Phytochemistry, Vol. 22, No. 12, pp. 2647–2651, 1983.
Printed in Great Britain.

0031-9422/83 $3.00 + 0.00

tRNA METHYLATION DURING THE GROWTH AND DIFFERENTIATION OF *CYLINDROCARPON IANTHOTHELE*

RÉGINE STEIMAN, JEAN LACHARME and FRANÇOISE SEIGLE-MURANDI

Laboratoire de Botanique, Cryptogamie, Biologie Cellulaire et Génétique, Faculté de Pharmacie, Avenue de Verdun, 38240 Meylan, France

(*Revised received* 23 *May* 1983)

Key Word Index—*Cylindrocarpon ianthothele*; Deuteromycotinae; tRNA methylation; fungal differentiation.

Abstract—tRNA formation in *Cylindrocarpon ianthothele* was studied during the various periods of its life cycle. The quantitative changes of tRNA were highly correlated with growth. tRNA methylation detected by labelling with [Me-^{14}C]-L-methionine, however, showed two maxima, at the end of the lag phase and at the beginning of conidia formation; one of these peaks was involved in the initiation of growth, the other in that of conidia formation.

INTRODUCTION

In studies on the hyphal differentiation of spores, the synthesis of proteins is rarely restricted to a study of proteins alone [1]; most of the investigations also include RNA [2–10], only tRNA [11–13], mRNA [14, 15], polysomes [16] or the synthesis of nucleotides [17]. Certain enzyme systems have been extensively studied, such as aminoacyl-tRNA synthetases [18] and tRNA methylases[19].

Most of the elements required for protein synthesis pre-exist in the conidia and the onset of germination appears to be preceded by a modification of these systems rather than by *de novo* synthesis. The events which may induce the synthesis of new proteins in spores are the subject of several hypotheses: activation of stored but non-functional mRNAs, ribosome-to-polysome initiation or the activation of initiation factors or the suppression of inhibition [20].

In the present research, we have concentrated on the study of tRNA, often suggested to play an important role in differentiation processes [21, 22].

RESULTS

Weight growth and morphological evolution

Microscopic observations were made at various intervals of the culture. This enabled three periods to be defined, characteristic of the development of the fungus (Figs. 1 and 2). The first was a lag phase, 24 hr long, which corresponded to spore germination (5 hr period) and to the beginning of the apical development of the germinative tube. This was generally produced by a terminal cell of the conidium, rarely by intermediate cells. The fresh weight did not change greatly; this phase corresponded to spore-filament differentiation. The second was an exponential growth phase until the 72nd hr. During this period, hyphal elongation occurred by apical growth and branching without apparent differentiation until the 35th hr. Conidiogenesis began at about the 36th hr. The stationary phase lasted until hr 120. Hyphal lysis of the primary thallus was compensated by the mycelial growth of newly formed conidia. After the first conidiogenesis, the different phases of vegetative multiplication overlapped and it was no longer possible to differentiate as clearly as could be done during the first 40 hr.

Figure 1 shows the changes in fresh and dry weights and total nitrogen during the first 50 hr of culture. [^{14}C]Leucine incorporation is also shown, the tracer having been added to the culture 2 hr before sampling.

Labelling experiments showed two periods during which protein synthesis was especially active (5 and 36 hr). These times corresponded to the end of germinative maturation and to the first conidiogenesis.

Quantitative changes of tRNA

The quantity of tRNA increased regularly up to hr 36, remained at a plateau until hr 50 and then decreased slightly during the second half of exponential growth (Table 1).

Synthesis of total RNA was rapid during the lag phase and then decreased to approach that of tRNA. The % ratio of tRNA/total RNA, however, showed that the pool was enriched in tRNA, and so these changes were not parallel.

