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The anatomy of the tRNA - rRNA region of the Neurospora crassa mitochondrial DNA

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ABSTRACT

To obtain more information about the arrangement of Hind III restriction fragments in the tRNA-rRNA region of the Neurospora crassa mitochondrial (mt) DNA we have cleaved the mtDNA with Hpa I and Hind II. We could construct additional cleavage maps for these enzymes. Hybridization of rRNAs to Hind II fragments confirmed the existence of an intervening region of about 2,300 basepairs in the 24S rRNA (Hahn et al., Cell, in press). About seven tRNA genes, among which the genes for $tRNA^{Ser}_{1}$ and $tRNA^{Met}_{M}$, are located in a segment of about 5,000 bp separating the 24S and 17S rRNA genes. Another cluster of 14 tRNA genes is found adjacent to the other end of the 24S gene. The genes for $tRNA^{Leu}_{1}$ and $tRNA^{Met}_{F}$ are located in this cluster.

INTRODUCTION

We have previously presented a restriction enzyme cleavage map of the 60,000 bp circular mitochondrial DNA of *Neurospora crassa*, using the enzymes Eco RI, Bam HI and Hind III[1-3]. The rRNA genes and most of the 25-27 tRNA genes were shown to be located in a segment of 16 kbp[3,4].

The exact positioning of the tRNA genes was complicated by the presence of two Hind III fragments H7 in this area (2,470 bp each). These contain 10 tRNA genes together, but it was not shown how many genes each of these H7 fragments contains individually. Moreover, uncertainty existed about the order of the fragments H12 and H13. H12 contains 4 or 5 tRNA genes, H13 none. To get a better insight in the relative position of the tRNA genes, we decided to cleave the DNA with restriction enzymes that do not yield too small fragments on the one hand and have enough cleavage sites in the tRNA-rRNA region on the other. Furthermore, we were interested in knowing the position of the genes for a number of specific tRNA-species. The aim of this article is to describe the relative position of the genes for the tRNAs and for 24S and 17S ribosomal RNAs. We will present experiments which show that the order of Hind III fragments as we have previously reported it, based on the assumption that the 24S rRNA gene is continuous, has to be revised. We will add

biochemical evidence to the existence of an intron in the 24S rRNA gene which was demonstrated electronmicroscopically by Hahn *et al.*[5]

METHODS AND MATERIALS

Strains of *Neurospora crassa*: For most of the experiments in this paper, we used wild type strain CBS 327.54, which was obtained from the Centraal Bureau voor Schimmelcultures (C.B.S.), Baarn, The Netherlands. We have always regarded this strain as Em 5256, based on information received from the C.B.S. However, in view of the data of Bernard and Küntzel[6] on the one hand and on unpublished observations of Mannella and Lambowitz (pers. commun.) on the other, it is clear that the mtDNA of our strain is of the "Lindegren(L)-type", whereas the real Em 5256 has "Abbott(A)-type" mtDNA. The differences between these two types of mtDNA are located in only two regions: E5 Lindegren is 3,500 bp, E5 Abbott is 2,500 bp; E9 L is 2,000 bp, E9 A is 2,100 bp. All other regions are identical as judged from cleavage patterns obtained with Eco RI[6], Bam HI[6], Hind II and Hpa I (our unpublished observations). For some experiments, mtDNA from strains *inl*-89601 (FGSC 497) and ANT-1[7], obtained from D.L. Edwards, was used. Both strains had the Lindegren-type of mtDNA.

Mitochondrial DNA was isolated from DNase-treated mitochondria as described by Terpstra *et al.*[2]

Digestion of mtDNA with restriction endonucleases, slabgel electrophoresis, visualization of the bands and photography of the gels was performed as described previously[2], for Hind II and Hpa I the same medium was used as for Bam HI. The DNA input per digest was 5 µg. For preparative purposes 100-150 µg were loaded on a 2.5 cm slot. Bands in agarose were reisolated by the KI procedure of Blin *et al.*[8] as described earlier[2]. Bands in polyacrylamide were extracted according to Van den Hondel *et al*[9].

Transfer of endonuclease fragments to nitrocellulose filters was performed according to Southern[10] after alkali denaturation of the DNA fragments.

Isolation of mitochondrial tRNAs was exactly as described previously[11].

