Nucleotide sequence of *Neurospora crassa* cytoplasmic initiator tRNA

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ABSTRACT

Initiator methionine tRNA from the cytoplasm of *Neurospora crassa* has been purified and sequenced. The sequence is: pAGCUGCAUm¹GGCGCAGCGGAAGCGCm²Gcy*GGGCUCAU⁶AACCCGGAGm⁷GU(or D)-CACUCGAUCGm¹AAACGAG*UUGCAGCU³ACCA^{OH}. Similar to initiator tRNAs from the cytoplasm of other eukaryotes, this tRNA also contains the sequence -AUCG- instead of the usual -T Ψ CG(or A)- found in loop IV of other tRNAs. The sequence of the *N. crassa* cytoplasmic initiator tRNA is quite different from that of the corresponding mitochondrial initiator tRNA. Comparison of the sequence of *N. crassa* cytoplasmic initiator tRNA to those of yeast, wheat germ and vertebrate cytoplasmic initiator tRNA indicates that the sequences of the two fungal tRNAs are no more similar to each other than they are to those of other initiator tRNAs.

INTRODUCTION

In an attempt to understand the relationship between their structure and their specialized functions, initiator tRNAs from several diverse prokaryotic and eukaryotic sources have been sequenced recently (reviewed in 1). An interesting finding from such work is that prokaryotic and eukaryotic cytoplasmic initiator tRNAs possess unique structural features which distinguish them from each other as a class and from most non-initiator tRNAs. The structural feature common to all prokaryotic initiator tRNAs, including those from gram negative and gram positive bacteria²⁻⁵, a mycoplasma⁶ and a blue green alga⁷, is the lack of a Watson-Crick base pair in the acceptor stem between the first nucleotide at the 5'-end and the fifth nucleotide from the 3'-end. The only exception is the initiator tRNA from *Halobacterium cutirubrum*, which, although a prokaryotic organism, differs from them in that it utilizes an unformylated Met-tRNA for the initiation of protein synthesis.

This tRNA contains an A:U Watson-Crick base pair at the end of the acceptor stem (B. Baumstark, S.T. Bayley, J.E. Heckman and U. L. RajBhandary, manuscript in preparation). Studies on yeast⁸, wheat germ⁹ and vertebrate¹⁰⁻¹⁴ cytoplasmic initiator tRNAs have shown that these tRNAs do not possess the above feature characteristic of prokaryotic initiator tRNAs. Instead they possess the sequence -AU(or Ψ)CG- in place of -TΨCG(or A)- found at the beginning of loop IV in most tRNAs which function in protein synthesis, including prokaryotic initiator tRNAs. It is interesting to note that the entire sequence of loop IV, -AU(or Ψ)CGm¹AAA-, has been conserved in the above eukaryotic initiator tRNAs. A finding of further interest is that initiator tRNAs from several diverse vertebrate sources, including those from salmon testes and liver¹², Xenopus¹⁴, rabbit liver¹¹, sheep mammary gland¹¹, mouse myeloma¹⁰ and human placenta¹³ all have identical nucleotide sequences.

We have examined the sequence of the cytoplasmic initiator tRNA from Neurospora crassa in order to determine whether this tRNA also contains the sequence -AU(or Ψ)CG- in loop IV and whether there is a similar strong conservation of initiator tRNA sequence within the plant kingdom. In addition, a knowledge of the sequence of N. crassa cytoplasmic initiator tRNA has been important in our studies (described elsewhere)¹⁵ on the sequence of the initiator tRNA from the mitochondria of N. crassa and for evaluating the overall relationship of the mitochondrial initiator tRNA sequence to those of prokaryotic and eukaryotic cytoplasmic initiator tRNAs. In this paper, we describe the purification and sequence analysis of the cytoplasmic initiator tRNA from N. crassa. We show that this tRNA, like other eukaryotic cytoplasmic initiator tRNAs, contains the sequence -AUCG- in loop IV.

In general, the N. crassa cytoplasmic initiator tRNA is as similar to the vertebrate initiator tRNAs as it is to yeast initiator tRNA. It is, however, quite different in sequence from that of the corresponding mitochondrial initiator tRNA.

MATERIALS AND METHODS

General. Most of the materials and methods are as described previously¹². Radioactive amino acids used for routine assays of column effluents were purchased from Amersham/Searle or New England Nuclear Corporation. The calcium salt of N⁵-formyltetrahydrofolic acid was purchased from Grand Island Biochemical Company. N²-hydroxyethylpiperazine-N²-ethanesulfonic acid (HEPES) was from Schwarz-Mann.

