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A point mutation in a mitochondrial tRNA gene abolishes its 3' end processing

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ABSTRACT

A temperature sensitive mutation mapping in the tRNA region of the mitochondrial genome of S.cerevisiae has been found to abolish 3' processing of $tRNA^{asp}$.

Mutant cells grown for a few generations at the non-permissive temperature were found to specifically lack mature $tRNA^{asp}$ and to accumulate 3' unprocessed precursors of this tRNA.

The accumulation of precursors of other mitochondrial tRNAs was also observed under the same conditions. After longer incubation times, a generalized decrease of mitochondrial transcripts could be observed.

The mutation was genetically mapped in a limited region surrounding the $tRNA^{asp}$ gene and found, by sequencing, to consist of a C->T transition at position 61 of the $tRNA^{asp}$ gene.

INTRODUCTION

The mitochondrial genome of S.cerevisiae codes for seven components of the respiratory chain complexes and for several components of the mitochondrial protein synthesis apparatus, namely the 21S and 15S rRNAs, a ribosomal protein and a full complement of tRNAs. Mitochondrial gene expression therefore requires the coordinate participation of nuclear and mitochondrial products (1).

The regulatory interactions between these components, and the cis acting signals necessary for the control of the mitochondrial translation and transcription are still only partially understood. We know however that mitochondrial protein synthesis is essential since a block in this function, either by mutation or by the use of mitochondrial inhibitors, often results in instability of the mitochondrial genome and in a high level of rho^- formation (2, 3, 4,).

Temperature sensitive mitochondrial mutants defective in

macromolecular syntheses can be very useful tools in analyzing the nucleo-mitochondrial interactions involved in these processes.

A series of temperature sensitive mutants mapping in the major tRNA gene cluster (a region in which transcription and processing have been extensively studied, ref. 5, 6) has been isolated and is under study. We report here a detailed genetic, physiological and molecular analysis of one of them, leading to the identification of a mutation which results in a defect in the biosynthesis of $tRNA^{asp}$.

Mitochondrial tRNA genes are transcribed into polygenic transcripts which are then processed at the 5' and 3'ends, to yield mature tRNAs. 5' end processing requires the presence in the mitochondrial genome of the tRNA synthesis locus which has been identified by Martin and Underbrink-Lyon (7); this locus codes for a 9S RNA necessary for the activity of the mitochondrial RNAse P, a ribonucleoprotein which also contains a nuclear-encoded protein (8). Rho$^-$ mutants lacking this locus therefore accumulate 5' unprocessed precursors (9). 3' end processing is mediated by a specific nuclear-encoded endonuclease which has been partially characterized (10). The case which is described here is the first defect observed in mitochondrial 3' tRNA processing and the first point mutation leading to a defect in tRNA biogenesis in this system. The present data also suggest the existence of a general control mechanism which regulates, at the level of tRNA processing, the concentration of mature mitochondrial tRNAs.

MATERIALS AND METHODS

Strains, media and growth conditions

S.cerevisiae strain FF1210-6C (ura1, ura2, rho$^+$) and its temperature sensitive derivatives ts 932 and ts 22b-16 were used. Strains were grown at 28°C or 37°C in YP medium (1% yeast extract, 1% peptone) containing 2% galactose (referred to as fermentable medium) or 2% glycerol (referred to as respiratory medium). For the isolation of the mitochondrial RNA, cells were grown at 37°C to stationary phase or for four generations on fermentable medium. At these stages the stability of the ts

phenotype was controlled by plating culture samples on YP-2% glucose at 28°C. The colonies formed after 2 days of incubation at 28°C were replicated on YP 2% glucose and YP 2% glycerol and incubated both at 28°C and 37°C. Cells which failed to grow on glycerol at both temperatures were considered rho⁻ mutants.

Isolation and genetic analysis of mutants

Mutant ts 932 and ts 22b-16 were isolated by $MnCl_2$ mutagenesis according to the method of Putrament et al., (11).