Labeling of tRNA with radioactive aminoacids: Total mt tRNA was charged with the appropriate [^{3}H]- or [^{35}S]-labeled amino acid as described by De Vries *et al.*[11]

Two-dimensional gel-electrophoresis of tRNA, either unlabeled, labeled

in one amino acid or [^{125}I]-labeled, was performed according to Fradin *et al*[12]. [^{125}I]-tRNA species were reisolated, after detection of the spots by autoradiography, from the slabgel according to Adams *et al.*[13] More than 90% of radioactivity was eluted. RNA was precipitated by ethanol.

Isolation of mitochondrial ribosomal RNAs was as described by Terpstra *et al.*[3]

Labeling of RNAs with [^{125}I]-iodide was performed exactly as described by Terpstra *et al.*[3]

Hybridization of labeled RNAs to stripfilters was performed in a glass tube closed on both sides with rubber stoppers and submersed in a shaking water bath. The hybridization medium contained 30% formamide, 2xSSC and 5-10 µg of labeled RNA. Hybridization was for 22-24 h at 36°5C. Filters were washed and ribonuclease-treated as described[3]. The dried filters were subjected to autoradiography with Kodak XR-1 X-omat film.

In the case of hybridization with amino acid-labeled tRNA the RNase treatment was omitted and the stripfilter was soaked in 20% PPO in toluene and dried before autoradiography (4 weeks at -70°C).

Restriction enzymes: Eco RI was from Boehringer, Hind III, Hind II, Hpa I and Bam HI from New England Biolabs.

Radioactive compounds were from the Radiochemical Centre, Amersham.

Nomenclature of restriction fragments: The following prefixes are used: E = Eco RI fragments, B = Bam HI fragments, Hp = Hpa I fragments, H = Hind III fragments, h = Hind II fragments. Bands are numbered in order of increasing mobility.

RESULTS

A. Cleavage maps of mtDNA with Hpa I and Hind II.

Hind II recognizes GTPyPuAC, Hpa I GTTAAC. Therefore, Hind II is expected to cut all Hpa I sites, a number of Hpa I fragments will be identical to Hind II fragments and redigestion of isolated Hpa I fragments with Hind II should only yield known Hind II fragments. Table I shows the cleavage products obtained by digestion of mtDNA with these two enzymes. Hpa I yields 8 fragments, Hind II 17 fragments. Tentatively, Hp3, Hp6, Hp7 and Hp8 were assumed to be identical to h1, h7, h10 and h17, resp.

To order the Hpa I fragments we have double-digested total mtDNA with Hpa I + Bam HI, and with Hpa I + Eco RI, and redigested isolated Hpa I frag-

TABLE I

CLEAVAGE PRODUCTS OBTAINED BY DIGESTION OF mtDNA WITH Hind II AND Hpa I

Hind II fragments	basepairs	Hpa I fragments	basepairs
h1	9,600	Hp1	14,600
h2	8,100	Hp2	13,600
h3	7,000	Hp3	9,600
h4	5,400	Hp4	7,900
h5	4,500	Hp5	7,150
h6	4,400	Hp6	4,300
h7	4,300	Hp7	2,600
h8	4,150	Hp8	200
h9	3,400		59,950
h10	2,550		
h11	2,300		
h12	1,350		
h13	1,000		
h14	540		
h15	400		
h16	250		
h17	200		
	59,490		

ments with Bam HI and Eco RI. In this way we could establish beyond doubt the order given in Fig. 1. To place the Hind II fragments on the map, we made use of the identity, mentioned above, of several Hpa I and Hind II fragments. Furthermore, digestion of isolated Hpa I fragments with Hind II allowed us to draw the following conclusions: Hp1 contains h5, h6, h9, h12 and h13; Hp2 contains h2, h8, h14 and h15; Hp3 is h1; Hp4 is composed of h4 and h11; Hp5 is composed of h3 and h16; Hp6 is h7; Hp7 is h10; Hp8 is h17. Of the fragments constituting Hp1, only h5 and h9 are cut by Eco RI in a double digest of total mtDNA, so each of these two must lie at an end of Hp1; of the fragments constituting Hp2, only h8 is cut by Eco RI, whereas both h2 and h8 are cut by Bam HI. The order of Hind II fragments as obtained in this way is given in Fig. 1.

