THE METHYLATION OF tRNA IN KB CELLS AFTER TREATMENT WITH ACTINOMYCIN D

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Received May 23, 1973

SUMMARY: Extensive shifts in the distribution of labeled methylated constituents of tRNA were observed in KB cells treated with actinomycin D for 30 min prior to a 90-min pulse with ^{3}H-CH_3-methionine. Although this treatment completely blocked the synthesis of tRNA, methylation continued to the extent of 12-15% of controls (pulsed without antibiotic). Under this condition the relative proportion of radioactivity incorporated into 3-methylcytosine, N^2-methylguanine and 2'-O-methylribose was markedly increased (170-235% of control values), it was moderately reduced in 1-methyladenine, 5-methylcytosine and 5-methyluracil (35-70% of controls) and markedly reduced in 1-, 7- and N^2N^2-methylguanines (15-30% of controls). These data suggest that specific types of methylations occur at particular times during the processing of pre-tRNAs.

INTRODUCTION: Evidence based on *in vitro* studies with heterologous tRNAs indicate that a number of tRNA methylases recognize specific sites of the tRNA molecule (1-6). Such specificity may account for the considerable uniformity in the location of particular methylated bases occupying discrete regions of sequenced tRNA molecules (7). For example, ribothymine is located exclusively in the TΨC loop, while 2'-O-methylguanosine occurs predominantly in the dihydrouridine (DHU) loop, although it is sometimes present at the 5'-end of the anticodon (7).

The present study represents an attempt to determine if the methylations that appear on newly synthesized tRNA *in vivo* are specific with regard to the time at which they are formed during the processing of pre-tRNAs. We have approached this question by comparing those types of methylations that occur during the last stages of pre-tRNA maturation with those that take place col-

lectively throughout the entire maturation process. To provide such a system for analysis, a 30-min actinomycin D-chase was employed initially to halt the synthesis of tRNA in KB cells and subsequently to chase the existing populations of pre-tRNAs into either fully matured forms or into their late stages of maturation prior to labeling with $^{3}H\text{-}CH_3$-methionine. The collective methylations of all maturation states were assessed by labeling in the absence of antibiotic.

EXPERIMENTAL: Exponentially growing KB cells (a strain of cultured human carcinoma cells) were harvested and resuspended in MEM spinner medium (GIBCO) at a density of 5×10^6 cells/ml and 10 ml aliquots preincubated at 37°C in a Dubnoff metabolic shaker maintained under 95% O_2:5% CO_2. After a 15-min preincubation, cells were pulsed with 100-500 μCi of ^{3}H-uridine (24 Ci/mM) or $^{3}H\text{-}CH_3$-methionine (5-9 Ci/mM) for 90 min, or treated with actinomycin D (Merck, Sharp and Dohme) for 30 min prior to initiating the 90-min radioactive pulse. Incubations were terminated by adding 20 ml of ice-cold MEM medium and the cells collected by low speed centrifugation and either processed immediately or stored overnight at -20°C.

Total RNA was extracted from cell homogenates with hot-phenol-SDS (8) and the tRNA purified by fractionation with 1.2 M NaCl (9) and isopropyl alcohol (10). Electrophoresis on 2.5 and 7.5% acrylamide gels indicated that the tRNA, prepared in this manner, was not contaminated with 28S and 18S rRNAs. The slight contamination with 5S RNA, which was sometimes observed, was of no consequence since it possesses no methylated constituents (11). At least 98% of the radioactivity of labeled tRNA preparations was present in the tRNA band of the gel. Specific activity determinations (Table I) were based on the above radioactivity measurements per μg of RNA applied to the

TABLE 1. EFFECTS OF ACTINOMYCIN D CONCENTRATION ON THE SYNTHESIS AND METHYLATION OF KB CELL tRNA.

Actinomycin D (μg/ml)	CPM/μg tRNA (% of Control)	
	^{3}H-CH_3-Methionine	^{3}H-Uridine
0 (control)	390 (100)	890 (100)
0.001	400 (103)	-
0.01	349 (91)	-
0.1	228 (59)	-
1.0	80 (21)	80 (9)
10.0	48 (12)	18 (2)
100.0	58 (15)	17 (2)

Labeling of KB cells, the isolation of tRNA and determination of its specific activity are described in experimental procedures and elsewhere (8). These values represent averages of two experiments.

gel.

Formic acid hydrolysis, as carried out by heating 1-2 OD_{260} units of tRNA dissolved in 0.5 ml of 90% formic acid in sealed pyrex tubes (1 x 30 cm) for 2 hr at 170°C (12), completely liberated the methylated bases without apparent destruction (unpublished observations). The hydrolysates were quantitatively transferred to test tubes and dried at 40°C under a stream of N_2. The residues were dissolved in 30 μL of a solution of formic acid containing 2-5 μg of the standard methylated bases listed in Table 2 and subjected to two-dimensional TLC (13). The adsorbent in the areas of the chromatogram occupied by the authentic methylated bases was removed for the determination of radioactivity. The 2'-O-ribose methylation was estimated by the loss of radioactivity (volatilized) during the hydrolysis and subsequent evaporation of another aliquot of the tRNA heated with 70% perchloric acid for 2 hr at 100°C (14).

