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Methylation of ribosomal and soluble RNA in Neurospora crassa

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Biochimica comparata. — *Methylation of ribosomal and soluble* RNA *in* Neurospora crassa (*). Nota (**) di EMMAPAOLA STURANI, MARIA GRAZIA MASSARI e FILIPPA A. M. ALBERGHINA, presentata dal Corrisp. E. MARRÈ.

RIASSUNTO. — Sono state determinate le velocità di metilazione dell'RNA solubile e ribosomale in miceli di *Neurospora crassa* in crescita esponenziale in quattro diversi mezzi di cultura che permettono velocità di crescita comprese tra 0.13 e 0.51 duplicazioni per ora. Le velocità di metilazione dell'rRNA e dell'sRNA sono state paragonate alle loro velocità di sintesi.

Per l'rRNA la velocità di metilazione è strettamente proporzionale alla velocità di sintesi, così che il suo grado di metilazione è praticamente uguale a tutte le velocità di crescita, e la misura della velocità di metilazione permette quindi di determinare correttamente la velocità di sintesi dell'rRNA. Ciò non è vero per l'sRNA il cui grado di metilazione varia al variare delle condizioni di crescita.

INTRODUCTION

A large number of methylated nucleotides is found both in soluble and in ribosomal RNA [1]. The specificity of the reactions yielding methylated sRNA [2], the effects of the growth conditions on the extent and the pattern of RNA methylation [1, 3], the biological function of the methylated sRNA [1] are still open questions.

We were interested in the methylation of RNA because during studies on the kinetics of RNA formation in *Neurospora* [4] we found that in experiments of pulse and chase the labeling of RNA can be conveniently done using $^{14}CH_3$-L-Methionine as a precursor, while the use of radioactive bases or of ^{32}P-orthophosphate is much less satisfactory because the precursor concentrations that have to be used are very high, and the periods of time required for pool equilibration somewhat longer.

Being primarily interested in the nutritional control of RNA synthesis in *Neurospora* [4], we have examined the effects of the growth conditions on the methylation of ribosomal RNA and soluble RNA in *Neurospora* cells in exponential growth on different media. The results we present here indicate that in *Neurospora* the rate of RNA methylation is closely proportional to the rate of RNA synthesis for ribosomal RNA but not for soluble RNA.

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MATERIALS AND METHODS

a) Growth conditions. Mycelia were grown in minimal Vogel's mineral medium [5] with one of the following carbon sources added: 2% glucose, 40 mM acetate, 2 % glycerol or 2 % ethanol.

750 ml flasks containing 200 ml medium were inoculated with $2 \cdot 10^7$-$6 \cdot 10^7$ conidia of wild type *Neurospora crassa* 74 A. In the case of growth on glucose, acetate or glycerol the flasks were incubated overnight in a Dubnoff water bath at 16 °C with shaking at about 100 RPM [6]; after 15 h the temperature of the bath was raised to 30 °C and the growth allowed to continue at this temperature until A_{450nm} was about 0.3. In the case of the growth on ethanol the low temperature incubation was omitted and the flasks were incubated overnight at 30 °C.

The growth was monitored as increase in A_{450nm} and the constant of the rate of growth, K (h^{-1}) was determined as previously reported [6]. The number of duplications per hour (μ) was calculated from: K/ln 2.

b) Incorporation of radioactive methionine by the culture. When the A_{450nm} of the cultures was about 0.3, 1 μ mole of $^{14}CH_3$–L-methionine (specific activity 11.5 Ci/mole) obtained from New England Nuclear Corporation, was added to a 200 ml culture so that its final concentration was $5 \cdot 10^{-6}$ M [4].

This concentration has been previously reported [4] to be not rate limiting for methyl incorporation into RNA under the experimental condition used. Besides the methyl incorporation into RNA has been shown to be a linear function of time for at least 0.25 duplication times [4]. After a labeling period corresponding to 0.08 duplication times in each of the growth conditions (10 min for glucose, 12 min for acetate, 19 min for glycerol and 37 min for ethanol) the cultures were rapidly collected by filtration washed with cold water and quickly used for RNA estraction.