B. Hybridization of mtRNAs to stripfilters of Hind II and Hpa I digests.

It was demonstrated by us through hybridization of [^{125}I]-labeled tRNA to Eco RI, Bam HI and Hind II stripfilters[4] that the total number of mt-tRNA

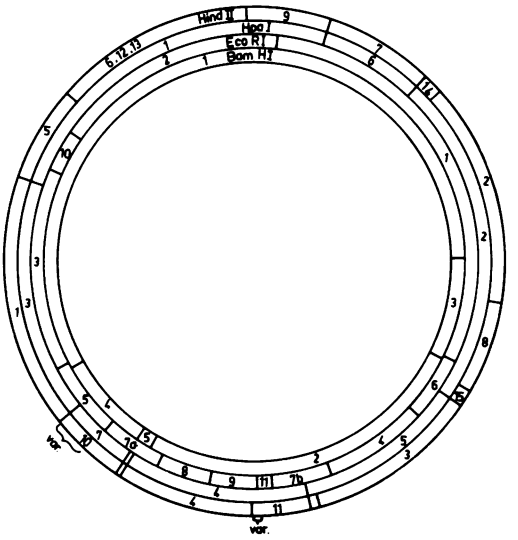

Fig. 1. Location of the Hpa I and Hind II fragments on the Eco RI-Bam HI map.

genes is 26-27. Plateau hybridization with [^{32}P]-labeled tRNA also indicated a value of 27 tRNA genes (unpublished results).

Fig. 2a shows the hybridization of total mitochondrial [^{125}I]-tRNA to a Hind II filter. It is evident that most of the radioactivity is located on fragments h2, h7, h8 and h9, with less hybridization on h1, h3 and h13. Quantitation of tRNA genes on each fragment by counting the separate bands is shown in Table II.

The fragments h2, h7, h8 and h9 should all lie on E1, which contains 21 tRNA genes. Hence, h9 lies on the righthand side of Hp1, and consequently h5 on the lefthand side. By digestion of h9 with Hind III (not shown) we found that it contains H10 (6 tRNA genes), and part of H7b. The two fragments H7 on E1 contain 10 tRNA genes together, so the remaining 2 tRNA genes of h9 are on H7b. Fragment h7 has 5 or 6 tRNA genes, which should also be on H7b, since h7 further contains only Hind III fragments that carry no tRNA genes: H13, H18, H20 and part of H15. The 6 to 7 tRNA genes on h2 should be those of H7a (2 genes), H12 (4 genes) and the one gene located on the B1/H1 overlap. The mapping of these tRNAs was confirmed by hybridization to a Hpa I stripfilter (Fig.2b): the RNAs hybridized mainly to Hp1, Hp2 and Hp6.

Fig. 3a shows the hybridization of the rRNA to a Hind II digest. It is

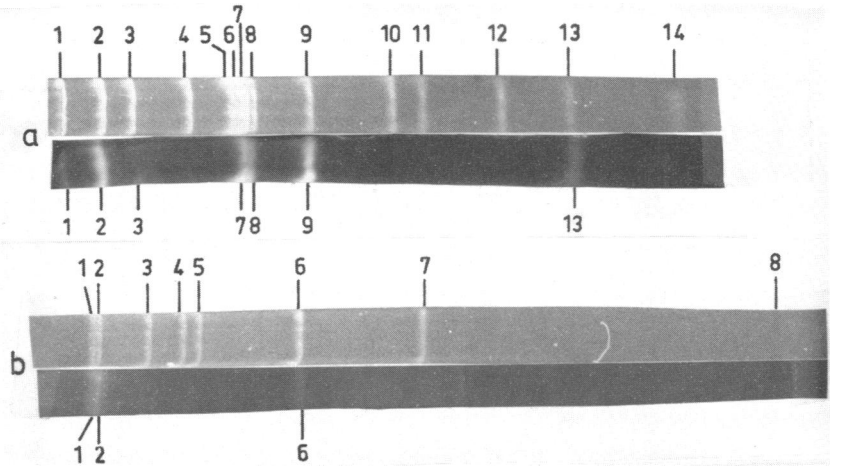

Fig. 2. Hybridization of total [^{125}I]-labeled mitochondrial tRNA to restriction fragments of mtDNA. (a). Hybridization to a Hind II digest. (b). Hybridization to a Hpa I digest. Hybridization was performed as described in Methods and Materials, in the presence of 40 µg of total mitochondrial ribosomal RNA.

