion of total radioactivity recovered in each fragment was determined from three independent sets of Lein 7A and poky fingerprints. As shown in Tables I and II, nearly all of the values differed by less than 1 standard deviation between the two strains. However, while the data can be used to estimate degree of redundancy of individual sequences, some of the standard deviations are fairly large, probably reflecting variable recovery of fragments during fingerprinting and the difficulty of excising closely spaced spots. For this reason, an additional experiment was carried out in which T1 plus phosphatase fingerprints were obtained from 32P-labeled Lein 7A mixed with 33P-labeled poky. In this experiment, the ratio of 32P/33P radioactivity recovered in each fragment is a sensitive indicator of quantitative differences between the strains. The data are included in Tables I and II, and again no significant difference was found between wild type and poky for either RNA. (For 19 S RNA the 32P/33P ratios cluster between 1.3 and 1.4 and for 25 S RNA around 0.4; the different ratios for 19 S and 25 S RNA reflect the different relative proportions of the RNAs in wild type and poky.) The data suggest that there are no changes in poky mt rRNA resulting in loss or addition of any sequence resolved in the T1 plus phosphatase fingerprints (i.e. all T1 fragments except G) or in the transposition of one sequence to another. Moreover, since all T1 fragments that occur in wild type are accounted for in poky, it can be concluded that wild type and poky mt RNA have the same overall length within a few nucleotides. The data do not exclude a small change (e.g. single base substitution) in a large fragment, particularly one occurring in the "smear" region at the origin of the second dimension.

Bethesda, Maryland 20014, and must be accompanied by a remittance to the order of the Journal in the amount of $1.65 per set of photocopies.

* M. B. Mitchell, personal communication.
FIG. 3. $T_1$ plus phosphatase fingerprints of 19 S RNA from poky and three wild type strains (Lein 7A, Lindegren a, and Abbott 4A). In $T_1$ plus phosphatase fingerprints, oligonucleotides are arranged in strict geometry determined by composition. The pattern consists of sections ("graticules") composed of fragments containing the same number of uridine residues, which is the primary determinant of mobility in the second dimension. The simplest oligonucleotide in each graticule, UG, UUG, UUUG, forms a "center of gravity" and is indicated in the figures for reference. Oligonucleotides containing no uridine residues form a "tail" (indicated by AG in the figures) and those containing more than 3 uridine residues migrate poorly in the second dimension and remain near the origin. The composition of an oligonucleotide may be approximately determined from its position. Within each graticule, displacement away from the center of gravity along the left- and right-hand edges corresponds to increasing numbers of adenosine and cytosine residues, respectively. Thus, the longer fragments, those containing 5 to 10 nucleotides, are found in the distal regions of the graticules. Fragments containing more than 10 nucleotides are generally found near the origin of the second dimension in the "smear" region.
The other wild type strains, Abbott 4A and Lindegren a, gave T₁ plus phosphatase patterns essentially the same as those for poky and Lein 7A. However, a few differences do appear in the Abbott 4A/25 S RNA pattern, in particular the absence of spot 108 and a possible shift in the position of spot 119 (Fig. 5).

T₁ Homochromatography—In order to resolve the very large fragments which are clustered near the origin in the standard T₁ plus phosphatase fingerprints, homochromatography was used as an alternative method of fractionating T₁ digests. Fig. 7 shows T₁ homochromatographic fingerprints of 19 S and 25 S RNAs from poky and Lein 7A. The fractionation is now roughly according to size with the longer fragments at the bottom (near the origin) and the shorter fragments (which are more poorly
FIG. 7. Homochromatographic fingerprints of T, digests of 19 S and 25 S RNAs from poky and Lein 7A. Fragments containing approximately 10 or more nucleotides as judged from position in the fingerprint are numbered for comparison between the two strains. The lowermost fragments in each fingerprint mark the origin of the second dimension.
resolved than in T$_1$ plus phosphatase fingerprints) near the top. Fragments judged from their positions to be longer than 10 nucleotides are numbered for comparison between the two strains. Although the individual fingerprints differ somewhat in resolution, the patterns are strikingly similar between the two strains for both 19 S and 25 S RNA. Again, no difference was revealed in $^{32}$P/$^{33}$P mixing experiments (data not shown). The Lein 7A/25 S RNA fingerprint in Fig. 7 does show an extraneous spot just under spot 14. This spot was not present reproducibly and so is not considered significant.