c) RNA purification and fractionation. RNA was extracted in the cold by the method previously reported employing the pH 7.4 buffer and a phenol-cresol mixture [4]. An aliquot of the purified RNA (about 10 A_{260nm} units) was layered on a 12 ml linear sucrose gradient (5–20 % *w/w*) prepared in 0.025 M Tris–Cl pH 7.4, 0.1 M NaCl, 1 μg/ml polyvinysulfate. The gradients were centrifuged at 1 °C at 24,000 RPM for 16 hrs in the SW 40 rotor or the Spinco L–2–65 B centrifuge, and fractionated on an ISCO density gradient fractionator connected to an UV analyzer. The acid–precipitable radioactivity of the fractions was determined as previously reported [4]. The top fractions of the gradient, containing sRNA, were deacylated by incubation at pH 8.8 [4] before the determination of the radioactivity.

d) Calculation of the rate of methylation of rRNA and of tRNA. The rates of methylation (expressed as number of methyl groups incorporated in rRNA or in sRNA, min^{-1}, per genome) were calculated as follows. The number giving the pmoles of methyl incorporated into 1 A_{260nm} unit of

RNA during a pulse 0.08 duplication times long was divided by the length (in minutes) of the period of labeling. The result was multiplied for the RNA/DNA (*w/w*) values characteristic of *Neurospora* cells in exponential growth in each medium [7] so as to obtain the pmoles of methyl groups incorporated, min^{-1}, per 1 A_{260nm} unit of DNA (45 μg of DNA) [8]. From this value, knowing the molecular weight ($2.3 \cdot 10^{10}$) of a haploid genome of *Neurospora crassa* [9], the number of methyl groups incorporated, min^{-1} per genome was determined.

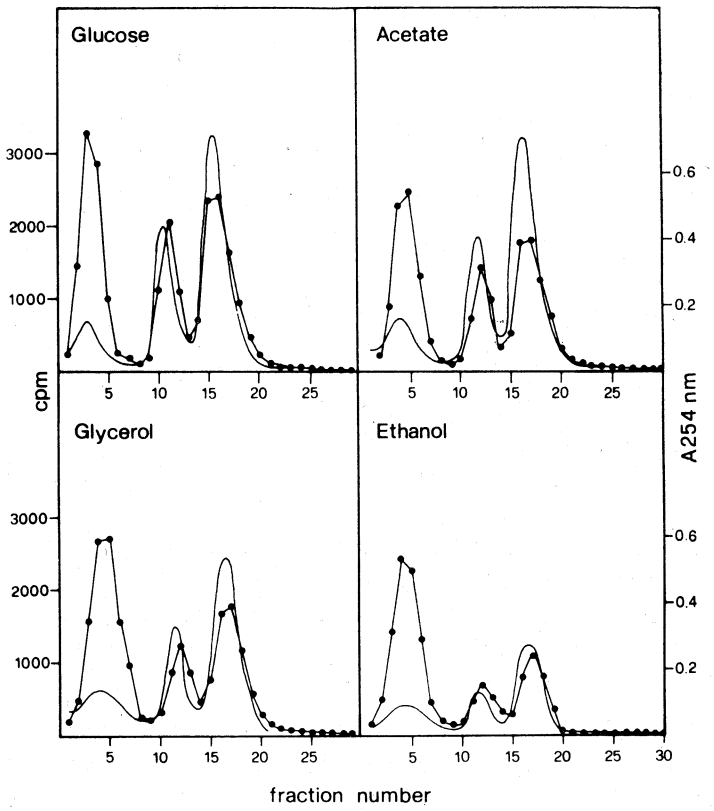

Fig. 1. – Sucrose gradient analyses of methyl-labeled RNA extracted from mycelia grown on different media. RNA was extracted from mycelia grown on glucose ($\mu = 0.51$), acetate ($\mu = 0.41$) glycerol ($\mu = 0.26$) or ethanol ($\mu = 0.13$), which were labeled for 0.08 duplication times (i.e. 10 min for glucose, 12 min for acetate, 19 min for glycerol and 37 min for ethanol), with L–$^{14}CH_3$–methionine ($5 \cdot 10^{-6}$ M, 11.5 Ci/mole). The direction of sedimentation is from left to right. —— Absorbance at 254 nm, ●—● cpm per fraction.

