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Dihydrouridine-deficient tRNAs in Saccharomyces cerevisiae

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ABSTRACT

A mutation in *Saccharomyces cerevisiae*, designated *mia*, is responsible for the production of isoaccepting tRNA molecules with reduced extents of nucleoside modifications. The *mia* isoacceptors of $tRNA^{Phe}$ and one of the mutant isoacceptors of $tRNA^{Tyr}$ were highly purified for nucleoside composition analyses. The data indicate that the mutant isoacceptors are lacking some of the dihydrouridine moieties. This is consistent with our previous hypothesis that the mutant isoacceptors were accumulated due to a defect in a modification process [Lo, R.Y.C. and Bell, J.B. (1981) *Current Genetics* 3, 73-82]. Data from *in vitro* poly-U translation experiments also support the previous results, suggesting *in vivo* biological activity of these mutant tRNAs.

INTRODUCTION

The primary transcripts of tRNA genes are precursor molecules which must undergo several processing steps to produce the mature tRNAs. These processing steps involve both size alterations and nucleoside modifications (1-4), and require the action of specific enzymes (5, 6). Such interesting problems as the temporal sequence of the nucleoside modifications and the functions of these modified nucleosides in the mature tRNAs remain largely unsolved (6, 7) although data are accumulating (6-8, 28). To solve such problems it will be useful to study mutants defective in tRNA nucleoside modifications.

Apparently, tRNAs lacking particular nucleoside modifications may still function normally during protein synthesis (9-12) and this makes it very difficult to isolate mutants defective in these modification processes. In yeast, only a few examples of such mutants have been reported (11, 12, and A. Hopper, personal communication). The *mia* mutation in *Saccharomyces cerevisiae* is responsible for the production of aberrant isoaccepting forms for several tRNA species and a partial characterization of the mutant revealed no additional phenotype (13-15). In this paper we present data that identify the molecular lesion in the mutant acceptor tRNAs. Results from nucleoside

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composition analyses of the purified isoacceptors of *mia* $tRNA^{Phe}$ and one mutant acceptor of $tRNA^{Tyr}$ indicate that the tRNAs affected by the *mia* mutation are missing some of the dihydrouridine modifications found in the cognate wild type tRNA molecules.

MATERIALS AND METHODS

Yeast strains and cultures

The yeast strains S288C (wild type), JB759-5A *MIA*$^+$, and JB759-5B *mia*, as well as the liquid YEPD media and the growth conditions, are as previously described (13).

Preparation of tRNA and aminoacyl-tRNA ligase

Crude tRNAs were prepared from yeast cells by phenol extraction and DEAE-cellulose chromatography (16). Crude aminoacyl-tRNA ligases were prepared as described by Nishimura *et al.* (17) with the following modifications: Wild type cells were disrupted by 3 passages through a French pressure cell at 17,000 psi; and 0.2 volumes of a 5% w/v streptomycin sulfate solution were added to the extract before the centrifugation step.

Purification of $tRNA^{Phe}$ and $tRNA^{Tyr}$ isoacceptors

Crude tRNAs were deacylated in 1 M Tris-Cl (pH 8) at 37°C for 2 hours, recovered by ethanol precipitation and fractionated by BD-cellulose chromatography as described by Wimmer *et al.* (18). The partially purified tRNA samples from the BD-cellulose chromatography step were then fractionated two to three times by RPC-5 chromatography (19) using a Tris buffer (pH 7.5) for unacylated tRNA and an acetate buffer (pH 4.5) for tRNAs acylated with either phenylalanine or tyrosine. The RPC-5 chromatography steps not only purify the tRNAs further, but also separate the various isoacceptors. The final amino acid acceptances of the purified isoacceptors were all greater than 1200 pmole/A_{260} nm unit.

For analytical RPC-5 chromatography, the tRNAs were aminoacylated with radioactive amino acids as described previously (15). The purified tRNAs were reacted for only 3 minutes due to their very rapid aminoacylation. Since <0.1 A_{260} nm units of purified tRNAs were used in the reactions, 10 A_{260} nm units of carrier tRNA were added to increase the recovery of the aminoacylated tRNAs. The carrier tRNA was added after the reactions were stopped by the addition of Na acetate (pH 4.5). RPC-5 chromatography of aminoacylated tRNAs was carried out as in Bell *et al.* (13). The salt gradients for Phe-$tRNA^{Phe}$ and Tyr-$tRNA^{Tyr}$ were 0.6-0.9 M and 0.6-0.8 M, respectively.