Enzymes and Assays. Nuclease P₁ from Penicillium citrinum was purchased from Yamasa Shoyu Co., Ltd., Tokyo. Calf intestinal alkaline phosphatase (grade I) was from Boehringer Mannheim Corp.

Crude Neurospora crassa aminoacyl-tRNA synthetases were prepared from freshly grown cells according to the procedure of Muench and Berg^{16,17} with the following modifications. Mycelia were broken by two passes through a Gaulin Press at 8000 psi. Cellular debris and ribosomes were removed by centrifugation at 55,000 xg for three hours. After chromatography on DEAE-cellulose, the protein-containing fractions were pooled and precipitated with ammonium sulfate at 100% saturation. The precipitate was resuspended in buffer containing 10 mM HEPES (pH 7.5), 10 mM β -mercaptoethanol, 100 mM KCl, 3 mM sodium azide and 25% glycerol. After chromatography on Sephadex G-25, the crude synthetases were frozen and stored as described previously¹⁸.

Crude E. coli aminoacyl-tRNA synthetases were prepared according to Muench and Berg^{16,17} from either E. coli B or E. coli Q13 (a gift of Dr. Salil K. Niyogi), which lacks RNase I and polynucleotide phosphorylase¹⁹.

Assays for aminoacylation of column effluents were performed at 30°C in 50 mM HEPES buffer (pH 7.5), 10 mM (for initial BD-cellulose columns) or 4 mM (for all subsequent columns) magnesium acetate, 1 mM ATP, 5 mM β -mercaptoethanol, 6 μ Ci/ml [³H]-methionine (5.3 Ci/mmol), 25 μ M of other unlabeled amino acids, and with a saturating amount of N. crassa or E. coli crude synthetases. After a ten-minute incubation, aliquots were spotted onto Whatman 3 MM filter paper discs and acid insoluble radioactivity was determined by a modification of Bollum's method^{20,21}.

Assay for the extent of formylation of pure N. crassa initiator tRNAs by crude E. coli enzymes was carried out as described previously^{12,17}.

Isolation of crude tRNA from Neurospora crassa. Neurospora crassa, wild type strain OR23-1A, was grown from a conidial inoculum in 350 liters of enriched Vogel's medium²² containing 2% glucose. The culture was stirred with an open paddle turbine at 140-450 rpm, and aerated by bubbling 180-400 liters of air per minute, depending upon culture density. Mycelia were harvested in late log phase, washed with distilled water, and stored in 1.5 kg cakes at -20°C.

RNA was obtained from mycelia as described previously²³ with the following modifications. Cakes of frozen mycelia were broken into small pieces, suspended in 5 volumes of buffer²³ and thawed while mixing in a Waring Blendor. The mycelia were then ruptured in a Gaulin Press at 8000 psi. After phenol extraction, only RNA soluble in 1.5 M NaCl was applied to DEAE-cellulose columns. Crude tRNA, which eluted between 0.2 and 0.7 M NaCl, was pooled and deacylated as described previously¹⁸.

Purification of Neurospora crassa cytoplasmic initiator tRNA_i^{Met}.

BD-Cellulose chromatography -- Approximately 60,000 A₂₆₀ units of crude deacylated N. crassa tRNA was applied on a column (6 x 50 cm) of BD-cellulose²⁴ previously equilibrated with 0.33 M NaCl, 10 mM Tris (pH 7.5), 10 mM magnesium acetate and 3 mM NaN₃, and eluted with a linear gradient (0.33-1.1 M) of NaCl (total volume of gradient, 20 liters) in 10 mM Tris (pH 7.5), 10 mM magnesium acetate and 3 mM NaN₃ (Fig. 1). Fractions containing the second major peak of methionine acceptance from two BD-cellulose column runs were pooled to obtain 14,500 A₂₆₀ units of partially purified cytoplasmic initiator tRNA (tRNA_i^{Met}).

DEAE-Sephadex A-50 chromatography -- Partially purified N. crassa tRNA_i^{Met} (14,500 A₂₆₀ units; see above) was chromatographed on DEAE-Sephadex A-50²⁵ and eluted with two linear gradients (0.375-0.47 M NaCl, total volume 7 liters; and 0.47-0.52 M NaCl, total volume 2 liters) containing 20 mM Tris

(pH 7.5) and 8 mM MgCl_2 . Fractions (25 ml) were collected and assayed for methionine acceptance.