The T$_1$ homochromatographic fingerprints of Fig. 7 reveal a comparatively large number of long T$_1$ fragments, presumably reflecting the low GC content of mt rRNAs (41). Fragments containing more than 20 to 25 nucleotides remain near the origin even in homochromatography. At least 6 such fragments are present in 19 S RNA and 15 in 25 S RNA. The fragments do not appear to be artifacts of incomplete digestion since the same homochromatographic patterns were obtained even if the enzyme to substrate ratio was increased 2- to 3-fold (data not shown). By contrast, T$_1$ digests of Escherichia coli 16 S RNA contain only 1 or 2 fragments which approach this length (42) and mouse cell 18 S and 28 S RNAs combined contain approximately 5 (43).

P-RNase Homochromatography—To exclude the possibility that some difference is overlooked by relying exclusively on T$_1$ digestion, homochromatographic fingerprints were also obtained for P-RNase digests. Since P-RNase cuts after pyrimidines, the longer fragments will now be those containing purine tracts which had probably been cut into small pieces by T$_1$. Fig. 8 shows the fingerprints for 19 S RNA. Initially, there was a promising difference: two independent preparations of poky 19 S RNA were deficient in spot 13 which was, in turn, present in 3 different wild types: Lein 7A, Abbott 4A, and Lindegren a. However, in a final experiment, spot 13 was found to be “light” in a fourth “wild type,” pan-1 al-2 and also in 19 S RNA prepared from mixed $^{32}$P-labeled Lein 7A and unlabeled poky cells (indicated by Mix in the figure). These findings negate the significance of the deficient spot 13 in the poky fingerprints. Otherwise, the pattern of longer fragments was identical for poky and Lein 7A except for spot 7 which, for unknown reasons, alternated between two positions in both strains. Some variability might be expected in P-RNase digests since the enzyme is known to cut slowly after purines (30). However, in control experiments, no difference in the patterns could be detected when the standard digestion time (30 min) was varied between 15 min and 1 hour. As shown in Fig. 9, P-RNase homochromatographic patterns for 25 S RNA were identical for poky and Lein 7A.

Methylation of mt rRNAs—As an extension of the fingerprint comparison, a second series of experiments was carried out comparing wild type and poky mt rRNAs with respect to levels and patterns of methylated nucleotides. In earlier work, Kuriyama and Luck (13), using procedures based on in vivo labeling with [methyl-$^3$H]methionine and [$^{32}$P]orthophosphate, reported that 19 S and 25 S RNAs from wild type had relatively high levels of methylation (approximately 1 methyl group/100 nucleotides) and that both mt rRNAs were grossly undermethylated in poky. Subsequently, however, we found that much of the $^3$H label incorporated into RNA under the conditions of Kuriyama and Luck (13) had been randomized directly into purine carbon skeletons (20). In other systems, such extra incorporation is suppressed by adding sodium formate (a purine precursor) to the labeling medium (44-46). Repeating the experiments of Kuriyama and Luck (13) with this additional precaution, much lower values were found for Neurospora mt rRNA methylation (0.05 to 0.16 methyl groups/100 nucleotides) and the difference between wild type and poky disappeared. The more recent data are summarized in Table III. In the case of the wild type (me-3) strain, the values correspond to approximately four methyl groups per molecule of 25 S RNA and one base methylation per molecule of 19 S RNA. The values for poky are in close agreement, particularly those in the Total column.
FIG. 9. Homochromatographic fingerprints of P-RNase digests of 25 S RNA from poky and Lein 7A. Larger fragments are numbered for comparison.

(Table III) based on gel analysis of several independent preparations. The values for poky base methylation do appear slightly higher than those for wild type, but the difference is not considered significant, since the very low levels of methylation are difficult to quantitate by conventional labeling techniques. In fact, as pointed out previously (20), it is still not clear whether any of the apparent base methylation is due to residual randomization of "H label or whether such randomization has obscured a small difference between wild type and poky. The initial studies also gave no information about patterns of methylated nucleotides in the two strains.