During the exponential phase, the various RNA species increased, with a predominance for tRNA, in correlation with increased metabolic activity associated with growth. We employed labelling techniques to determine the qualitative variations occurring in tRNA during these differentiations. The degree of tRNA methylation was chosen as an index of this.

tRNA methylation levels after prolonged labelling

[Me-^{14}C]-L-Methionine was added to cultures 13 hr before harvesting mycelia. An initial experiment was performed with only this radiolabel. A second double label experiment was performed with ^{14}C and with $^{32}PO_4HNa_2$, added to the medium under the same conditions as was ^{14}C. The maximum quantity of tRNA was observed between hr 36 and 50, consistent with the

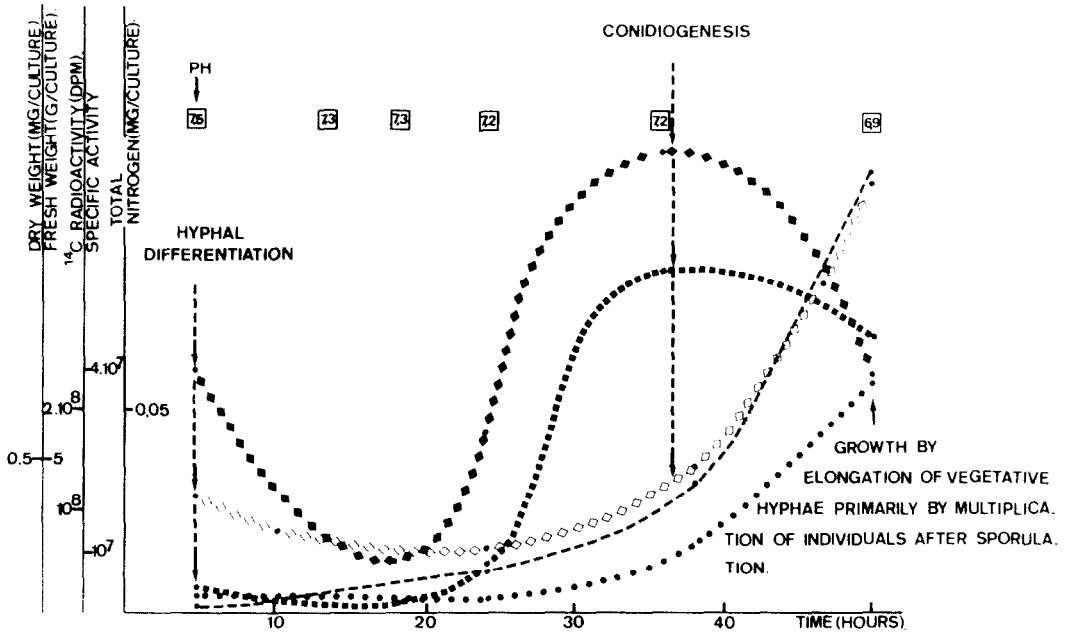

Fig. 1. Weight changes of a *C. ianthothele* culture in Czapek–Dox medium and [^{14}C]leucine incorporation during the first 50 hr. Weight changes are expressed as fresh weight (●), dry weight (◇) and total nitrogen (– – –). Labelled leucine was added at 2 μCi/l. 2 hr before sampling. (■), absolute radioactivity of proteins. (◆), specific radioactivity.

Table 1. Changes in tRNA and total RNA during the first 70 hr of culture, expressed in mg/100 g fresh weight

	Time (hr)							
	5/6	13	18	24/27	36	38/39	48	69/70
Total RNA	89	255	105	113	193	189	—	153
tRNA	17	27	37	43	88.5	86	87.5	74
% tRNA/RNA	19	10.6	35.4	38	45.8	45.5	—	48
Fresh weight (g)/culture	0.58	0.49	0.51	0.86	1.60	2.07	4.64	7.4

Results are the mean of three experiments for all times except 48 hr (one test).

above results. The degree of methylation, however, expressed as absolute radioactivity, evolved differently. Thus, a maximum was observed at hr 36, followed by a relatively rapid decrease of radioactivity. The specific radioactivity demonstrated a second period of increased methylation, around hr 60. These two activity increases coincided with two microscopically observed periods of conidiogenesis (Fig. 2).