RPC-5 chromatography -- Material isolated from the DEAE-Sephadex A-50 column (3500 A_{260} units) was divided into three equal portions and each subjected to RPC-5 chromatography²⁶. The column was run at 37°C and eluted with a linear gradient (0.475-0.55 M NaCl, total volume of gradient 2 liters) containing 10 mM Tris (pH 7.0), 10 mM MgCl_2 , 1 mM β -mercaptoethanol, and 3 mM NaN_3 . A total of 956 A_{260} units of $\text{tRNA}_i^{\text{Met}}$ was isolated from 3 such columns. This partially purified $\text{tRNA}_i^{\text{Met}}$ was chromatographed once more on BD-cellulose and then on RPC-5.

Sephacrose 4B chromatography -- Final purification of $\text{tRNA}_i^{\text{Met}}$ (340 A_{260} units) obtained after the above steps was carried out on two Sepharose 4B columns²⁷. Each column was loaded with 170 A_{260} units of partially purified $\text{tRNA}_i^{\text{Met}}$ and eluted with a linear gradient [1.3-0.0 M $(\text{NH}_4)_2\text{SO}_4$, total volume 2 liters] containing 10 mM sodium acetate (pH 4.5), 10 mM MgCl_2 , 5 mM β -mercaptoethanol, and 1 mM EDTA (Fig. 2). A total of 215 A_{260} units of pure *N. crassa* cytoplasmic $\text{tRNA}_i^{\text{Met}}$ was obtained.

Analysis of modified nucleotides in *N. crassa* $\text{tRNA}_i^{\text{Met}}$. About 1.5-2.0 A_{260} units of tRNA were digested to 3'-mononucleotides with T_2 -RNase, and the digest was analyzed by two-dimensional thin layer chromatography as described previously^{6,13}.

Chemical cleavage of tRNA at the site occupied by $m^7\text{G}$. This was performed on 5.7 A_{260} units of *N. crassa* $\text{tRNA}_i^{\text{Met}}$ ²⁸. The reaction products were separated by gel electrophoresis and recovered as described¹³.

End-group labeling of tRNA fragments and intact tRNA with [^{32}P]. The procedure for complete T_1 - or pancreatic RNase digestion of tRNA and subsequent labeling of resulting oligonucleotide fragments with [^{32}P] at the 5'-end with T4 polynucleotide kinase was as described previously¹². Details of 5'- and 3'-end labeling of intact tRNA and purification and recovery of the end-labeled tRNAs were as described elsewhere^{15,29}.

Partial digestion of 5'-[^{32}P] labeled oligonucleotides with snake venom phosphodiesterase. Conditions for partial di-

gestion of 5'-end labeled fragments with snake venom phosphodiesterase were as described¹².

More recently, for the sequence analysis of long 5'-[³²P] labeled oligonucleotides, particularly those containing modified bases, we have used the relatively non-specific endonuclease P₁ from Penicillium citrinum^{30,31} in obtaining partial digests²⁹. This nuclease cleaves phosphodiester bonds in RNA or DNA to produce a free 3'-hydroxyl and a 5'-phosphate³⁰. Reaction mixtures for partial digestion of oligonucleotides are described in an accompanying paper²⁹.

Analyses of partial digestion products by electrophoresis on DE-81 paper³² or by the two-dimensional hcmochromatography technique³³ were as described previously^{12,34}.

RESULTS

Purification of *N. crassa* tRNA_i^{Met}. Starting from the crude tRNA, purification of *N. crassa* cytoplasmic tRNA_i^{Met} required six steps of column chromatography. Figure 1 shows the A₂₆₀ and methionine acceptance profiles obtained upon chromatography of *N. crassa* tRNA on BD-cellulose. Assay with the homologous *N. crassa* aminoacyl-tRNA synthetases yielded four peaks of methionine acceptor activity. Using crude extracts of *E. coli* for aminoacylation with methionine, peak II could be quantitatively aminoacylated, and peak IV to about 40%, whereas neither peak I nor III was aminoacylated to any extent. Since further studies have shown that tRNA_i^{Met} peak I differs from peak II only in lacking the 3'-terminal A, it is likely that the crude *E. coli* extract used in these assays lacked the tRNA nucleotidyl transferase necessary for addition of 3'-terminal A to tRNA_i^{Met} peak I prior to its aminoacylation.

In the presence of *N. crassa* aminoacyl-tRNA synthetases, peaks I and II could be formylated by *E. coli* met-tRNA formylase, while peaks III and IV were not. Peaks I and II, therefore, were considered most likely to represent the cytoplasmic initiator tRNA species (tRNA_i^{Met}) and were chosen for purification and further study.