Polyacrylamide-urea gel electrophoresis

20% polyacrylamide gels (acrylamide:bisacrylamide 39:1) containing 7 M urea were prepared according to Peacock and Dingman (20) and cast in a vertical slab gel apparatus (15 x 12 x 0.15 cm) from Aqueboque. The tRNA samples, mixed with a dye solution (50% sucrose, 7 M urea, 0.1% xylene cyanol FF), were loaded on the gels and electrophoresed for 16 hours at 150 V. The gels were then fixed for 15 minutes in 1 N acetic acid and stained in 0.05% methylene blue in 0.2 M Na acetate (pH 4.6) for 20 minutes. Destaining was achieved in distilled water.

Analysis of the nucleoside composition of tRNA isoacceptors

The nucleoside composition of purified tRNA isoacceptors was analysed by the tritium derivative method described by Randerath *et al.* (21). [^{3}H] KBH_4 (3.1 Ci/mmole), and thin layer cellulose sheets were from Amersham and Eastman, respectively. The fluorograms were exposed for 2-4 days on Kodak XRP-1 film at -60°C, after which the films were developed and the spots were quantitated by liquid scintillation counting.

In vitro poly-U translation

An S-30 extract was prepared from wheat germ as in Roberts and Paterson (22) except that the pre-incubation step was omitted. The translation assays were also set up as described, with the following modifications: The assays were in a final volume of 100 µl and contained, in addition to the described ingredients (22), 15 mM Mg acetate, 60 mM NH_4Cl, and 20 mM KCl. Poly-U and [^{3}H] L-phenylalanine at 2500 Ci/mole (New England Nuclear) were added to start the reactions, after the rest of the ingredients and 0.1 µg of a purified $tRNA^{Phe}$ isoacceptor were pre-incubated at 30°C for 10 minutes. The reactions were carried out at 30°C for 1 hour, with 10 µl samples removed at timed intervals.

RESULTS

Purification of tRNA isoacceptors

It was necessary to isolate the isoacceptors for nucleoside analyses in order to characterize the actual molecular lesion produced by *mia*. The three isoacceptors of *mia* $tRNA^{Phe}$, as well as the $tRNA^{Phe}$ from *MIA*$^+$ (13), were individually purified as described in Materials and Methods. The final phenylalanine acceptance levels of the purified tRNAs are listed in Table 1, and an RPC-5 chromatogram of each purified isoacceptor from *mia* is presented in Figure 1. These levels monitor the purity of the tRNA samples with respect to contamination by other tRNA species, while the RPC-5 chromatograms monitor

Table 1. Amino acid acceptance levels of the purified tRNA isoacceptors. The aminoacylation assays were carried out for 3 minutes as described in Lo and Bell (15). [^{14}C]-phenylalanine and [^{14}C]-tyrosine were present at a concentration of 4.75 μM and 15 μM, and final specific activities of 500 Ci/mole and 1000 Ci/mole, respectively.

$tRNA^{Phe}$ isoacceptors	pmoles phenylalanine/A_{260} nm
MIA$^+$	1287
mia isoacceptor A	1257
mia isoacceptor B	1542
mia isoacceptor C	1589
$tRNA^{Tyr}$ isoacceptors	pmoles tyrosine/A_{260} nm
MIA$^+$	1437
mia isoacceptor D	1565

the purity with respect to contamination by the other isoacceptors. The data in Table 1 also support previous results which show that there is no deficiency in the ability of mutant acceptors to be aminoacylated (15).

In addition to the $tRNA^{Phe}$ isoacceptors, one of the four isoacceptors of $tRNA^{Tyr}$ from *mia* and the wild type acceptor of $tRNA^{Tyr}$ from *MIA*$^+$ (14) were also purified for nucleoside analysis. Only the last isoacceptor to elute from RPC-5 (called isoacceptor D) was purified, since it was expected to differ most from wild type $tRNA^{Tyr}$. It was not necessary to obtain completely purified isoacceptor D since contaminating $tRNA^{Tyr}$ isoacceptors are also mutant and we were not looking for the absolute quantitative differences between isoacceptor D and wild type $tRNA^{Tyr}$ (see Discussion). The tyrosine acceptor activities of the purified $tRNA^{Tyr}$ samples are also presented in Table 1, while an RPC-5 co-chromatogram of $tRNA^{Tyr}$ isoacceptor D and total *mia* $tRNA^{Tyr}$ is presented in Figure 2.