The double label experiment (Table 2) involved 13 hr labelling periods with ^{14}C and ^{32}P, except for the first sample in which, by definition, it was only 5 hr. The specific radioactivity of ^{32}P demonstrated an intense tRNA synthesis during the lag phase. This synthesis continued until hr 18 and then decreased. The level of ^{14}C-methyl labelling apparently evolved similarly, but the ^{14}C/^{32}P ratio showed that the maximum occurred at hr 38, rather than hr 18, i.e. during conidiogenesis.

These two experiments demonstrated a qualitative change in the tRNA pool. Increased tRNA methylation was greater at the beginning of conidiogenesis.

tRNA methylation after short labelling

Prolonged labelling indicated net anabolism and catabolism, but it was difficult to determine the relative proportions of each. We thus performed an additional single label experiment with [M-^{14}C]methionine with a 30 min labelling period before each sampling. In addition, more samples were taken over a 72 hr period. The results (Table 3) were more suitable for indicating the tRNA methylation rates during fungal development.

Increased methylation activity at hr 39 was confirmed at the beginning of conidiogenesis. In addition, the increased levels of absolute and specific radioactivities at hr 13 and 18 reflected a similar phenomenon at the end of the lag phase. This finding was masked when labelling was for 13 hr.

DISCUSSION

The purpose of the present work was to determine the events concerning tRNA synthesis during the develop-

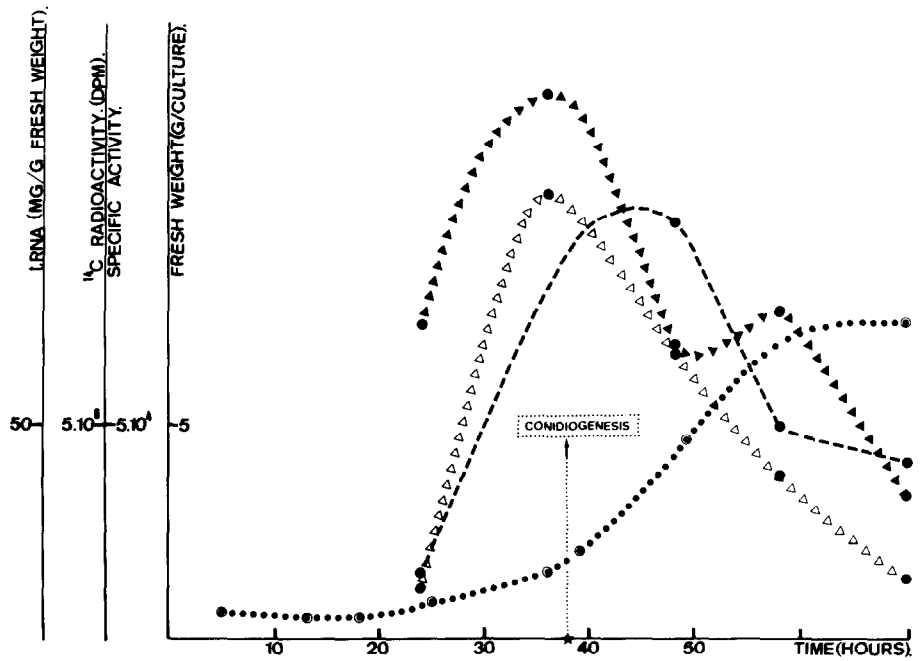

Fig. 2. Labelling of tRNA by [Me-^{14}C]-L-methionine added at 5 μCi/l. 13 hr before sampling. The growth curve, in g fresh weight/culture, is indicated by solid circles. Quantities of tRNA (mg/100 g fresh weight) are indicated by the dashed curve. (△), total radioactivity. (▲), specific radioactivity.