Mitotic segregation of the mutations was analyzed in the progeny of crosses between mutants and appropriate tester strains using the "random cross" technique of Coen et al., (12). Their localization on the mitochondrial map was determined by deletion mapping using characterized rho⁻ strains as tester strains for crosses. Experimental details can be found in Bolotin-Fukuhara et al., (13).

Preparation and labeling of nucleic acids

Preparation of mtDNA and mtRNA, gel electrophoresis of nucleic acids and DNA labeling by nick translation have been described previously (14). RNA blotting to diazobenzyloximethyl-paper (DBM-paper) was performed following the method of Alwine et al.,(15) as modified by Wahl et al., (16). Transcripts of mitochondrial genes were identified by hybridization with mtDNAs from the following rho⁻ strains retaining different genes: DS6/A407 (oxi3) (17); DS400/A3 (oli1) (18); DS502 (seven tRNA genes from $tRNA^{cys}$ to $tRNA^{gly}$) (19); DS200/A102 ($tRNA^{asp}$) (20); DS200/A5 (tRNA genes from $tRNA^{lys}$ to $tRNA^{met}$) (20); DS401 ($tRNA_1^{ser}$; var) (21); DS504 ($tRNA_2^{thr}$, $tRNA^{cys}$, $tRNA^{his}$) (22). S1 nuclease protection experiments were performed following the Weaver and Weissmann modification (23) of the Berk and Sharp method (24).

DNA cloning and sequencing

AsuII fragments (see Fig. 1) of 3044 bp from both wt and ts 932 mtDNAs were cloned into the AccI site of plasmid pTZ19R (Pharmacia). After transformation of E.coli strain JM109, the recombinant plasmids were selected on LB medium containing 100 ug ml of ampicillin in the presence of X-gal and IPTG (25).

The DNA prepared from white colonies was digested with appropriate enzymes to verify the presence of the correct fragment. These inserts were subcloned for sequence analysis. The

3044bp AsuII fragment from the w.t. mtDNA was excised from pTZ19, cut into two TaqI fragments of 1789bp and 1255bp which were separately cloned in the same plasmid. The 1789bp fragment was digested with MboI and three fragments (785, 622 and 377bp in length) were generated. The 622bp fragment containing the $tRNA^{asp}$ gene and the 785bp fragment containing the upstream flanking sequence were cloned separately.

The recombinat DNA plasmid containing the 3044bp AsuII fragment from ts 932 mtDNA was treated with DNAseI following the procedure described by Lin et al., (26). The deletion subclones were identified by restriction analysis. Sequences were determined by the dideoxy chain termination procedure (27). A 17-mer M13 reverse primer and Sequenase^{tm} as DNA polymerase, were used as described by the manufacturer (United States Biochemical Corp.). Samples were run on 8% or 6% acrylamide gels in the presence of 7M urea and tris-borate buffer. Gels were dried and exposed to Kodak X-5 films.

RESULTS

Localization of the ts 932 mutation

The ts 932 mutant was selected after Mn Cl_2 mutagenesis as a strain unable to grow on glycerol-containing medium at 37°C, while its growth was close to the wild-type level at 28°C. Studies of its genetic determinism showed that the mutant phenotype was due to a mitochondrial mutation: (a) a mitotic segregation (32% of heat-sensitive and 68% of heat-resistant diploid colonies) was observed when the mutant strain was crossed to a wild-type tester strain, while the whole diploid population was heat-sensitive when the tester strain was transformed into a *rho*°. (b) recombination data (not shown) with a collection of defined *rho*$^-$ strains (13) confirmed the mitochondrial determinism of the mutation and localized the mutation in the region around the $tRNA^{asp}$ gene. This localization was refined by the use of *rho*$^-$ mutants retaining specific sections of the tRNA region (Fig. 1): ts 932 recombined with the DS200/A102 and the ME121 *rho*$^-$ genomes while it did not with MA91. Restriction studies by Wesolowski et al., (28) have shown that the difference between