Sizing of tRNA molecules

The purified tRNAs were analysed by polyacrylamide-urea gel electrophoresis to determine if the mutant isoacceptors are the same length as the cognate wild type molecules. Under the denaturation conditions employed for electrophoresis (7 M urea), the molecules are resolved primarily according to their size differences. The lengths of $tRNA^{Phe}$ and $tRNA^{Tyr}$ are 76 and 78 nucleotides, respectively (23). It can be seen from Figure 3 that these two

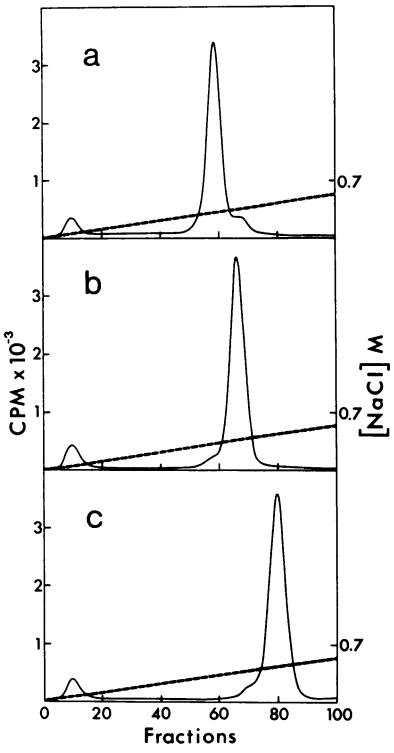

Fig. 1 RPC-5 chromatograms of the purified isoacceptors of $tRNA^{Phe}$ from 759-5B *mia*. The three isoacceptors of $tRNA^{Phe}$, a) isoacceptor A, b) isoacceptor B, and c) isoacceptor C, were named according to their elution order during RPC-5 chromatography of bulk *mia* tRNA (13). The small peak at the beginning of each chromatogram is free amino acid derived from deacylation of the tRNA samples between the time of preparation and application to the RPC-5 columns.

species of tRNA are resolved. Further, the four purified $tRNA^{Phe}$ isoacceptors all migrate to one position, and the two purified $tRNA^{Tyr}$ isoacceptors both migrate to another position. This suggests that there are no size differences between the mutant tRNA isoacceptors and the respective wild type molecules. It is also apparent from Figure 3 that the purified tRNA isoacceptors are free of any major contaminating materials.

Translational activity of isoacceptors *in vitro*

The purified $tRNA^{Phe}$ isoacceptors were assayed for the ability to mediate the production of poly-phenylalanine in an *in vitro* translation system using poly-U as the template. It can be seen in Figure 4 that there are only slight differences in the kinetics of tRNA-dependent poly-phenylalanine for-

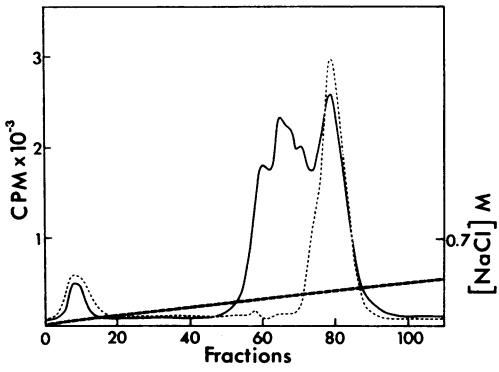

Fig. 2 RPC-5 co-chromatogram of the purified isoacceptor D of *mia* $tRNA^{Tyr}$ together with bulk *mia* $tRNA^{Tyr}$ from 759-5B *mia*.
..... represents [^{14}C]-tyrosyl-$tRNA^{Tyr}$ of purified *mia* $tRNA^{Tyr}$ isoacceptor D.
——— represents [^{3}H]-tyrosyl-$tRNA^{Tyr}$ of bulk $tRNA^{Tyr}$ from 759-5B *mia*.

mation when each purified isoacceptor is used individually in the assays, and other similar results (not included) indicate the differences are not significant.