The final chromatography profile of tRNA_i^{Met}, peak II on Sepharose 4B is shown in Figure 2. Subsequent analysis indi-

Figure 1. Chromatography of crude *N. crassa* tRNA on BD-cellulose. Methionine acceptor activity was assayed using the homologous *N. crassa* crude aminoacyl-tRNA synthetases.

cated that this tRNA was at least 90% pure. Using a similar purification scheme, tRNA_i^{Met} purified from peak I of Fig. 1 was also obtained about 80% pure. Most of the work described below was carried out on the tRNA_i^{Met} purified from peak II of Figure 1.

Formylation of *N. crassa* tRNA_i^{Met} with *E. coli* met-tRNA formylase. The ability of pure *N. crassa* tRNA_i^{Met} to be aminoacylated and subsequently formylated by crude *E. coli* enzymes was determined as before^{6,12}. Under standard assay conditions, in which control initiator tRNAs from *E. coli* and salmon liver were formylated to greater than 90%, the *N. crassa* cytoplasmic initiator tRNA was formylated to only 16%. This was not due to a decreased rate of aminoacylation of the *N. crassa* tRNA by *E. coli* enzyme since incorporation of methionine into the tRNA was complete within the first few minutes. Assays of [¹⁴C]-formyl incorporation from [¹⁴C]-formyltetrahydrofolate showed

Figure 2. Final purification of *N. crassa* cytoplasmic $\text{tRNA}_i^{\text{Met}}$ on Sepharose 4B. Methionine acceptance profiles are identical when either *N. crassa* or *E. coli* crude aminoacyl-tRNA synthetases are used.

that formylation of the *N. crassa* $\text{tRNA}_i^{\text{Met}}$ occurred at a slower rate than that of *E. coli* $\text{tRNA}_i^{\text{fMet}}$ (data not shown).

Analysis of modified nucleotide content of *N. crassa* $\text{tRNA}_i^{\text{Met}}$. $\text{tRNA}_i^{\text{Met}}$ was digested to mononucleotides with T_2 -RNase and the digest then analyzed by two-dimensional thin layer chromatography. Figure 3 shows the pattern of ultraviolet absorbing spots obtained. Noteworthy is the absence of spots corresponding to Tp and Ψ p, two nucleotides which are present in virtually every non-initiator tRNA, and of spots corresponding to m^2Gp and m^5Cp , modified nucleotides which are present in other eukaryotic initiator tRNAs. The tRNA does contain m^1Gp , m^7Gp , t^6Ap , m^2Gp and m^1Ap , modified nucleotides also found in other eukaryotic initiator tRNAs. Spot D_1 is believed

Figure 3. Two-dimensional thin layer chromatography of a T_2 -RNase digest of *N. crassa* cytoplasmic tRNA_i^{Met}. Solvent 1: isobutyric acid:concentrated NH_4OH :water (557:38:385, v/v, pH 4.3). Solvent 2: *t*-butanol:concentrated HCl:water (70:15:15, v/v). Open circles represent spots that were fluorescent at acid pH.

to be a dinucleotide partially resistant to T_2 -RNase (see below). Its slow mobility, bluish fluorescence at acid pH, and ultraviolet spectrum taken after elution suggests that it is a dinucleotide containing G or a modified G. Since dihydro-uridine phosphate (Dp) does not absorb UV light around 260 nm, the above method of modified nucleotide composition analysis can not be used to detect Dp. 5'-End-group analysis of oligonucleotides showed that this tRNA does contain D (see below, Table I, oligonucleotide t9a).

Analysis of oligonucleotides present in complete T_1 -RNase and pancreatic RNase digests of tRNA_i^{Met} and their localization in the 5'- or 3'-terminal fragments produced by cleavage of the tRNA at m^7G . The presence of m^7G in this tRNA permitted the selective chemical cleavage of the polynucleotide backbone at this residue^{28,35}. The 5'- and 3'-terminal fragments thus produced were separated by electrophoresis on a polyacrylamide gel. The intact tRNA_i^{Met} and the two fragments were each com-

pletely digested with either T_1 or pancreatic RNase, and the resulting mixture of oligonucleotides were labeled with [^{32}P] at their 5'-ends^{11,12,34} and separated by two-dimensional electrophoresis³².