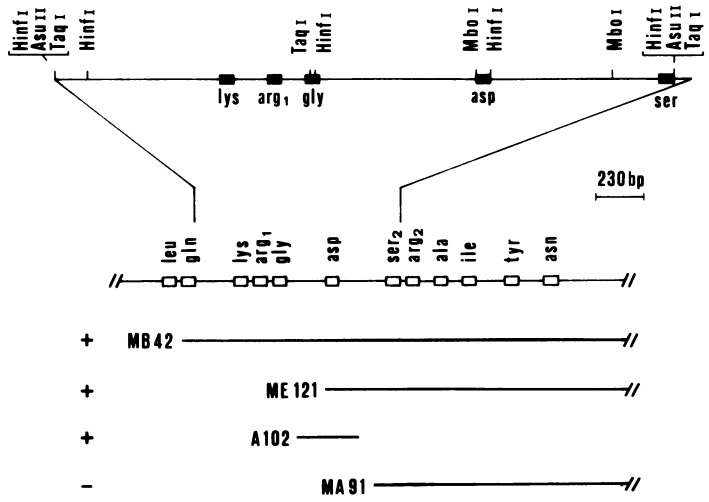

Figure 1: Diagrammatic representation of the localization of the ts 932 mutation. + or - indicate the retention or loss of the ts 932 genetic marker in crosses with different rho⁻ strains according to M.Bolotin-Fukuhara et al., (13).
The deletion structure of the rho⁻ mutants (20, 28) and the restriction map of the $tRNA^{asp}$ region are also indicated.

ME121 and MA91 resides in a 620bp MboI fragment which contains the $tRNA^{asp}$ gene.

Physiological effects of the ts 932 mutation

Analysis of the electrophoretic pattern of mt RNA prepared from ts 932 cells grown at 37°C on fermentable medium, revealed the complete absence of 21S RNA (Fig. 2A). Production of rho⁻ cells after growth at this temperature was checked and found to be between 10 and 20%, a percentage too low to account for the complete absence of 21S rRNA.

We then investigated whether growth at the non-permissive temperature produced a generalized decrease in the level of transcripts; we therefore looked for the presence of other RNA species (mRNAs, tRNAs) by hybridization of specific mt DNA probes to Northern blots of mtRNA. The RNA species analyzed (21S rRNA, oli1 mRNA, oxi3 mRNA, six tRNA genes of the major tRNA cluster) were absent when the mutant was grown to stationary phase at non-permissive temperature, and present in the wt FF 1210-6C grown at