Nucleoside composition analysis

The nucleoside composition of each of the purified tRNA isoacceptors was quantitatively determined as described in Materials and Methods, and the results are presented in Tables 2 and 3. The data were not corrected for recoveries of the labile minor nucleosides since quantitative comparisons amongst the isoacceptors were deemed sufficient for this analysis. Furthermore, we have no evidence the tRNAs we are studying are identical in sequence to those published. The 2'-O-methyl-nucleosides are not detected in this analysis; however, identical fingerprints were obtained from each of the purified $tRNA^{Phe}$ isoacceptors after RNase T_1 digestion and subsequent two-dimensional electrophoresis (24, 25), thus eliminating a 2'-O-methylguanosine deficiency as the lesion in *mia* tRNAs (results not shown). Since 2'-O-methylcytidine is not present in mature $tRNA^{Tyr}$ (23), which is also affected by *mia* (15), this eliminates it as the lesion in *mia* tRNAs. A representative fluorogram of the separated nucleoside trialcohol derivatives from the purified wild type $tRNA^{Phe}$ of MIA^{+} and each $tRNA^{Phe}$ isoacceptor from *mia* is presented in Figure 5.

It can be seen from Table 2 that the most significant difference amongst the nucleoside compositions of the $tRNA^{Phe}$ isoacceptors is in the molar

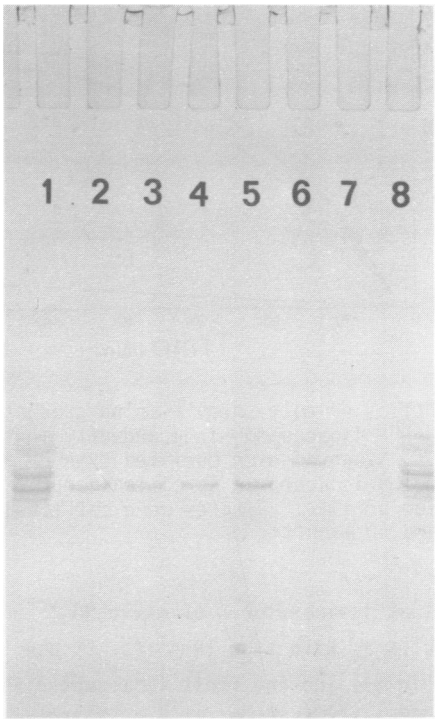

Fig. 3 A 20% polyacrylamide-urea gel after electrophoresis of tRNA samples. Slots 1 and 8 contain 3 μg of bulk tRNA from wild type S288C and 759-5B *mia*, respectively. About 0.3 μg of purified tRNA samples were applied to the remaining slots; 2) wild type $tRNA^{Phe}$, 3) *mia* $tRNA^{Phe}$ isoacceptor A, 4) *mia* $tRNA^{Phe}$ isoacceptor B, 5) *mia* $tRNA^{Phe}$ isoacceptor C, 6) wild type $tRNA^{Tyr}$, and 7) *mia* $tRNA^{Tyr}$ isoacceptor D.

amounts of dihydrouridine, which agrees with the impression one gets from Figure 5. Transfer RNA^{Phe} from MIA^{+} and isoacceptor A from *mia* are essentially identical in their nucleoside composition, which is consistent with previous observations that isoacceptor A is wild type $tRNA^{Phe}$ (13). Isoacceptor B differs from isoacceptor A or wild type $tRNA^{Phe}$ in having only half the amount of dihydrouridine, while isoacceptor C does not have any detectable dihydrouridine. There are two molecules of dihydrouridine in wild type $tRNA^{Phe}$ (23) and it appears that *mia* is defective in the production of some of the dihydrouridine moieties on tRNA molecules.