To address these questions directly, experiments were carried out in which ["H]methyl-labeled RNA was subject to fingerprint analysis. In these experiments, wild type (me-3) and poky (f+ me-3) mt rRNAs were labeled in vivo with [methyl-"H]methionine and ["P]orthophosphate. Methionine-requiring strains were used to ensure quantitative incorporation of ["H]methyl label and for the purpose of comparison with previous studies. Sodium formate (10 mM) and adenosine and guanosine (0.1 mM) were added to the culture medium to suppress randomization of "H label. An additional problem in these experiments was the low yield of mt rRNAs (compared, for example, to cytoplasmic rRNAs). As a result, we were always faced with low recovery of expensive "H counts and it was necessary to use the entire preparation of each labeled RNA to obtain a single T, plus phosphatase fingerprint. All of the spots in the fingerprint were cut out and counted in toluene plus 2,5-diphenyloxazole (PPO). Spots showing significant "H label (more than 25 cpm) were recovered from the scintillation vials, extracted with triethylamine carbonate and recounted for quantitation. Moreover, because of the low counting efficiency of "H in paper (approximately 5 to 10%), all other spots which from the initial counting could be suspected of harboring "H radioactivity were also eluted (50 to 75 spots in all for each fingerprint). In other experiments, an attempt was made to locate methyl groups by autoradiography using ["C]methyl-labeled RNA. Unfortunately, because of the low yield of mt rRNAs, it was not possible to obtain sufficient "C counts per min.

The results of the "H/"P experiment are summarized in Table IV which lists all T, plus phosphatase fragments found to contain "H label. For each fragment, the table also shows the ratio of "H/"P radioactivity and the number of methyl groups per molecule to which the "H radioactivity corresponds. In this type of experiment, randomized "H label would appear concentrated in small purine-containing fragments, e.g. A-G, which are highly redundant and so contain a relatively large number of bases. In addition, since each individual base contains only a small amount of randomized "H label, the ratio of "H/"P radioactivity in such fragments would be low and, in general, the overall "H radioactivity would correspond to less than one methyl group per RNA molecule. By these criteria, only one of the fragments in Table IV, 25 S RNA/spot 118, contains a "real" methyl group with a ratio of "H/"P radioactivity strikingly higher than those for the other fragments and with total "H radioactivity corresponding to approximately one methyl group per RNA molecule in both the wild type (me-3) and poky experiments. From the position of spot 118 in the fingerprints (Figs. 5 and 6), its composition may be approximated as (ACU)-G. The "H label associated with the remaining fragments appears to be due to randomization, emphasized in particular by the uniformly low ratios of "H/"P radioactivity. In the experiment of Table IV, the extent of randomization as judged by this ratio appears to be greater in wild type than in poky. However, in the previous study (20), in which analyses were carried out for several independent preparations from both strains, apparent levels of methylation were sometimes higher for poky (note the higher apparent base methylation for poky in Table III). This variability was previously attributed to small differences in the extent of "H label randomization in individual experiments, and the present results seem to confirm this conclusion.

For the me-3 strain, the level of randomization as judged by the fingerprint experiment is quantitatively sufficient to ac-
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count for all of the \(^4\text{H}\) label attributed to base methylation in both 19 S and 25 S RNA in Table III. This conclusion probably holds for the poky mt RNAAs as well. Although the level of randomization does appear lower in the poky fingerprint experiment, it should be emphasized that there is variability between preparations and that the data in Table IV actually underestimate randomized label, since a substantial proportion must also be associated with T-A and C-G which do not appear in these fingerprints. Moreover, in poky as well as in me-3, only one "real" methylated fragment could be identified, a finding which itself implies that much of the apparent base methylation is artifactual. Thus, considered together, the results suggest that all of the apparent base methylation in both mt RNAAs in both strains is due to \(^4\text{H}\) label randomization. On this basis, 25 S RNA contains only two ribose methylations and 19 S RNA contains no methylated nucleotides.