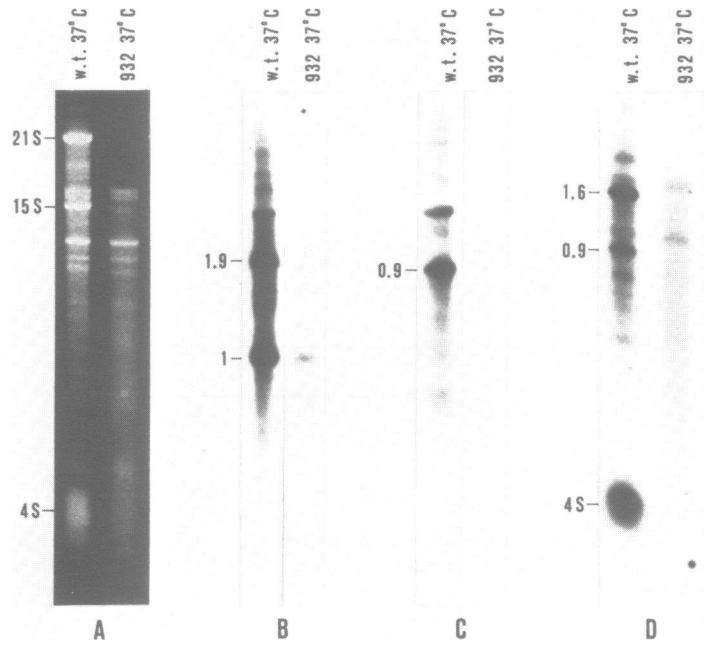

Figure 2: Electrophoretic analysis of mitochondrial transcripts from strains FF1210-6C and ts 932 grown to stationary phase at 37°C. A) Ethidium bromide staining B), C) and D). Hybridization patterns obtained with nick translated mtDNA from rho⁻ mutants DS6/A407 DS400/A3 and DS502 In B) the 1,9 and 1 bands correspond respectively to mRNA and intron 5 of the oxi3 gene. In C) the 0,9Kb band corresponds to oli1 mRNA. In D) the major precursors of the tRNA genes probed are indicated.
The RNA sizes are indicated in nucleotides X 10^{-3}. Mitochondrial 21S rRNA and 14S rRNA were used as size calibration markers.

the same temperature. Examples of these results are shown in Fig. 2.

We therefore concluded that in strain ts 932, the mutation affected a function essential for the maintenance of the mitochondrial biosynthetic apparatus.

A study of the mitochondrial translation products synthesized after the shift from the non-permissive to the permissive temperature (28°C), suggested that the mutant might be affected in the biogenesis of some component of the mitochondrial protein synthesizing machinery. In fact, one hour after the shift, the mutant was unable to perform mitochondrial protein synthesis at

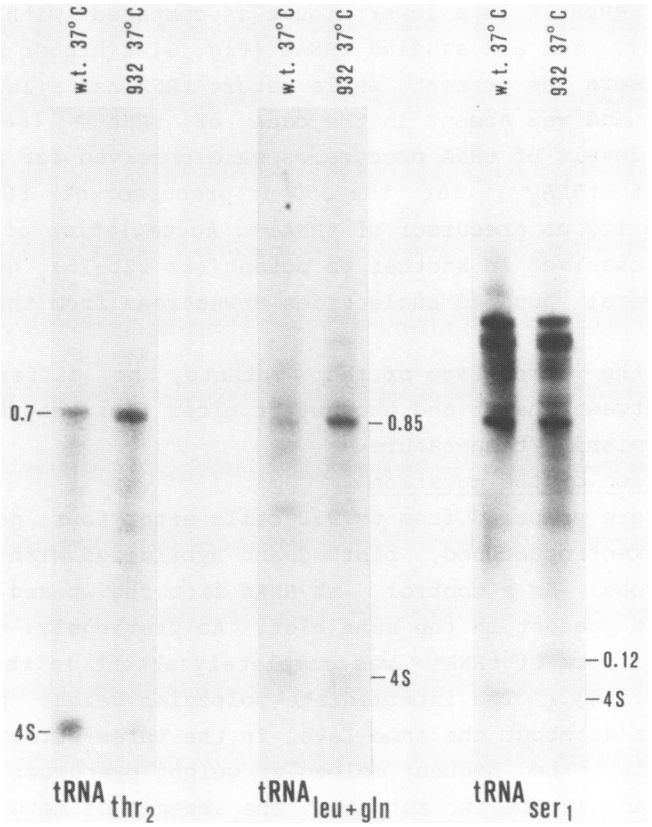

Figure 3: Transcripts of mitochondrial tRNA genes of wt and ts 932 strains grown for four generations at 37°C.
The probes used were as follows: a MboII fragment from DS504 mtDNA retaining the $tRNA_2^{thr}$ gene; a TaqI fragment from DS502 mtDNA retaining the $tRNA^{leu}$ and $tRNA^{gln}$ genes; a HaeIII fragment from DS401 mtDNA retaining the $tRNA_1^{ser}$ gene (see ref. 20, 21, 22). Other details are as in Fig. 2.