The data from the $tRNA^{Tyr}$ isoacceptors support this hypothesis. There are six molecules of dihydrouridine in wild type $tRNA^{Tyr}$ (23), and the data

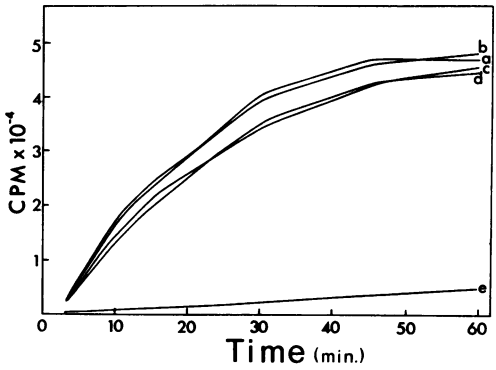

Fig. 4 *In vitro* kinetics of poly-phenylalanine production using each purified $tRNA^{Phe}$ isoacceptor independently in the wheat germ translation assays. 2 µg of each purified $tRNA^{Phe}$ sample were used: a) wild type, b) isoacceptor A, c) isoacceptor B, d) isoacceptor C, e) no addition of tRNA. Samples were collected at 3, 6, 9, 12, 15, 20, 30, 45 and 60 minutes.

in Table 3 indicate that isoacceptor D of *mia* $tRNA^{Tyr}$ has only half as many dihydrouridine molecules as wild type $tRNA^{Tyr}$. Since there are four isoacceptors of $tRNA^{Tyr}$ in *mia* and the first isoacceptor is wild type (14), we expect the fourth isoacceptor to have only three dihydrouridine modifications if each isoacceptor is produced by the cumulative defect of lacking an additional dihydrouridine modification.

Another nucleoside that seems to vary in amount amongst the isoacceptors is pseudouridine. Isoacceptor C of $tRNA^{Phe}$ appears to have an increased amount of pseudouridine. The data regarding uridine, dihydrouridine, and pseudouridine for the $tRNA^{Phe}$ isoacceptors were further analysed in Table 2. It can be seen that the total amount of uridine and dihydrouridine remains the same amongst the $tRNA^{Phe}$ isoacceptors, while the total amount of uridine, dihydrouridine, and pseudouridine is slightly higher for isoacceptor C. Since dihydrouridine and pseudouridine are derivatives of uridine, this suggests that the increased amount of pseudouridine in isoacceptor C is probably due to contaminants not originating from the $tRNA^{Phe}$ molecules.

DISCUSSION

Purified tRNA isoacceptors from *mia* are the same size as cognate tRNAs from wild type yeast and, furthermore, no substantial differences can be detected in the abilities of individual $tRNA^{Phe}$ isoacceptors to participate in *in vitro* translation under the conditions employed. These results support

Table 2. Nucleoside compositions of the purified $tRNA^{Phe}$ isoacceptors*.

Nucleoside	Expected†,‡	*MIA*+	*mia* isoacceptor A	*mia* isoacceptor B	*mia* isoacceptor C
A	17	14.5§	14.7	15.0	14.1
U	12	14.0	14.4	15.0	16.5
G	18	16.7	18.5	17.9	15.8
C	15	17.8	17.4	17.2	16.9
m^2G	1	0.58	0.63	0.52	0.54
m_2^2G	1	1.66	1.13	1.28	2.07
m^7G	1	0.79	0.76	0.74	0.80
m^5C	2	1.30	1.33	1.27	1.41
m^1A	1	0.46	0.42	0.49	0.43
Y	1	0.48	0.51	0.58	0.55
T	1	1.00	0.85	0.87	0.97
Ψ	2	2.82	2.28	2.44	3.77
D	2	1.67	1.32	0.65	-¶
U+D	14	15.7	15.7	15.6	16.5
U+D+Ψ	16	18.5	18.0	18.1	20.3

* Relative molar amounts of the nucleosides calculated according to equation 3 in Randerath *et al.* (21). The average recovered cpm/chromatogram is 9×10^5.

† See reference 23.

‡ The 2'-O-methyl-nucleosides can not be detected by this analysis.

§ All values are averages of three independent determinations with an average coefficient of variation of 11.58% ± 9.4%.

¶ No detectable amounts were obtained.

our previous supposition (15) that the mutant isoacceptors are biologically functional.

Comparisons of the nucleoside compositions of the purified $tRNA^{Phe}$ isoacceptors indicate that the mutant isoacceptors have a reduced amount of dihydrouridine compared to wild type $tRNA^{Phe}$. This analysis identifies the lesion causing the mutant acceptors, and the results are consistent with the hypothesis that *mia* is a leaky mutation resulting in incomplete conversion of the appropriate uridines to dihydrouridines. Previous results (15) which suggested a precursor-product relationship between mutant acceptors and wild

Table 3. Nucleoside composition of the purified $tRNA^{Tyr}$ isoacceptors*.