RESULTS AND DISCUSSION: Since it has been reported (15, 16) that a rela-

TABLE 2. EFFECTS OF ACTINOMYCIN D ON THE DISTRIBUTION OF RADIOACTIVE METHYLATED CONSTITUENTS OF KB CELL tRNA[a].

Actinomycin D treatment	+	- (Control)
Methylated Constituents	% of Radioactivity Applied to TLC Plates	
1-methylhypoxanthine	ND[b]	1.2
1-methyladenine (1A)	3.4	5.5
2-methyladenine	ND	ND
N^6-methyladenine (MA)	5.0	8.7
N^6N^6-dimethyladenine	ND	ND
1A + MA = 1A[d]	8.4 (59)[c]	14.2
1-methylguanine	1.7 (29)	5.8
7-methylguanine	0.9 (18)	5.1
N^2-methylguanine	27.0 (188)	14.4
N^2N^2-dimethylguanine	2.1 (19)	11.3
3-methylcytosine	5.9 (169)	3.5
5-methylcytosine	12.0 (67)	17.9
3-methyluracil	ND	ND
5-methylruacil	3.6 (36)	9.9
2'-O-methylribose	40.7 (235)	17.3
CPM applied	3090	9400
CPM recovered (%)	102.3	100.6

[a]The labeling of KB cells and determination of the amount of radioactive methylated constituents of tRNA are as described in experimental procedures. ND[b] = not detected, i.e. less than 0.5% of applied radioactivity. ( )[c] = % of control value. 1A[d] During hydrolysis 1A is partially converted to MA (13); this is of no consequence since only 1A occurs in mammalian tRNA (17). These values represent averages of three experiments. Standard deviation (not shown) was less than ± 10% of each value listed.

tively large concentration of actinomycin D is required to inhibit the synthesis of tRNA, it was necessary to determine the level of antibiotic required for this purpose in our system. As shown in Table 1, actinomycin D in concentrations of 10 and 100 μg/ml effectively inhibited the incorporation of ^{3}H-

uridine into the tRNA. The small amount of label incorporated into tRNA was attributed to the turnover of the CCA terminus of tRNA, due to a partial conversion of ^{3}H-uridine to cytidine derivatives (15). Under these same conditions, the labeling of tRNA by ^{3}H-CH_3-methionine was reduced to 12-15% of control values. We have interpreted this incorporation to reflect those methylations that occur on pre-tRNAs during their late stages of maturation. By extending the actinomycin D-chase to 60 min, the incorporation of label was reduced to approximately 2% of control values (not shown) and indicated that the methylation of pre-tRNAs is completed under these conditions within 1 hr after synthesis. This is in agreement with the findings of Bernhardt and Darnell (16).

Analysis of the methylated constituents of tRNA preparations isolated from KB cells incubated with antibiotic (10 μg/ml) for 30 min prior to a 90-min ^{3}H-CH_3-methionine-pulse revealed extensive shifts in the distribution of ^{3}H-CH_3-groups incorporated into various methylated bases as well as into the 2'-O-position of the ribose moiety (Table 2). In all instances, the recovery of radioactivity as methylated constituents accounted for 95-105% of the radioactivity of the tRNA. It is our interpretation that the disproportionate increases in the radioactivity incorporated into 3-methylcytosine, N^2-methylguanine and 2'-O-methylribose of antibiotic-treated preparations, relative to controls, represents those methylations that take place predominantly during the later stages of pre-tRNA maturation. Similarly, the disproportionate decreases observed in 1-methyladenine, 5-methylcytosine and 5-methyluracil (moderately reduced) and in 1-, 7- and N^2N^2-methylguanines (markedly reduced) reflect those methylations that occur during the intermediate and early stages of maturation, respectively. It should be stressed that the terms "early", "intermediate" and "late" stages of pre-tRNA matu-

ration, as used in this communication, are defined solely on the basis of methylation and exclude all other types of modification that may occur.

It is possible that actinomycin D may act directly to inhibit specific methylase activities or, indirectly, by inhibiting the synthesis of mRNAs that code for rapidly turning-over methylases. However, preliminary results with the tRNA-methylating enzymes in 100, 000 x g supernatant fractions obtained from KB cells previously exposed to actinomycin D for 2 hr indicate that neither the rate of methylation of heterologous tRNA substrates in vitro nor the methylated products differ significantly from that obtained with control preparations.

It is of interest that a comparison of 23 sequenced tRNA molecules (7) indicates that the methylations classified here as "early", appear on or near the junctions of the three major loops; "intermediate", in the region of the TΨC loop, and "late", in the regions of the DHU and anticodon loops.

ACKNOWLEDGMENTS: The authors are deeply indebted to Dr. Maurice Green, St. Louis University Institute of Virology, for generously providing the KB cells used in this study and to Miss Janice Hummel and Miss Sharon Weber for excellent technical assistance. This work was supported by grants from the American Cancer Society (Institutional Grant IN-63I) and the U.S. Public Health Service (CA 13178).

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