28° (N.El Ely-Fridhi, C.Niederst, R.Zelikson, M.Bolotin-Fukuhara in preparation). To determine which was the earliest function affected by the mutation, mitochondrial RNAs were prepared from wt and ts 932 cells after growth for four generations at 37°C. This condition was found to allow the identification of specific effects and was used in all subsequent work. In fact, at this stage hybridization of specific probes on Northern blots of mtRNA showed that all the transcripts which were probed were present in

the mutant even if in a lower amount as compared with wt cells (not shown). For all studied tRNAs (Fig. 3) a higher amount of some precursors was present, while mature tRNA had a lower steady state level and was absent in the case of $tRNA^{asp}$ (see below). Increased levels of tRNA precursors were observed for the 700 nt precursor of $tRNA_2^{thr}$, for the 850 nt precursor of tRNAleu+gln and for the 120 nt precursor of $tRNA_1^{ser}$. Accumulation of the same bands was observed in another ts mutant (ts 22b-16) genetically mapped several hundred nucleotides downstream from the ts 932 mutation.

As for the percentage of rho$^-$ mutants, no difference was observed between the wt and the mutant after four generations at the non-permissive temperature.

Identification of the mutation

MtRNAs were prepared from ts 932 cells after four generations at 37°C, electrophoresed, blotted and hybridized with a $tRNA^{asp}$ specific probe. As a control, mt RNAs from the wt and ts 22b-16 mutant were present in the same blot. As previously mentioned, the mature form of $tRNA^{asp}$ was completely absent in the ts 932 mutant (Fig. 4A). The intermediate molecular weight precursors were present at about the same level in the three strains, while, interestingly, the highest molecular weight precursor was much more abundant in the two mutants. The absence of mature $tRNA^{asp}$ in the mutant suggested that the mutation might affect some step of the maturation of this tRNA. A faint band corresponding to the size of mature $tRNA^{asp}$ was observed in Northern blots of mt RNA from cells grown at 28° (not shown), suggesting that a small amount of mature $tRNA^{asp}$ is produced at this temperature, and is apparently sufficient to allow mitochondrial protein synthesis and growth on glycerol.

To identify the defective processing step, we mapped the 5' and 3' termini of the $tRNA^{asp}$ transcripts by S1 protection experiments. MtRNAs from wt and ts 932 cells were hybridized with 5' and 3' end labeled single stranded probes, which contained portions of the $tRNA^{asp}$ gene, and then digested with S1 nuclease. Results, reported in figure 4B, clearly showed that the 5' end of $tRNA^{asp}$ was correctly processed in both strains,