Nucleoside	Expected†,‡	*MIA*+	*mia* isoacceptor D
A	15	12.8§	13.1
U	7	10.2	13.9
G	20	21.1	21.0
C	20	22.1	21.1
m^2G	1	0.55	0.45
m^2_2G	1	1.07	-¶
m^5C	1	0.57	0.42
m^1A	1	0.51	0.42
i^6A	1	0.34	0.29
T	1	0.87	0.79
Ψ	3	2.56	2.35
D	6	4.48	1.96

*, †, ‡, § - As in Table 2, except that the average recovered cpm/chromatogram is 7.3×10^5.

¶ Not determined due to the presence of contaminating materials over that nucleoside.

type tRNA can be explained by the present data. Two dihydrouridine moieties are found in mature wild type tRNA (23) and *mia* isoacceptor A; isoacceptors B and C are accumulated, and identified, due to the defect of lacking one or two dihydrouridine modifications, respectively. The undermodified tRNA molecules are still substrates for the defective modification enzyme, which slowly converts them to mature molecules (15). However, the conversion is never complete, so either the enzyme is not active at the later stages of a cell culture, or the tRNA molecules are no longer suitable substrates if they are not modified at the proper time. In the case of the $tRNA^{Tyr}$ isoacceptor D, the data indicate that it is probably missing three dihydrouridine moieties.

The production of mutant isoacceptors in *mia* was found to be influenced by the growth condition of the yeast cultures (14). These results can now be rationalized. The production of dihydrouridine on tRNA is probably a simple enzymatic reduction of the 5,6 double bond of the uracil moiety. Cultures grown at a fast-shaking rate have a higher level of dissolved oxygen; as a result, the enzymatic reduction of uridine is inhibited and mutant isoaccepting tRNAs lacking dihydrouridine moieties are accumulated. This is supported by the observation that *mia* cultures grown at the fast-shaking condition, but