The presence of two ribose methylations in 25 S RNA was deduced in the previous study by a method which is not vulnerable to randomization of \(^4\text{H}\) label (i.e., \(^4\text{H}\) radioactivity in a separated alkali-stable dinucleotide fraction; see Ref. 20 and legend of Table III). Presumably, one of these ribose methylations is present in spot 118. The other appears to have been missed in the fingerprint analysis, possibly casually overlooked, but more likely associated with a "light" spot outside of the regular T, plus phosphatase pattern which would necessarily have been overlooked. Such a spot could consist of a 5' or 3' terminus or of a fragment containing an internal G with a methylated ribose moiety which would confer resistance to T, hydrolysis.

It should be emphasized that the one methylated fragment which was found, 25 S RNA/spot 118, was present in both wild type and poky, so again there were no differences between the strains.

DISCUSSION

During the initial stage of this work, the most likely hypothesis was that the deficiency of small ribosomal subunits in poky mitochondria could be attributed to a defect in 19 S RNA. To test this hypothesis, the primary structure of wild type (mainly Lein 'A') and poky mt RNAAs were compared in three different fingerprinting systems and no differences were found. The analysis of T, plus phosphatase fingerprints rules out a change in poky mt RNAAs resulting in loss or addition of any sequences resolved in the fingerprints or in the transposition of one sequence to another. This conclusion is emphasized by the similar \(^3\text{P}/\(^3\text{P}\) ratios in all T, fragments when \(^3\text{P}\)-labeled Lein 'A' was co-fingerprinted with \(^3\text{P}\)-labeled poky. Moreover, since all T, fragments appear to be conserved in poky, the experiment suggests that there is no change in the overall size of the mt RNAAs which might have resulted from deletion, insertion, or misprocessing. The homochromatographic fingerprints support this conclusion and also display a striking similarity in the primary structure of very long T, and P RNAAs fragments from wild type and poky mt RNAAs.

Overall, the data do not exclude certain types of modifications of long fragments, e.g. single base substitutions, which might have occurred without altering sites of enzymatic cleavage or affecting the mobility of the fragments. Similarly, changes occurring in "light" spots outside of the regular fingerprint patterns, for example 5' or 3' termini, could have been overlooked. Apart from these possibilities, however, the results strongly suggest that the poky mutation does not affect the primary structure of the mt RNAAs.

A second aspect of the present work was the comparison of mt RNA methylation in wild type and poky. In nonmitochondrial systems, RNAAs contain 1 to 2% methylated nucleotides, and there is some evidence correlating methylation with RNA processing and function. In HeLa cells, the RNAAs are transcribed in tandem as a single 45 S precursor molecule which is, subsequently cleaved and trimmed to yield mature 18 S and 28 S RNAAs (47). Nearly all of the methylations occur on the 45 S RNA and are confined to sequences conserved during processing (32). More suggestive, when HeLa cells are deprived of methionine, methylation of 45 S RNA is inhibited and the undermethylated 45 S molecules accumulate and are not processed further (48). In hepatoma cells, polynucleosinate, an inhibitor of RNA methylases brings about a reduction in the ratio of 18 S to 28 S RNA (49), suggesting that defects in methylation may lead to a selective loss of small subunit RNA in this system. By contrast, E. coli also contain a 30 S precursor RNA, analogous to HeLa cell 45 S RNA, but the E. coli precursor is poorly methylated and seems to be processed normally in methionine auxotrophs grown with ethionine (50). Nevertheless, methylation of E. coli 16 S and 23 S RNAAs has been shown to be absolutely required for ribosome function (55,56). Thus, considered together, the results suggest that methylation may play a direct role in RNA processing or function and, for this reason, studies on mt RNA methylation in wild type and poky Neurospora were initiated. Surprisingly, however, levels of methylation of Neurospora mt RNAAs were found to be extremely low, apparently consisting of a total of two ribose methylations both found on 25 S RNA and no difference could be detected between wild type and poky. The very low levels of methylation agree closely with those reported for HeLa and hamster cell mt RNA (57-59), but contrast sharply with those for RNAAs from mammalian mitochondrial sources, for example HeLa cell cytoplasmic 18 S and 28 S RNAAs, which contain 46 and 71 methyl groups, respectively (32).