Table 2. Evolution of tRNA in *Cylindrocarpon ianthothele* during the first 50 hr of culture with labelling for 13 hr before harvest

	Time (hr)				
	5	13	18	38	50
Fresh weight (g)	0.58	0.62	0.46	2.30	7.48
tRNA (mg/100 g fresh weight)	18	28	43.5	111	97
^{32}P Radioactivity (dpm) per 100 g fresh weight ($\times 10^{-4}$)	4940	9300	16480	32400	7100
^{32}P/tRNA $\times 10^{-2}$	27400	33150	37870	29190	7330
^{14}C Radioactivity (dpm) per 100 g fresh weight ($\times 10^{-4}$)	860	1550	3590	8700	1790
^{14}C/tRNA $\times 10^{-2}$	4780	5540	8260	7800	1850
^{14}C/^{32}P	0.17	0.16	0.21	0.27	0.25

Table 3. Evolution of methylated tRNA of *Cylindrocarpon ianthothele* with labelling for 30 min before harvest

	Time (hr)								
	5	9	13	18	24	39	42	48	72
Fresh weight (g)	0.40	0.40	0.53	0.50	0.55	1.60	1.50	3.20	6.30
tRNA (mg/100 g of fresh weight)	25	24	27	27	48	83	96	73	70
^{14}C Radioactivity (dpm)/100 g of fresh weight ($\times 10^{-4}$)	46	33	302	400	173	444	433	312	96
^{14}C/tRNA $\times 10^{-2}$	184	137	1119	1481	360	535	451	427	137

ment and differentiation of a fungus, *C. ianthothele*, between spore germination and the formation of the first conidia. Nevertheless, a certain number of biochemical processes existed before hyphal differentiation—those already present in the spore. This aspect has been extensively studied [2–4, 12, 13, 16].

Considering these results, it was consistent to believe that the conidium was mostly equipped with the nucleic

acid components required for the protein syntheses which subsequently occur during germination. This was precisely what we observed during spore germination: [^{14}C]leucine labelling demonstrated a high level of protein synthesis from the onset of hyphal differentiation and which decreased sharply between hr 15 and 25, i.e. until the end of the lag phase. At the morphological level, this period corresponded to the development of the germinative tube from one terminal cell of the spore. This development occurred only apically, with no dichotomous ramifications.

A new wave of protein synthesis occurred between hr 24 and 36 during the first part of exponential growth until the appearance of conidiophores. Mycelial hyphae developed during this period not only by apical growth but also by dichotomous ramification without apparent differentiation until hr 36. This synthesis can only be related to the production of the various nucleic acids which are its support, since if we follow the changes in nucleic acids, especially tRNAs, their absolute quantity increased regularly, beginning at hyphal development during the lag phase and during the first two-thirds of exponential growth. Levels subsequently stabilized and then decreased at the end of this phase. This decrease became even greater during the stationary phase, i.e. after hr 70. The absolute or specific ^{32}P radioactivity of tRNA showed that general synthesis was especially intense until hr 38 and then decreased considerably. The findings concerning tRNA are in agreement with those published about spore germination of *Rhizopus stolonifer* [5], *Aspergillus oryzae* [11] and *Peronospora tabacina* [3].

Results furnished by determination of tRNA or general ^{32}P labelling yielded a quantitative, but not qualitative, evaluation of the tRNA pool. Correlations exist between the modified nucleoside composition of certain tRNAs and the different physiological states of a cell [21]. The constitution of tRNA has been considered by some workers [19]; studying tRNA function with the aminoacyl tRNA synthetases, they concluded that these enzymes existed in the conidiospores of *Botryodiplodia theobromae* and that their activities increased during germination [18]. The study of the composition of tRNAs showed that tRNA methylase activities were different in the conidia and the vegetative cells [19]. Reciprocal *trans*-methylation with the enzymes of both types of cells showed that the methylation of mycelial tRNA was more intense than that of conidial tRNAs. The authors did not specify the physiological state of the mycelia, i.e. immediately developed from the spore or already differentiated. It thus becomes difficult to state if this property is permanent and distinguishable between mycelial and spore forms.