Figure 4 shows the fingerprints of T_1 -RNase digests of the intact $\text{tRNA}_{\text{i}}^{\text{Met}}$ and the 5'- and 3'-terminal fragments. Particularly noteworthy is the absence of a spot due to [^{32}P]-T Ψ CG, a fragment present in T_1 -RNase digests of most tRNAs. Every spot present in the intact tRNA fingerprint (Fig. 4A) is represented uniquely in the corresponding digests of either the 5'- (Fig. 4B) or 3'- (Fig. 4C) fragment, with the exception of spots t2 and t9. Spot t2 occurs in both fragments. Spot t9 has been eliminated by the chemical cleavage reaction and is replaced by a new spot, t9a, in the T_1 -RNase digests of the 3'-fragment (Fig. 4C).

Figure 5 shows the fingerprints of the pancreatic RNase digests of intact $\text{tRNA}_{\text{i}}^{\text{Met}}$, and the 5'- (Fig. 5B) and 3'- (Fig. 5C) terminal fragments. Spots p3 and p4 occur in both fragments, and spot p12 is missing after the cleavage reaction and is replaced by a new spot p10a in the fingerprint of the 5'-terminal fragment (Fig. 5B).

The 5'-[^{32}P] labeled oligonucleotide spots were cut out, counted and eluted. The sequence of the oligonucleotides was then established by partial digestion with snake venom phosphodiesterase and/or nuclease P_1 and analysis of the partial digestion products by two-dimensional homochromatography^{12,29,34,36}. Tables I and II summarize molar yields and sequences determined for oligonucleotides present in complete T_1 or pancreatic RNase digests, respectively. The presence of oligonucleotides pAUCG (spot t8) and pm¹AAACG (spot t5) in the T_1 -RNase digests of the 3'-terminal fragment suggests that the sequence of Loop IV of this *N. crassa* cytoplasmic initiator tRNA is -AUCGm¹AAA- and, hence, is identical to that in other eukaryotic initiator tRNAs.

Two of the modified nucleosides present in *N. crassa* $\text{tRNA}_{\text{i}}^{\text{Met}}$ have not been identified. The first occurs in oligonucleotide t7a, which is present in fingerprints of T_1 -RNase digests of *N. crassa* $\text{tRNA}_{\text{i}}^{\text{Met}}$ and of the 5'-terminal fragment and which

Figure 4. Autoradiographs of fingerprints of oligonucleotides present in T₁-RNase digests of A) intact *N. crassa* trRNA₁^{Met}, B) 5'-terminal, and C) 3'-terminal fragments of *N. crassa* trRNA₁^{Met} produced by cleavage at m⁷G. (B) surrounded by dots, xylene cyanole blue tracking dye. B and P indicate locations of blue and pink tracking dyes, respectively, in the first dimension.

Figure 5. Autoradiographs of fingerprints of oligonucleotides present in pancreatic RNase digests of A) intact *N. crassa* tRNA^{Met}, B) 5'-terminal and C) 3'-terminal fragments of *N. crassa* tRNA^{Met} produced by cleavage at m⁷G. (B) surrounded by dots, xylene cyanole blue tracking dye. B and P indicate positions of blue and pink tracking dyes in the first dimension. X represents non-nucleotidic material occasionally observed in fingerprints of pancreatic RNase digests¹¹.

Table I: Sequence and molar yield of oligonucleotides present in fingerprints of a T_1 -RNase digest of *N. crassa* tRNA^{Met}₁ (Fig. 4).

Fingerprint Spot ^(a)	Sequence of Oligonucleotide	Molar ratio Found ^(b)
1	pCG	3.0(3)
1a	pCm ² G ^(c)	0.2(1)
2	pCAG	1.9(2)
3	pAG	1.8(2)
4	pAAG	1.0(1)
5	pm ¹ AAACG	0.7
5a	pm ⁶ AAACG ^(d)	0.2 (1)
6	pCUACCA	0.5(1)
7	pCUG	1.0(1)
7a	pCY*G ^(c)	0.4(1)
8	pAUCG	0.9(1)
9	pm ⁷ GU(or D)CACUCG	0.7(1)
9a	pU(or D)CACUCG ^(e)	0 (0)
10	pCAUm ¹ GG	0.5(1)
11	pAG*UUG	1.0(1)
13	pCUCAUt ⁶ AACCCG ^(c)	0.25(1)
2a	pCCG ^(f)	