clear that 17S rRNA hybridizes to h2 and h8, which, in combination with the data obtained with Hind III and Bam HI digests[3], leads us to assume that h2

TABLE II

QUANTITATION OF THE NUMBER OF tRNA GENES ON Hind II FRAGMENTS

Bands were cut out and counted. After correction for background, the percentage of total measured radioactivity was calculated for each band. This percentage was then divided by 3.75, since the total number of genes is 26-27[4].

Hind II fragment	% of total radioactivity	number of tRNA genes
h1	3.65	1
h2	23.90	6.5 (6-7)
h3	5.56	1
h7 + h8	30.44	8*
h9	31.25	8
h13	5.20	1

*By comparison of the intensities it can be estimated that h7 contains 5 or 6, and h8 2 or 3 genes.

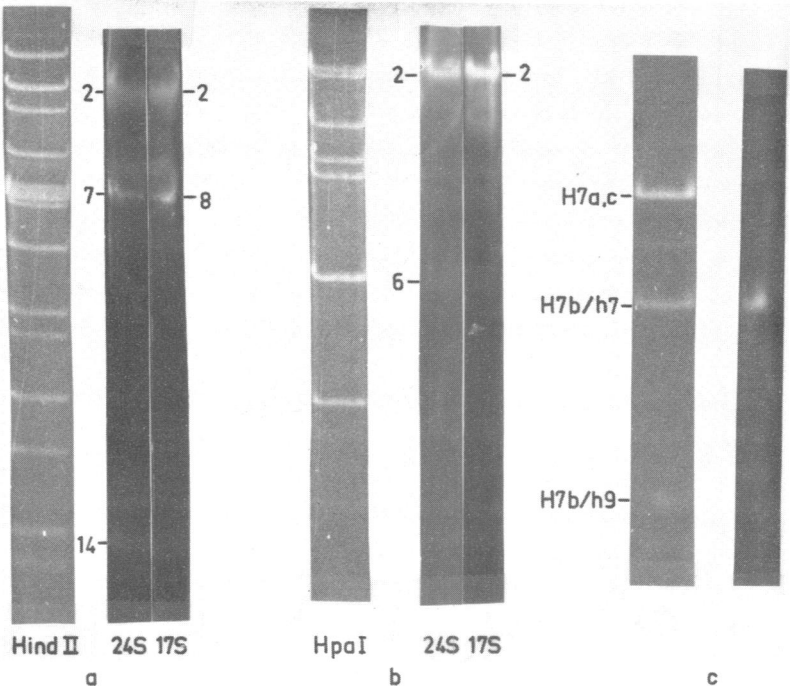

Fig. 3. Hybridization of [^{125}I]-labeled mitochondrial 24S and 17S rRNA to restriction fragments of mtDNA. (a). Hybridization to a Hind II digest. (b). Hybridization to a Hpa I digest. (c). Hybridization of 24S RNA to a Hind II digest of H7(a+b+c). For experimental details, see under Methods and Materials. For 24S hybridization 40 µg of unlabeled 17S rRNA was present, whereas with 17S hybridization 40 µg of unlabeled 24S rRNA was added.

and h8 are adjacent. From the radioactivity of the bands it can be estimated that about 1,300 bp of the 17S gene are on h2, the remaining part (about 700 bp) on h8. With 24S rRNA, fragments h2, h7 and h14 are labelled. Of the total radioactivity, 65% is found on h2, 20% on h7 and 15% on h14. To check the Hind II hybridization data we have also hybridized the rRNAs to Hpa I filters: as expected 24S hybridizes mainly to Hp2 and Hp6, whereas 17S only hybridizes to Hp2 (Fig. 3b). These hybridization results are incompatible with the arrangement of the 24S gene proposed earlier by our group[3]. The ordering of Hind III fragments in E1 was based on partial analysis and on the assumption - at that time the only reasonable one - that the fragments hybridizing to 24S RNA

a continuous gene. In this earlier paper[3] it was found that 24S

RNA hybridizes to H1, H7, H14, H15 and H19. The hybridization to H1 was assumed not to be due to 17S contamination, since the same 24S RNA preparation did not hybridize to B3. However, we found that the hybridization to H1 could be competed out by a 10-fold excess of cold 17S RNA, whereas the hybridization to H7, H14, H15 and H 19 remained (not shown). Although we have no explanation for the earlier lack of hybridization to B3, we conclude now that if there is any 24S sequence on H1, it is extremely short.