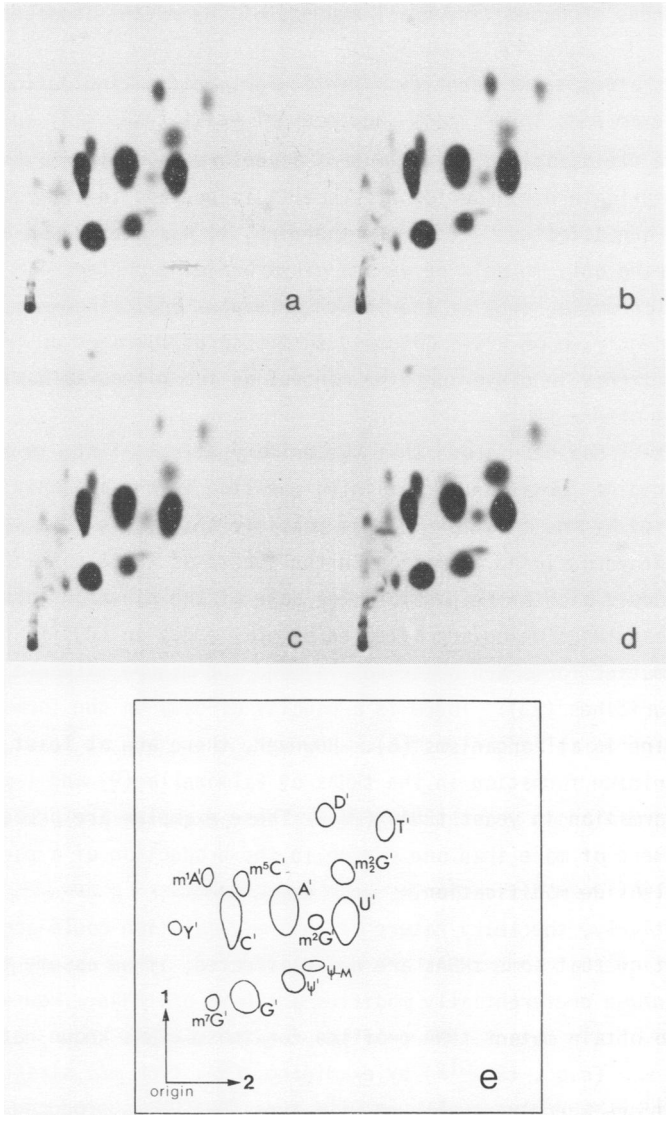

Fig. 5 Nucleoside composition analysis by two-dimensional thin layer chromatography of nucleoside trialcohols from the purified $tRNA^{Phe}$ isoacceptors. The fluorograms were developed as described in Materials and Methods: a) wild type, b) isoacceptor A, c) isoacceptor B, d) isoacceptor C, and e) a diagramatic description of the fluorograms and identification of the nucleoside derivatives. Ψ-M is a monoaldehyde derived from Ψ (21). See Table 2 for quantitative measurements. The spots which are variably present in panels a-d but which have no counterpart in panel e are probably due to minor impurities.

under nitrogen, produced very small amounts of the mutant isoacceptors (data not shown).

Previous attempts to identify a particular modification deficiency in *mia* strains by examining the primary sequences of yeast tRNAs (23) and comparing these to the pleiotropic array of mutant acceptors found in *mia* (13, 15) failed to implicate dihydrouridine, since it is present in all yeast tRNAs and not all are affected by *mia*. Furthermore, it has been reported (6) that tRNAs differing only in uridine versus dihydrouridine content can not be resolved by chromatography of the intact molecules and, since *mia* tRNAs are routinely identified on RPC-5 columns, this also discouraged us from considering a deficiency in dihydrouridine content as the molecular basis for the mutant forms of *mia* tRNAs.

Now that it has been shown that the primary effect of *mia* is a deficiency in dihydrouridine formation, a remaining question is why are only some of the tRNAs affected by the mutation. It is possible that there is a separate enzyme for dihydrouridine formation in the subset of tRNAs not affected by *mia*, which could also be responsible for some of the dihydrouridine modifications in those tRNAs which are affected by *mia*, e.g., in $tRNA^{Tyr}$ from *mia* only three mutant forms are observed (14) and the mature molecule contains six dihydrouridines (23). There is a paucity of data on the formation of dihydrouridine in all organisms (6). However, there are at least two enzymes for pseudouridine formation in the tRNAs of Salmonella (9) and 1-methylguanosine formation in yeast tRNAs (26). These examples are precedents for the involvement of more than one enzyme in the production of a particular type of nucleoside modification.

Alternatively, the leaky nature of this *mia* mutation could account for the observation that some tRNAs are never affected, if we assume that the defective enzyme preferentially modifies a subset of tRNAs. However, we have attempted to obtain mutant tRNA profiles for those tRNAs known not to be affected by *mia* (e.g., $tRNA^{Gly}$) by examining tRNAs from *mia* strains grown under conditions where virtually no wild type $tRNA^{Phe}$ is produced (15). These results show that the $tRNA^{Gly}$ profile is still wild type (data not shown). Moreover, the $tRNA^{Tyr}$ profile in this case has demonstrably higher proportions of mutant isoacceptors but no new mutant acceptors are evident; only three mutant forms appear, but they represent a much higher proportion of total $tRNA^{Tyr}$.

It is also possible that all tRNAs are affected by *mia*, but not all dihydrouridine-deficient tRNAs are resolved from the respective wild type accep-

tors by RPC-5 chromatography. This possibility can be tested by analysing the nucleoside composition of one of the tRNA species apparently not affected by *mia* (e.g., $tRNA^{Gly}$). It has already been ascertained (15) that *mia* strains do not overproduce tRNA; rather, the mutant forms accumulate at the expense of normal levels of the respective wild type acceptors.

Further experiments with *mia* could address the problem of determining the function of dihydrouridine in tRNA since this is presently unknown (6, 7). If *mia* were grown under conditions favouring the maximum production of dihydrouridine-deficient tRNAs, the yeast cells could then be examined to ascertain whether or not any particular cellular functions, in which tRNAs are known to be involved (27), are impaired. The existence of a species of tRNA (isoacceptor C of *mia* $tRNA^{Phe}$) completely devoid of dihydrouridine also provides a promising substrate for studying the enzymology of dihydrouridine biosynthesis.

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