- (a) Fingerprint spots and corresponding oligonucleotides derived from T_1 -RNase digests are referred to by number in the text and with the prefix 't'.
- (b) Based on Cerenkov counting of fingerprint spots from one in vitro labeling reaction. Molar yields predicted by the final tRNA sequence are given in parentheses.
- (c) Low yields may be due to incomplete T_1 -RNase digestion of the anticodon stem and loop.
- (d) Produced by partial conversion of m¹A to m⁶A during digestion of the tRNA and subsequent analyses.
- (e) New fragment produced as a result of cleavage of tRNA at m⁷G. Molar ratio of 0.5/1 found in fingerprint of 3'-terminal fragment.
- (f) Probably derived from pCY*G during alkaline conditions used for cleavage of tRNA at m⁷G.

migrate slightly slower than oligonucleotide t7 (pCUG) in the first dimension (Figs. 4A and 4B). The following lines of evidence suggest that oligonucleotide t7a is pCY*G in which Y* is probably a N⁴-acyl derivative of C: (a) The 5'-end group of t7a was identified as pC. Partial digestion with snake venom phosphodiesterase indicated that oligonucleotide t7a is a trinucleotide, and that the second nucleoside must be modified, since the dinucleotide produced migrates more slowly upon electrophoresis in DE-81 paper than either pCC or pCU

Table II: Sequence and molar yield of oligonucleotides present in fingerprints of a pancreatic RNase digest of *N. crassa* tRNA^{Met}₁ (Fig. 5).

Fingerprint Spot (a)	Sequence of Oligonucleotide	Molar ratio Found (b)
1	pAC	2.5 (2)
2	pm ² ₂ GC	0.9 (1)
3	pGC	4.6 (4)
4	pAGC	3.3 (3)
5	pAU	1.8 (2)
6	pGm ¹ AAAC	
6a	pGm ⁶ AAAC (c)	0.6 (1)
7a	pt ⁶ AAAC (d)	
7b	pm ¹ GGC (d)	1.9 (2)
8	pGAU	1.0 (1)
9	pGGAAGC	0.3 (1)
10	pGGGC	0.4 (1)
11	pGAG*U	0.6 (1)
12	pGGAGm ⁷ GU (or D)	0.4 (1)
10a	pGGAG-ribose fragment (e)	0 (0)

- (a) Fingerprint spots and corresponding oligonucleotides derived from pancreatic RNase digests are referred to in the text by number and with the prefix 'p'.
- (b) See Table I, footnote (b).
- (c) See Table I, footnote (d).
- (d) These oligonucleotides were later separated by one-dimensional chromatography on PEI-cellulose, using 2 M pyridinium formate (pH 3.4) as described previously¹³.
- (e) See Table I, footnote (e). Molar ratio of 0.3/1 found in fingerprint of 5'-terminal fragment.

at pH 3.5, or pCC, pCU or pCA at pH 1.9 (data not shown).

(b) The modified nucleoside cannot be a purine, since none of the fragments present in pancreatic RNase digests contains a modified A at the 5'-end (Table II), and both fragments that contain a modified G at the 5'-end are accounted for in other fragments of T₁-RNase digests. (c) Among the more common modified pyrimidines, the modified nucleoside in t7a cannot be D, Ψ, or m⁵C. As discussed before, Ψp and m⁵Cp are absent in T₂-RNase digests of this tRNA (Fig. 3). Also, the electrophoretic mobility of pCD or pCm⁵C is known to be higher than that of pCU. (d) The location of oligonucleotide t7a on T₁-RNase

fingerprints requires that Y* behave like U in the sense that it must not be significantly protonated at pH 1.9. All the above evidence is consistent with Y* being N⁴-acyl C³⁷. The modified nucleoside N⁴-acetylcytidine is present in the sequence Cac⁴CG in T₁-RNase digests of yeast tRNA₃^{Leu} 38,39, and its location in a two-dimensional electrophoretic fingerprint is similar to that of oligonucleotide t7a. The oligonucleotide [³²P]-Cac⁴CG was, therefore, isolated from a fingerprint of T₁-RNase digests of yeast tRNA₃^{Leu} (a gift of Dr. Simon H. Chang). Chromatographic comparisons of this trinucleotide to oligonucleotide t7a from *N. crassa* tRNA_i^{Met} showed, however, that they were not identical (B. de Weerd, unpublished observation). Therefore, Y* is not ac⁴C, but may be another N⁴-acyl derivative of cytidine.