To establish the correct order of Hind III fragments, we have digested isolated Hind II and Hpa I fragments with Hind III, as already mentioned above. Fragment h2 yields H7a, H12 and H19, the overlap H1/h2 (2,900 bp) and a fragment of about 800 bp. Fragment h14 is cut into two small fragments of 400 and 200 bp. Fragment Hp6 (=h7) yields H13, H18, H20 and fragments of 1,600 bp (H7b/h7) and 650 bp. Fragment h9 is cleaved into H10, a fragment of 700 bp (H7b/h9) and several very small fragments. The order of Hind III fragments given in fig. 4 is derived from these data and from partial digestions of h2, h9 and Hp6. To establish which of the two fragments H7a and H7b is complementary to 24S RNA, we made use of the fact that fragment H7b is cleaved by Hind II, whereas H7a and H7c (located on h1) are not. In fig. 3c it is shown that 24S RNA hybridizes only to the 1,600 bp fragment H7b/h7 obtained after Hind II digestion of isolated H7(a+b+c) and not to the undigested H7a. This experiment shows that the 24S RNA gene is discontinuous. Most of the gene is on the stretch H19-H15-H14 and also on H20 as can be concluded from the fact that 24S hybridizes to a partial fragment composed of H13 + H20 (not shown), but about 500 bp are located on the H7b/h7 overlap. The exact position of this part of the gene on H7b could not be determined in this way, but according to the electronmicroscopical data of Hahn *et al.*[5] the intron is 2,300 bp long, so we have placed the smaller part of the gene at a distance of 2,300 bp from the larger part.

The arrangement of genes and restriction fragments in the E1 area is shown in Fig. 4. Although the rRNAs are not as close as we thought before[3] there is still a considerable clustering of genes for rRNAs and tRNAs. Two clusters of tRNA genes are especially evident, one of 14 genes on H10 + H7b and one of 7 genes between the rRNAs. The gene closest to the 17S gene is $tRNA^{Tyr}$ (U.L. RajBhandary, pers. comm.).

C. Localization of the gene for some specific tRNAs.

We have shown earlier[11] that the mitochondrial tRNAs can be separated by two-dimensional gel electrophoresis into about 26 species, which have different mobilities from the cytoplasmic tRNAs. Fig. 5 shows the electro-

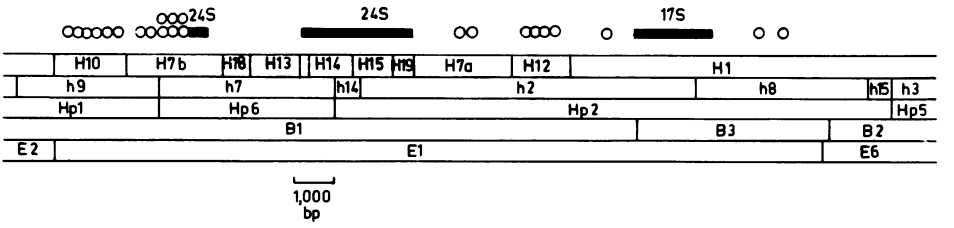

Fig. 4. Fine structure restriction and transcription map of the E1 area of the mitochondrial DNA. The rRNA genes are presented by solid bars, the tRNA genes by open circles.

pherogram, the arrows indicate the place of the Leu, Ser, Phe and Met-species. To locate the genes for the leucine species, we have hybridized total mt-tRNA, charged with [^{3}H]-leucine, to Hind III + Bam HI stripfilters. The result is shown in Fig. 6: one species hybridizes to H7, the other to H10. To decide which gene is for Leu_1 and which for Leu_2, we have made use again of the gel-electrophoretic technique to separate [^{125}I]-labeled total mt-tRNA. Autoradio-