Long-term labelling of *C. ianthothele* by [^{14}C]methionine in the present work demonstrated that tRNA methylation was especially elevated just before mycelial hyphae differentiated into the conidiogenic apparatus, since ^{14}C radioactivity was greatest during these periods. The ^{14}C/^{32}P ratios were also highest at these times. Short labelling (30 min) demonstrated another peak of methylation activity, at the end of the lag phase just before the onset of the growth phase. Thus, two tRNA methylations could be observed which preceded a rearrangement of certain nucleic acid fractions at the beginning of a change of physiological state of the fungus. At the end of the lag phase, it was possible that methylation was one of the elements initiating this change in nucleic acids, required for the synthesis of proteins involved in the growth of mycelial hyphae. The second wave of methylation, observed during exponential growth (hr 36), would have another physiological significance. It would assure the differentiation of the nucleic acids required for the formation and maturation of conidiospores. Enzymes and nucleic acids would be relatively non-functional in the dormant spore and would undergo an increase in their metabolic activities during spore germination and the lag period. These various aspects of the differentiation cycle are represented in Fig. 3, from hyphal differentiation up to conidiospore formation.

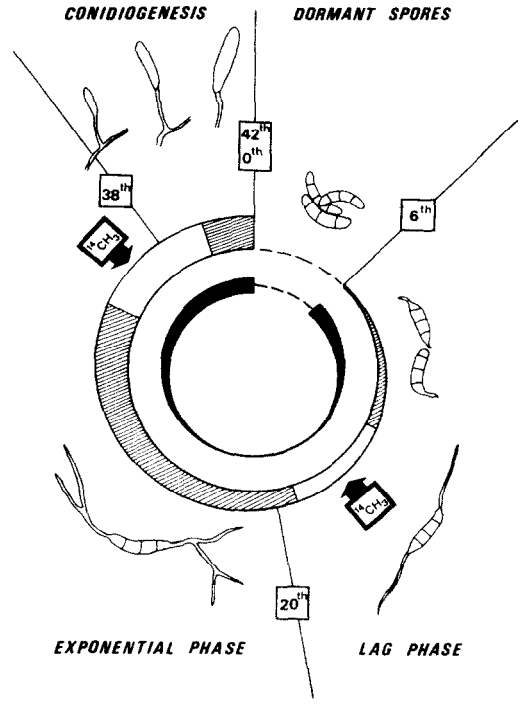

Fig. 3. Representation of cellular events from hyphal differentiation up to conidiogenesis in *C. ianthothele*. The inner circle represents the intensity of protein synthesis, proportional to the thickness of the black circumference. The outer circle corresponds to tRNA formation, with the thickness of the hatched zone proportional to the quantities synthesized. Clear zones correspond to periods of intense methylation, pointed out by the squares and arrows.

EXPERIMENTAL

Cylindrocarpon ianthothele is included in the *Cylindrocarpon* group described and classified in ref. [23]. Among the strains in our collection, the one presenting the most regularity in its growth characteristics is strain No. 78. This is a *C. ianthothele* var. *majus* Wollenw, isolated from the soil in the Cormoran grotto in 1977 [24].

Culture conditions. The stock culture was stored on 2% malt agar at 4°. Cultures for expts were raised by growing in 2 l. Czapek–Dox liquid medium (pH 7.4) in 5 l. conical flasks with agitation and at 22°. Seeding was with spores, whose density was determined by nephelometry at 600 nm (2×10^6 spores/l.).

Sampling times were chosen as a function of the fungal growth curve. This was routinely determined by the fr. and dry wts of mycelia and also by assaying protein nitrogen or more simply total nitrogen in the samples. Protein nitrogen is defined as the insoluble fraction after precipitating homogenates with 10% trichloroacetic acid (TCA). In certain expts, only total nitrogen assays were used, which are in fact quite close to protein nitrogen assays.

Total and protein nitrogen. Aliquots of the protein ppt. were mineralized in H_2SO_4 with Se–Cu catalyst (40 g $CuSO_4$, 11.2 g SeO_2). Assays were performed in Parnas and Wagner apparatus: distilled NH_3 was assayed by acid titrimetry.

Proteins. The sample was ground several times in liquid N_2 in a stainless steel pestle. The homogenate was precipitated twice with 10% TCA, followed by centrifugation at 10000 *g* for 15 min. The pellet was dissolved in 2 ml 0.1 M NaOH and protein was assayed by colorimetry [25].

tRNAs. These were extracted [26], fractionated as described [27], purified on DEAE-Sephadex A50 [28] and deaminoacylated [29].