Results and discussion

Exponentially growing *Neurospora* cultures were labeled with $^{14}CH_3$-L-methionine under conditions which ensure, as indicated in the Methods, that the amount of radioactivity incorporated into RNA allows correct

TABLE I

Methylation of rRNA *and* sRNA *in* Neurospora *cells in different growth conditions*

Growth condition	Distribution of ^{14}C–methyl groups in stable RNA (*) pmoles $^{14}CH_3$ into rRNA	pmoles $^{14}CH_3$ into sRNA	Ratio $^{14}CH_3$ in sRNA/ $^{14}CH_3$ in rRNA
Ethanol	63.1	96.3	1.58
	60.6	96.6	1.59
Glycerol	60.8	52.4	0.86
	55.0	51.2	0.84
	57.0	50.3	0.92
Acetate	55.2	38.7	0.70
	55.0	39.0	0.71
Glucose	72.1	45.8	0.61
	74.7	47.4	0.63
	72.0	46.7	0.64

(*) These values give the pmoles of methyl groups incorporated during a pulse 0.08 duplication times long into 1 A_{260} units of RNA resolved by sucrose gradient into rRNA and sRNA (see Methods for details).

TABLE II

Rate of methylation and rate of synthesis of rRNA *and* sRNA *in* Neurospora *cells grown in different media*

Growth medium	rRNA			sRNA		
	Rate of methylation (*)	Rate of synthesis (**)	Extent of methylation (***)	Rate of methylation (*)	Rate of synthesis (**)	Extent of methylation (***)
Ethanol	1.24	1.4	0.89	1.97	0.46	4.40
Glycerol	2.75	3.3	0.83	2.5	1.16	2.19
Acetate	6.66	9.2	0.72	4.7	1.96	2.44
Glucose	12.5	15.2	0.82	8.15	1.84	4.52

(*) Expressed as methyl group (10^{-4}) incorporated, min^{-1}, per genome.
(**) Expressed as nucleotides polymerized (10^{-6}), min^{-1}, per genome [7].
(***) Expressed as methyl groups per 100 nucleotides.

calculation of its rate of methylation. The RNA was extracted with phenol and analyzed on sucrose gradients in order to resolve the different kinds of RNA. In fig. 1 the distribution of the radioactivity between the soluble and the ribosomal RNA is shown as it takes place in cells exponentially growing in different media. The sRNA peak is more heavily labeled in slow growing cells. To quantitate these results the counts of the fractions containing respectively rRNA or sRNA were added together and referred as indicated to a given amount of total RNA. In Table I the amounts of methyl groups which during the pulse were incorporated into rRNA and into sRNA by cells in different growth conditions are reported (column 1 and 2), as well as the ratio between these two values (column 3). These data clearly confirm that the distribution of the radioactivity between rRNA and sRNA is dependent upon the growth condition: the ratio between methyl groups incorporated into sRNA and methyl groups incorporated into rRNA considerably decreases by increasing the rate of growth, being about 1.6 in minimal ethanol ($\mu = 0.13$) and 0.6 in minimal glucose ($\mu = 0.51$).

The heavier methylation of sRNA observed at lower growth rates could be explained by the fact that, as has been shown [7] the sRNA represents an increasing fraction of the total RNA for μ lower than 0.5. To find out whether this was the case or the change in methylation reflects also a change in the extent of methylation, the rates of RNA methylation were estimated in all growth conditions and compared with the respective rates of RNA synthesis.

The rates of methylation of both rRNA and sRNA (expressed as methyl incorporated, min^{-1} per genome) were calculated as referred in the Methods and are reported in Table II (columns 1 and 4). Together are reported (columns 2 and 5) the rates of synthesis of rRNA and sRNA (expressed as nucleotides polymerized, min^{-1} per genome) in the four growth conditions, taken from a previous paper [7].

It appears that the extent of methylation of rRNA (column 3) is practically not affected by the growth conditions and that it is constantly about 0,8 methyl groups every 100 nucleotides polymerized, in good agreement with the values reported for rRNA of other species [1].

More complex appears to be the situation for sRNA, whose extent of methylation (column 6 of Table II) appears to be affected by the growth condition and fluctuates between 2.2 and 4.5 methyl groups every 100 nucleotides.

It follows that, while for rRNA the rate of methylation is strictly proportional to the rate of synthesis and therefore can offer an accurate way to measure its rate of synthesis, this is not true for the methylation of sRNA.

As for the varying extent of sRNA methylation in differently grown cells, it may be relevant to recall that extensive processes of cytodifferentiation have been shown to occur in *Neurospora* cells in exponential growth on different media [10] and that in several systems the composition of the tRNA population and its pattern of methylation seems to be affected by differentiation [11]. Thus we suggest that the relatively small changes in the extent of methylation of sRNA observed in *Neurospora* may depend upon differentiating events.

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