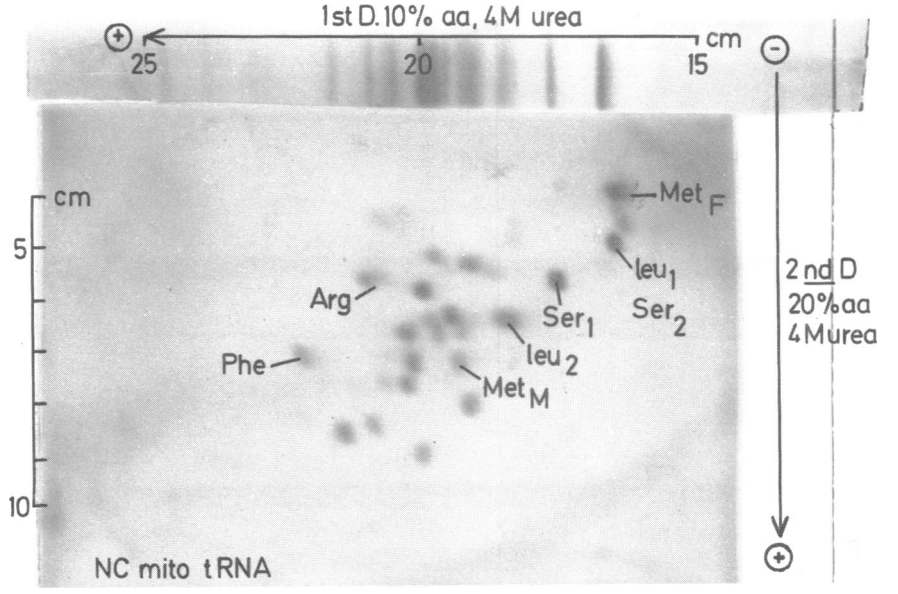

Fig. 5. Two-dimensional electrophoresis of mitochondrial tRNAs. The arrows indicate the place of isoacceptors carrying the labeled aminoacids. Experimental details are as described under Methods and Materials.

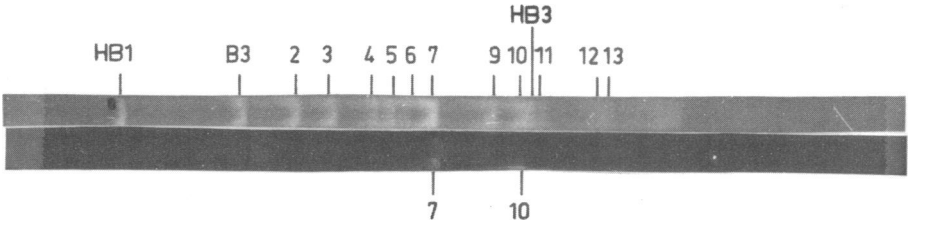

Fig. 6. Hybridization of [^{3}H]leucine-charged mitochondrial tRNAs to (Hind III + Bam HI) fragments of mtDNA.

graphy was used to visualize the spots pattern, which was completely identical to the pattern obtained with unlabeled tRNA. The two Leu spots were cut out, and the tRNAs eluted and hybridized again to Hind III + Bam HI stripfilters. Fig. 7 shows that the tRNA found in the Leu_1 spot hybridizes to H7 and H10, whereas the Leu_2 species is complementary to H7. The Leu_1 spot also contains Ser_2, so combined with the data of Fig. 6 the conclusion is that Leu_1 is on