Before assessing the significance of the low levels of methylation of mt RNAAs, it should be considered that nearly all of the results are based on in vivo labeling with [methyl-\(^3\text{H}\)]methionine. This procedure, which has been used routinely to study methylation of other RNAAs, assumes that [methyl-\(^3\text{H}\)]methionine equilibrates with intramitochondrial S-adenosylmethionine and, indeed, that S-adenosylmethionine is the primary substrate for mt RNA methylases. With regard to the first assumption, the direct experiment with Neurospora has shown that intramitochondrial S-adenosylmethionine is efficiently labeled by [methyl-\(^3\text{H}\)]methionine in vivo (20) (and also, in the case of yeast, no discrepancy exists in the specific activity of the methyl group in 25 S RNA/spot 118, since the \(^3\text{H}\) counts correspond to one methyl group per RNA molecule). Similarly, Dubin and Taylor (60) showed that [methyl-\(^3\text{H}\)]methionine is incorporated with equal efficiency into \(^N^\text{N}\)-methyladenine from mitochondria and cytoplasmic RNAAs in eukaryotic cells. With regard to the second assumption, there is, strictly speaking, no direct proof that S-adenosylmethionine is the substrate for all mt RNA methylases. However, the same labeling procedures did reveal substantial base methylation in Neurospora mitochondrial 4 S RNAAs (approximately 1.7 methyl groups/100 nucleotides; Ref. 20) and, on this basis, low
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levels of methylation appear to be a unique property of mt rRNAs and not artifacts of the labeling procedure.

The results for Neurospora mt rRNA methylation have direct relevance to the postulated role of methyl groups in rRNA processing. In Neurospora mitochondria, rRNAs arise from cleavage of a 32 S precursor molecule which is analogous to the HeLa cell 45 S RNA (12). From previous work, it is known that 32 S RNA, like the mature mt rRNAs, appears poorly methylated (20). It can be concluded, therefore, that large numbers of methyl groups are not required for processing of ribosomal precursor rRNAs. In addition, from work with HeLa cells, and by analogy with prokaryotic restriction-modification systems, it has often been postulated that methyl groups function as recognition signals for specific nucleases and other rRNA processing enzymes (47). In the case of the Neurospora mt rRNAs, there are clearly too few methyl groups for this to be the case. Rather, the results are consistent with the hypothesis that methyl groups in other rRNAs merely facilitate specific conformations which are required for processing or ribosome formation and that these conformations can be attained without substantial methylation in Neurospora mt rRNAs.

Since the experiments in this report do not support the hypothesis that the primary lesion in poky involves mt rRNA, it now seems reasonable to adopt an alternate view in terms of future work. From other studies, it is known that poky’s mt ribosomes are active in protein synthesis since they are of the primary defect in Neurospora. In that case, we may focus on two alternate possibilities: (a) that the primary defect lies in the unconserved regions of 32 S precursor RNA and (b) that the defect is in a mitochondrially synthesized assembly protein associated with the 32 S RNA. The first possibility could be approached experimentally by fingerprinting 32 S RNA from wild type and poky. However, at the present time, it is not possible to isolate sufficient pure 32 S RNA from poky in which there is a high background of pulse-labeled heterogenous RNA (13). Subsequent investigations focused on the second possibility, by isolating mt ribosomal precursor particles and comparing their protein composition in wild type and poky. These investigations led to the identification of a mitochondrially synthesized ribosomal protein (S-4a; apparent M, = 52,000) which could conceivably be the site of the primary defect in poky.4

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Addendum—After this manuscript was submitted for publication, Kloczewiak et al. (44) reported identical conclusions about methylation of mitochondrial ribosomal rRNAs from the yeast S. carlsbergensis. 21 S RNA was found to contain two ribose methylations and 15 S RNA, no methylated nucleotides.

REFERENCES

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Figure 4. Oligonucleotide map for T₁ plus phosphatase fingerprints of 19 S RNA. Map is a tracing of the Abbott 4A fingerprint in Fig. 3. The position of spots in the "smear" region (numbers 1 to 22) was approximated from a lightly exposed autoradiogram in which the spots appear better resolved than in Fig. 3.

Figure 5. Oligonucleotide map for T₁ plus phosphatase fingerprints of 25 S RNA. Map is a tracing of phyki fingerprint in Fig. 5. The position of spots in the "smear" region was deduced from a lightly exposed autoradiogram.