The second unidentified nucleoside occurs in oligonucleotide t11 of T₁-RNase digests (Figs. 4A and 4C) and also in oligonucleotide p11 of pancreatic RNase digests (Figs. 5A and 5C) of intact tRNA and of the 3'-terminal fragment. In the case of oligonucleotide t11, partial digestion with snake venom phosphodiesterase showed it to be a pentanucleotide with pA at the 5'-end. The second nucleoside is modified since the dinucleotide produced has an electrophoretic mobility greater than pAG, but less than pAA, pAU, or pAC on DE-81 paper at pH 3.5 (data not shown). Similarly, partial snake venom phosphodiesterase digestion of oligonucleotide p11 showed it to be a tetranucleotide; in this case, the trinucleotide thus generated (pGAX) has an unusual electrophoretic mobility. For both oligonucleotides t11 and p11, two-dimensional homochromatography of the partial digests gave identical G-like angular mobility shifts³³ for the loss of the modified nucleotide, and led to the sequence assignments of pAG*UUG (Table I) and pGAG*U (Table II), respectively. Thus G* is believed most likely to be a derivative of G which is resistant to T₁-RNase digestion. This could also account for the presence of the G-containing dinucleotide usually found in T₂-RNase digests of this tRNA (spot D₁, Fig. 3), which is most probably G*pUp. Although identification of G* is not complete, it is worth noting that the position of spot D₁ in our two-dimensional

chromatography system is close to that of GmpUp determined by Hashimoto *et al.*⁴⁰ using a similar two-dimensional chromatography system⁴¹.

Derivation of the nucleotide sequence of *N. crassa* tRNA_i^{Met}.
The oligonucleotides listed in Tables I and II were aligned into a unique sequence using the overlaps of nucleotide sequences as indicated on Figure 6. Much of the information necessary for this was derived by application of a procedure described in an accompanying paper²⁹ for sequencing of 5'- or 3'-termini of [³²P]-end-group labeled tRNAs using nuclease P₁. This allowed us to derive the sequence of 24 nucleotides from the 5'-end (fragment c) and of 15 nucleotides from the 3'-end (fragment d) of the tRNA. Based on the sequences of fragment (d) and oligonucleotide t9, the sequence of the 30-nucleotide long fragment (b) produced by cleavage of the tRNA at m⁷G could be deduced except for the relative order of AUCG (t8) and m¹AAACG (t5) within it. This was assigned based on the unique location of m¹A in loop IV of those tRNAs which contain m¹A. Furthermore, since this fragment is preceded by m⁷G in the tRNA, overlap of fragment (b) with GGAGm⁷GU(or D) (oligonucleotide pl2) provided the sequence of 35 nucleotides from the 3'-end. Within the 5'-terminal fragment (a), the only oligonucleotides still left unassigned at this stage were Cm²G (t1a), CY*G (t7a), GGGC (pl0) and CUCAUt⁶AACCCG (t13). These could be placed within the tRNA sequence by making two simple assumptions: (i) that the oligonucleotide CUCAUt⁶AACCCG is partly in the anticodon loop, which is quite likely since of the four fragments this is the only one which contains the methionine anticodon sequence -CAU- followed immediately by t⁶A, a modified nucleoside found only adjacent to the anticodon; and (ii) that m²G is located in the interstem region between the D and anticodon stems, as it is in every tRNA which contains m²G¹.

Figure 7 shows the sequence of *N. crassa* tRNA_i^{Met} in the cloverleaf form. Like all other eukaryotic cytoplasmic initiator tRNAs, the *N. crassa* cytoplasmic tRNA_i^{Met} is 75 nucleotides long. A summary of molar yields of oligonucleotides present in T₁- and pancreatic RNase digests compared to those predicted by the final sequence is given in Tables I and II.

Figure 6. Sequence of *N. crassa* cytoplasmic tRNA^{Met} in linear form (top). Fragments (a-d) are explained in the text. Other oligonucleotides are from complete T₁-RNase (with the prefix "t") and pancreatic RNase (with the prefix "p") digests. Dashed lines indicate that the fragments concerned contained additional nucleotides, the sequence of which was not completely deduced from the particular experiment described.

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DISCUSSION

The N. crassa cytoplasmic tRNA_i^{Met} provides another example of a eukaryotic tRNA which contains the sequence -AU(or Ψ)CG- instead of -TΨCG(or A)- present in virtually every tRNA active in protein synthesis, and has in its loop IV the sequence -AUCGm¹AAA-. Thus, the unusual sequence -AU(or Ψ)CG-m¹AAA- previously found in yeast⁸, wheat germ⁴⁴ and vertebrate cytoplasmic initiator tRNAs¹⁰⁻¹⁴ may be common to all eukaryotic cytoplasmic initiator tRNAs.