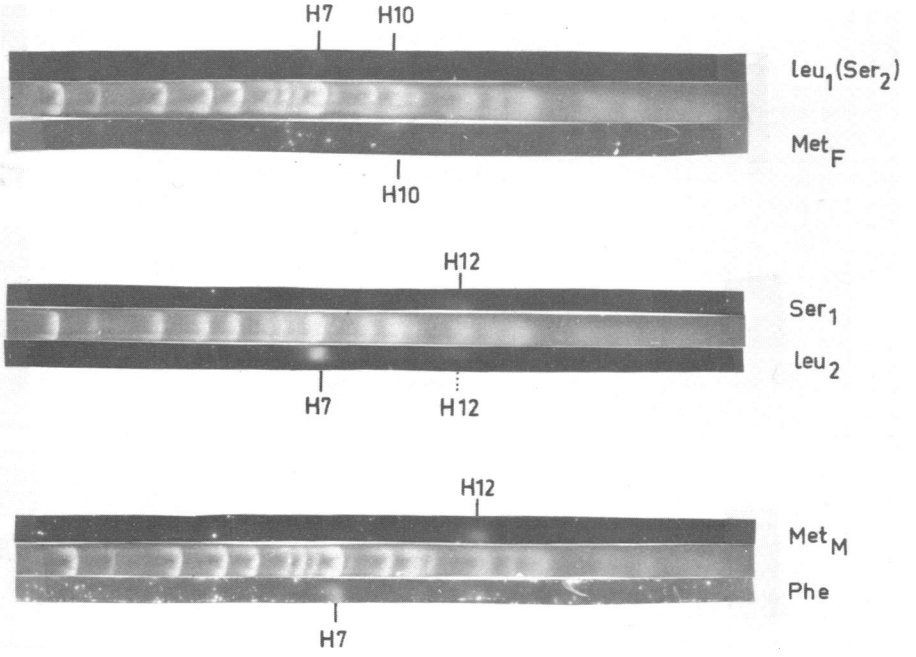

Fig. 7. Hybridization of [^{125}I]-labeled tRNA species to (Hind III + Bam HI) fragments of mtDNA.

H10, Leu_2 on H7 and Ser_2 on H7. Fig. 7 further shows that Phe is on H7, Met_F is on H10, Met_M is on H12 and Ser_1 is on H12.

DISCUSSION

The transcription mapping data obtained by the hybridization of mitochondrial ribosomal and transfer RNAs to the Hpa I and Hind II fragments confirmed the strong clustering of the genes for rRNAs and tRNAs in a 16 kbp segment, or 27%, of the mitochondrial DNA. In animal mtDNAs the ribosomal RNA genes are located adjacently, but the tRNA genes are scattered over the genome[14,15], in yeast mtDNA, there is clustering of tRNA genes[16,17] but the rRNA genes are located almost opposite to each other.

The hybridization of mitochondrial transfer RNAs to Hind II stripfilters confirm our previously reached conclusion[4] that *Neurospora crassa* mtDNA codes for about 26 tRNAs, the same number as found for yeast mitochondria[16,18] and for bean[19] and *Euglena* chloroplasts[20]. Since the number of genes corresponds excellently with the number of spots obtained after two-dimensional electrophoresis, there can be no doubt that import of tRNAs from the cytoplasm, as proposed for *Tetrahymena* by Chiu *et al.*[21], is either absent or negligible in *Neurospora*.

An intervening region in the 24S rRNA gene was postulated first by Hahn *et al.*[5] based on electronmicroscopical data. As shown here, we could confirm their conclusion by hybridization of 24S RNA to Hind II and Hpa I fragments. Intervening sequences of about 1,000 bp in the large ribosomal RNA gene have been found in mitochondrial DNA of ω^+ strains of *S. cerevisiae*[22,23] and in *Chlamydomonas* chloroplast DNA[24]. The intervening sequence in *Neurospora crassa* mtDNA evidently is much longer, about 2,300 bp. It would be interesting to investigate different strains or species of *Neurospora* for absence or variation in size of the intron. In *Saccharomyces* the intron is absent in *S. carlsbergensis* and also in ω^- strains of *S. cerevisiae*[22]. The exact length of the intron in our Lindegren-mtDNA has not yet been determined, but it does not seem likely that a difference with the Abbott-type used by Hahn *et al.*[5] will be found, since the only differences between these two types are found in other regions. The exact length of the intron is presently under investigation by electron microscopy. Other important questions concerning the organization of this region are whether the tRNAs are transcribed from the same strand as the rRNAs or from opposite strands or from both, and how the processing of transcripts occurs. Only one precursor is known so far

which contains the two rRNAs and an additional 1,000 nucleotides of spacer RNA[25]. The rRNA genes are separated by about 5,000 bp, which means that to arrive at this precursor not only the intron region but also at least 4,000 bp of the region between the 24S and 17S genes have to be removed. It would be interesting to know if this spacer RNA in the precursor still contains tRNA sequences and, if so, whether those sequences are excised during processing and used as functional tRNA molecules. Finally, the direction of transcription still has to be determined. Some of these questions are presently under investigation in our laboratory.

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