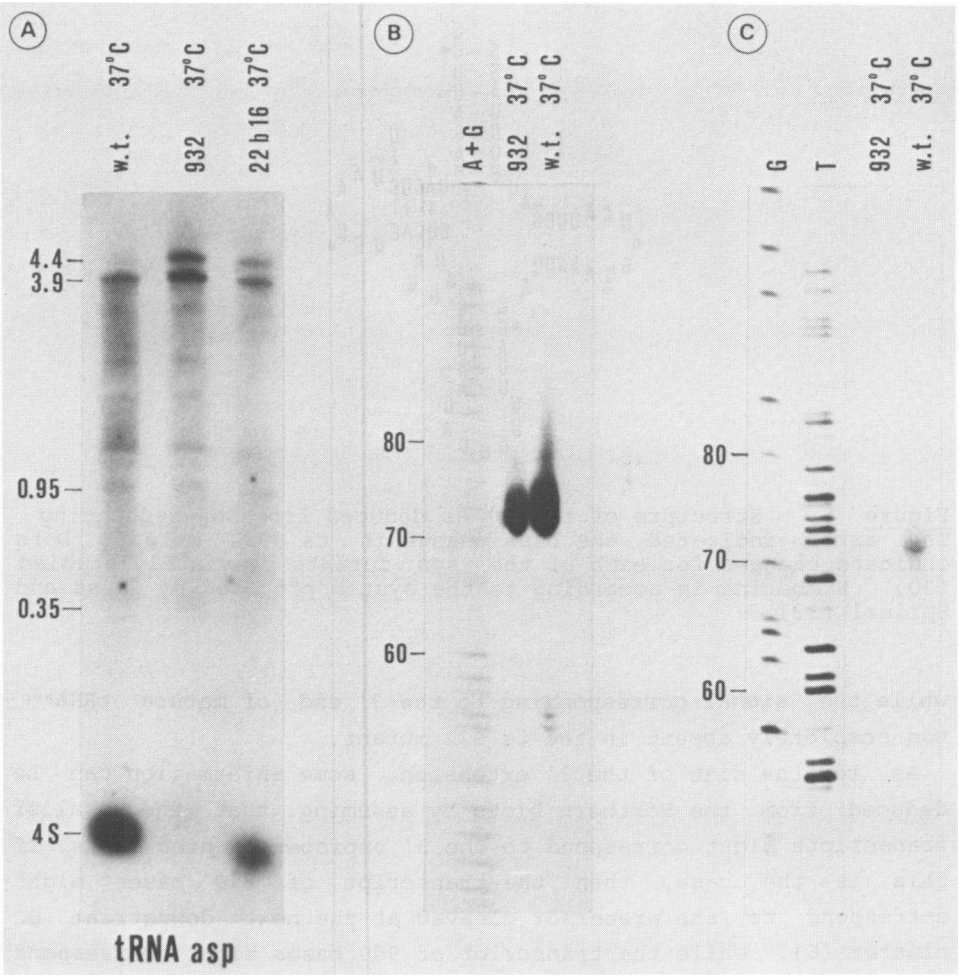

Figure 4: Analysis by Northern blotting and S1 mapping of 5' and 3' termini of tRNA^{asp} gene transcripts. A) hybridization of blotted mtRNA from ts 932 and ts 22-16 mutants with nick translated DS 200/A102 mtDNA retaining the tRNA^{asp} gene. B) the 850bp Hinf1 fragment from rho$^-$ strain DS200/A5 was 5' end labeled; after strand separation the template strand was hybridized to mtRNA prepared from wt and ts 932 cells grown for four generations at 37°C. After nuclease S1 treatment, samples were loaded on a 10% acrylamide 8M Urea sequencing gel and electrophoresed. The S1 signals correspond to the 5' end of mature tRNA^{asp}. C) The 3' end labeled 620bp MboI fragment of mtDNA from strain DS200/A5 was used following the protocol described above. The S1 signal in the wt track corresponds to the 3' end of tRNA^{asp}.

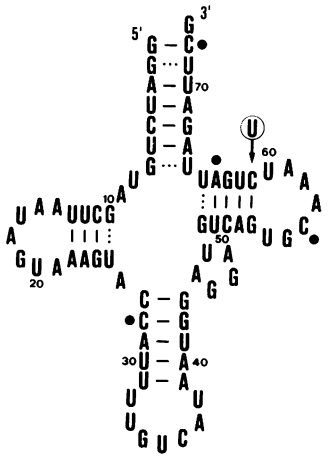

Figure 5: Structure of tRNA^{asp} as deduced from DNA sequencing. The arrow indicates the base change in ts 932 mutant. Dots indicate changes for each of the syn$^-$ mutants previously studied (30). Numbering is according to the system proposed by Gauss and Sprinzl (32).

while the signal corresponding to the 3' end of mature tRNA^{asp} was completely absent in the ts 932 mutant.