Aside from the sequence of loop IV, there are other interesting features in the N. crassa cytoplasmic tRNA_i^{Met}. The guanosine at position 10 from the 5'-end is unmodified, whereas the other sequenced eukaryotic initiator tRNAs have m²G at this position. Also, the N. crassa tRNA_i^{Met} contains G*, an unidentified derivative of G at position 63 from the 5'-end in the TΨC stem. Since yeast⁸ and wheat germ⁴⁴ initiator tRNAs also contain a modified purine at this position, the presence of a modified purine here may be a feature common to fungal and plant initiator tRNAs.

The sequence of N. crassa cytoplasmic tRNA_i^{Met} may also provide some information concerning the highly specific recognition of tRNAs by E. coli met-tRNA formylase. Figure 8 shows a composite structure of those tRNAs which are efficient substrates for the met-tRNA formylase. The N. crassa cytoplasmic tRNA_i^{Met}, which is a relatively poor substrate, contains all the nucleotides present in the composite structure except the G₇:C₆₇ and G₅₃:C₆₃ base pairs of Figure 8, which are replaced by A:U and C:G base pairs, respectively. It is, therefore, possible that the absence of one or both of these base pairs in the N. crassa cytoplasmic tRNA_i^{Met} results in the slower rate of formylation of this tRNA by the E. coli met-tRNA formylase. We have recently found¹⁵ that the N. crassa mitochondrial initiator tRNA also lacks the G₇:C₆₇ base pair and yet is a good substrate for the E. coli met-tRNA formylase. Thus, it may be possible to correlate the reduced rate of formylation of N. crassa cytoplasmic tRNA_i^{Met} to the alteration of a specific base pair, in this case the G₅₃:C₆₃ base pair.

Our reasons for selecting the N. crassa cytoplasmic

**Composite Structure of tRNAs Recognized
by *E. coli* Methionyl-tRNA Transformylase**

Figure 8. Composite structure of tRNAs recognized by *E. coli* methionyl-tRNA formylase based on the sequences of *E. coli*, yeast, *B. subtilis* and vertebrate initiator tRNAs. Nucleotides found in the same position in all tRNAs are indicated by small letters. Those uniquely common to these initiator tRNAs are indicated by large letters.

tRNA_i^{Met} for sequence studies were two-fold: (a) As an Ascomycete, *N. crassa* may be closely related to baker's yeast, for which the cytoplasmic initiator tRNA sequence is known⁸. A comparison of initiator tRNA sequences from these two fungi would indicate whether the degree of evolutionary conservation of initiator tRNA sequences among the non-photosynthetic plants is comparable to that observed among the vertebrates¹⁰⁻¹⁴.

(b) A considerable background in the study of *N. crassa* cytoplasmic and mitochondrial tRNAs and aminoacyl-tRNA synthetases had been accumulated⁴⁵⁻⁴⁷. This facilitated the purification of the *N. crassa* cytoplasmic initiator tRNA and the preparation of large quantities of *N. crassa* mitochondria for tRNA and enzyme isolation, which made *Neurospora* the organism of choice for comparative studies of cytoplasmic and mitochondrial initi-

ator tRNAs. As discussed earlier, the initiator tRNAs from both prokaryotes and eukaryotic cytoplasm display unusual structural features that distinguish them from the non-initiator tRNAs and from each other. Our aim was, therefore, to determine whether initiator tRNAs from mitochondria have any unusual features, and if so, whether they are prokaryotic, eukaryotic, or fall into a category of their own. As described elsewhere¹⁵, we have now also sequenced the N. crassa mitochondrial initiator tRNA and found it to have a sequence quite different from that of the corresponding cytoplasmic tRNA_i^{Met}. Interpretation of some of the interesting features of the mitochondrial initiator tRNA has depended upon a knowledge of the sequence of the corresponding cytoplasmic initiator tRNA, as presented herein.

Finally, a comparison of the N. crassa cytoplasmic tRNA_i^{Met} sequence with those of yeast, wheat germ and vertebrate cytoplasmic tRNA_i^{Met} shows that, disregarding differences in post-transcriptional modification, the extent of homology of the N. crassa tRNA to these tRNAs is: Yeast (76%), wheat germ (73%) and vertebrate (79%). Thus, the two fungal initiator tRNA sequences are no more closely related to each other than to the other eukaryotic initiator tRNAs. Evidently, conservation of initiator tRNA sequence within the ascomycetes has been less stringent than within the vertebrate subphylum of the animal kingdom¹⁰⁻¹⁴.

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