Radioactive labelling. Proteins were labelled with [2-^{14}C]-L-leucine (CEA, France). Sp. act. was 52.5 mCi/mmol and it was added to the culture medium at a final concn of 2 μCi/l. Nucleic acids were labelled with $Na_2H^{32}PO_4$ (CEA, France). Sp. act. was 20 mCi/mg and the final concn in the medium was 0.3 mCi/l. Methylation was determined by adding [$^{14}CH_3$]-L-methionine (CEA, France), sp. act. 55 mCi/mmol, to the medium at a final concn of 5 μCi/l. Labelling periods are given in the legends.

REFERENCES

1. Gong, C. S. and Lovett, J. S. (1977) *Exp. Mycol.* **1**, 138.
2. Murphy, S. M. N. and Lovett, J. S. (1966) *Develop. Biol.* **14**, 68.
3. Hollomon, D. W. (1970) *J. Gen. Microbiol.* **62**, 75.
4. Van Assche, J. A. and Carlier, A. R. (1973) *Arch. Mikrobiol.* **93**, 129.
5. Roheim, J. R., Knighi, R. H. and Van Etten, J. L. (1974) *Develop. Biol.* **41**, 137.
6. Alberghina, F. A. M. and Sturani, E. (1975) *J. Biol. Chem.* **250**, 4381.
7. Mirkes, P. E. (1977) *Exp. Mycol.* **1**, 271.
8. Nickerson, K. W., MacCune, B. K. and Van Etten, J. L. (1977) *Exp. Mycol.* **1**, 317.
9. MacLeod, H. and Horgen, P. A. (1979) *Exp. Mycol.* **3**, 70.
10. Rao, P. V., Aggarwal, A. K. and Singhal, G. S. (1979) *Indian J. Exp. Biol.* **17**, 1027.
11. Tanaka, K., Ono, T. and Yanagita, T. (1966) *J. Gen. Appl. Microbiol.* **12**, 329.
12. Van Etten, J. L., Koski, R. K. and El Olemy, M. M. (1969) *J. Bacteriol.* **100**, 1182.
13. Merlo, J., Roker, H. and Van Etten, J. L. (1972) *Can. J. Microbiol.* **18**, 949.
14. Van Etten, J. L. and Freer, S. N. (1978) *Exp. Mycol.* **2**, 301.
15. Freer, S. N. and Van Etten, J. L. (1978) *Exp. Mycol.* **2**, 313.
16. Mirkes, P. E. (1974) *J. Bacteriol.* **117**, 196.
17. Van Laere, A. J., Van Assche, J. A. and Carlier, A. R. (1980) *Exp. Mycol.* **4**, 96.
18. Van Etten, J. L. and Brambl, R. M. (1968) *J. Bacteriol.* **96**, 1042.
19. Wong, R. S. L., Scarborough, G. A. and Borek, E. (1971) *J. Bacteriol.* **108**, 446.
20. Lovett, J. S. (1974) *Int. Spore Symp.* **2**, 189.
21. Söll, D. (1971) *Science* **173**, 293.
22. Seigle-Murandi, F. and Steiman, R. (1981) *Phytochemistry* **20**, 2093.
23. Booth, C. (1966) *Mycol. Pap.* **104**, 1.
24. Lacharme, J., Seigle-Murandi, F., Steiman, R., Nicot, J. and Pinel, Cl. (1978) *Actes 6ème Congr. Suisse Spéléol.* Porrentruy, Sept. 1978, pp. 63–70.
25. Zack, B. and Cohen, J. (1961) *Clin. Chim. Acta* **6**, 665.
26. Beck, G. (1966) Thèse Doctorat ès-Sciences, Strasbourg.
27. Zubay, G. J. (1962) *J. Mol. Biol.* **4**, 347.
28. Nishimura, S., Harada, F., Narushima, V. and Takeshi, S. (1967) *Biochim. Biophys. Acta* **142**, 133.
29. Weil, J. H. (1964) Thèse Doctorat d'Etat, Strasbourg.