As for the size of the 3' extension, some information can be deduced from the Northern blots by assuming that the smaller transcripts might correspond to the 3' unprocessed precursors. If this is the case, then the transcript of 350 bases might correspond to the precursor cleaved at the next downstream GC cluster (6), while the transcript of 950 bases might correspond to a precursor having a 3' extension to the 5' end of the next downstream tRNA gene sequence. To identify the mutation at the sequence level, mt DNA corresponding to the common segments retained in ME121 and DS200/A102 and containing the tRNA^{asp} gene were isolated from wt and ts 932 mutant, cloned and sequenced as described in Materials and Methods. Sequencing of the gene and of the flanking regions revealed a single base change within the structural gene (Figure 5).

This change consisted in a C 61-> U61 transition in the T pseudouridine stem. The sequence analysis of the tRNA^{asp} flanking regions revealed the existence of limited polymorphism between

the FF 1210-6C strain and the published sequence of strain D273-10B (3 different bases in over 700 sequenced nucleotides).

DISCUSSION

We have reported data concerning the characterization of a mitochondrial temperature sensitive mutation mapping downstream from the 21S rRNA gene and we have identified this mutation as being in the $tRNA^{asp}$ gene.

It is interesting to note that the primary defect of the mutation, i.e. the absence of mature $tRNA^{asp}$, could only be detected if the growth at the non-permissive temperature (on a fermentable carbon source) was relatively short; if the strain was grown to stationary phase under the same conditions, a general decrease in the amounts of mature forms of all RNAs species (mRNAs, rRNAs and tRNAs) was observed.

This result points to the necessity to carefully set up the physiological conditions for experiments involving mitochondrial mutations affecting very general systems like macromolecular syntheses.

The general absence of mature RNAs observed after extended growth at 37°C is probably a consequence of the arrest of mitochondrial protein synthesis due to the lack of mature $tRNA^{asp}$. Although we know from previous experiments (2, 3, 4) that the arrest of mitochondrial protein synthesis often leads to the transformation of the original mutant into a $\underline{rho}^-/\underline{rho}^\circ$ state, and in turn to the possible absence of transcripts, the results observed here cannot be due to this drastic mitochondrial modification, since a maximum of 20% $\underline{rho}^-$ cells appeared in the cultures grown to stationary phase.

After four generations, specific effects could be observed: a lower steady state level of most transcripts, absence of $tRNA^{asp}$ and accumulation of precursors of tRNAs; this accumulation might suggest that a general regulative control on mature tRNA levels might take place through the control of specific steps of the maturative process, depending on the efficiency of the mitochondrial biosynthetic machinery.

This hypothesis is supported by the observation that the same precursors accumulate in the ts 22b-16 mutant, in which the defect

has not yet been identified but which maps in a different region. In this respect, it is also interesting to note that alterations in the precursor patterns have also been shown to occur during metabolic changes like glucose repression (29).

Sequencing of the $tRNA^{asp}$ gene region in the ts 932 mutant has lead to the identification of the defect: a C->U transition at position 61 which is a strictly invariant nucleotide in all tRNAs sequenced. Several other mutations, indicated by dots in Fig. 5, have been mapped in the same gene (and one of them in the T loop) but all of them are correctly processed (30). This is therefore the first point mutation found in mitochondria which affects tRNA processing.

The C->U transition produces a G-U base pair which is usually considered capable of H bonding. However in this case, the mutated base pair is located at the beginning of a stem containing only three standard base pairings and the mutation probably produces a structural alteration which allows a limited processing at 28° C, but the absence of mature $tRNA^{asp}$ at 37°C. The fact that a mutation in a strictly invariant nucleotide is compatible with some function, even if at low temperature, is somewhat unexpected, but probably even a low level of mature $tRNA^{asp}$ is sufficient to support mitochondrial protein synthesis at 28°C.

Only one other similar processing defect has been reported for human $tRNA^{fmet}$ (31) but in that case the mutation (a G->T transversion) was located in the loop. The lesion in strain ts 932 completely prevents 3' processing and therefore defines for the first time a structural feature of mitochondrial tRNA which is required for the recognition and/or the activity of 3' pre tRNAase.

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