[RNA Biology 3:1, 49-53, January/February/March 2006]; ©2006 Landes Bioscience

Research Paper

The Prokaryotic Origin of the Pathways for Synthesis and Post-Synthetic Modification of Deoxyribonucleic Acid

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Received 03/13/06; Accepted 04/11/06

Previously published online as a *RNA Biology* E-publication:
http://www.landesbioscience.com/journals/rnabiology/abstract.php?id=2794

KEY WORDS

evolution, N^6-methyladenine vs. 5-methylcytosine, deoxycytidylate and deoxyadenylate methyltransferases, type-I, type-II and type-III restriction-modification endonucleases

ABBREVIATIONS

CGC	culture growth cycle
M9	minimal medium
LB	Luria-Bertani medium
pfu	plac forming units
m^6A	N^6-methyladenine
m^5C	5-methylcytosine
dam	deoxyadenylate
dcm	deoxycytidylate
RM	restriction-modification
DNAmet	DNA methyltransferase
AdoMet	S-adenosyl-L-methionine
[^{14}C]Met	[^{14}C]methyl-L-methionine

ACKNOWLEDGEMENTS

Sincere gratitude is expressed to Tamilla Eremenko, of the former Institute of Experimental Medicine, CNR, Rome, for her advice and help in providing a number of facilities for this study. Many thanks are also due to Enrico Bertoli, of the Institute of Biochemistry of the University of Ancona, for his valuable help. The investigation was supported by the University of Rome "Tor Vergata", the University of Ancona, the Ministry of the University and Scientific and Technological Research and the National Research Council.

ABSTRACT

A previous study showed that in mammals the pathways leading to synthesis and post-synthetic modification of DNA employ methionine as common donor of atoms: the carbon coming from the methyl group of this amino acid is needed for replication; its entire methyl group is needed to build m^5C on semiconservatively newly replicating chains. This work showed that the two pathways originate in bacteria where an enzymatic system forms, on DNA, m^6A in addition to m^5C. The formation rate of m^6A gradually decreased during the bacterial CGC, while that of m^5C reached an optimum in its middle. This shift suggested that the *dcm* and *dam* methyltransferase activities, as well as the activities of the methyltransferase moieties of the RM enzymes, are uncoupled.

INTRODUCTION

Evolution of living matter is accompanied by that of post-synthetic modification of DNA, implying the formation of two classes of DNA methylated bases: m^6A and m^5C in bacteria,[1] m^6A, but not m^5C, in ciliates[2] and m^5C only in mammals.[3] In concomitance with the disappearance of the "archaic" systems combining *dcm* and *dam* DNAmets[4] and RM endonucleases,[5-7] both characterizing the bacterial world,[8] molecular selection led to the loss, in vertebrates, of m^6A not only in nuclear[9] but also in mitochondrial[10,11] DNAs. The m^5C was preserved in these two DNA species by a novel methyltransferase protein family likely generated by alternate splicing.[12-14] In bacterial DNA, while the presence of m^6A was shown to be due partly to the activity of the *dam* DNAmets[4,15,16] and partly to the activity of type I, type II and type III methylating RM endonucleases involved in the digestion of the infecting m^6A-free phage DNA,[5-7,17] the presence of m^5C was shown to be due in part to the activity of the *dcm* DNAmets[4,18,19] and in part to the methyltransferase activity of the sole type II RM system involved in the digestion of the infecting m^5C-free phage DNA.[5] The m^5C in bacterial DNA was related not only to the RM anti-phagic defense.[20-22] By complementing the induction-repression control,[23] to some extent it was also associated with an auxiliary regulation of transcription.[24] Such an association was in harmony with the idea that, in the genomic DNA of mammals, the well documented concentration of m^5C in the promoters and introns[9,25,26] would be involved in a mechanism modulating gene expression.[9,26-31]

This research pointed to two main purposes. The first was an attempt to verify whether bacteria are able to use methionine as common donor of atoms for a convergence of DNA synthesis and methylation, as mammalian cells do.[9,32,33] The second was an effort to verify whether, in bacterial DNA, the timing for optimal formation of the m^5C residue coincides with that for optimal formation of the m^6A residue.

MATERIALS AND METHODS

Bacterial growth. *E. coli* MRE 600 cultures were grown on *Petri* dishes by inoculating 0.5–1 ml of M9 (with 1.10^{11} cells/ml) into 100 ml of LB. The M9 contained 65 g $Na_2HPO_4.7H_2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_3, 20 ml 20% glucose, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 50 mg/ml thyamine, in 1 liter of water, adjusted at pH 7.5 with 10 N NaOH. The LB contained 10 g Bacto trypton, 5 g Bacto yeast extract and 10 g NaCl, in 1 liter of water, also adjusted at pH 7.5 with 10 N NaOH. At 30-min intervals through the CGC, portions of suspension were taken and adequately diluted with 9 g/liter NaCl to detect the corresponding bacterial density. This was estimated in arbitrary units of OD by measuring the absorbance of the samples at 600 nm in a Beckman spectrophotometer.

DNA Labelling. At intervals of time during the CGC, the DNA carried by 1.10^8–1.10^{10} bacteria in a 2-ml LB suspension was labelled with 2 µCi [^{14}C]Met for 40 min at 37°C. A small number of bacteria (harvested at 6,000 rev./min for 5 min and resuspended in 1 ml of 15 g/litre NaCl) were tested to verify the entity of incorporation of radioactivity into a crude DNA extract. This was precipitated on 0.45 µm Millipore filters (HAWP) with 100 µl of 12% trichloroacetic acid. The yielded radioactivity was measured in a Packard Tri-Carb 2100 TR radiospectrometer, after dissolution of the filters in the Quicksafe N liquid scintillator.

DNA extraction. The ^{14}C-labelled DNA was purified with minor modification of the original method described by Marmur.[34] A 100-mg bacterial pellet was washed through centrifugation at 6,000 rev./min for 10 min with 9 g/liter NaCl and resuspended in 0.8 ml of solution A (50 mM Tris-HCl at pH 8, 50 mM EDTA and 15% sucrose) to which one added: 18 µl of 10 mg/ml lysozyme (for 10 min); 1.6 µl of 10 mg/ml deoxyribonuclease-free RNAse A (for 1 hr at 37°C); 1.2 ml of solution A; 40 µl of 10% sodium dodecylsulphate (for 20 min at room temperature and for 5 min at 70°C); 2 µl of 20 mg/ml proteinase K (for 4 hrs at 42°C). Then, the mixture was treated with 1 ml of TE buffer (10 mM Tris-HCl at pH 7.4 plus 1 mM EDTA) and 1.2 ml of liquid phenol. After centrifugation at 7,000 rev./min for 20 min, the upper aqueous layer was collected and the lower phenolic layer was treated again with 2 ml of TE buffer at pH 7.4 and recentrifuged at 7,000 rev./min for 20 min. The upper aqueous layer was added to the one collected earlier. Some other liquid phenol (1.3 ml) was added to the unified supernatants. A last centrifugation at 7,000 rev./min for 20 min completed the elimination of proteins (which remained in the phenolic layer). An 1/10th volume of 3 M sodium acetate at pH 5.2 and 3 volumes of 100% ethanol were added to the new aqueous supernatant. The DNA, sedimenting at the water/ethanol interphase, was collected with a glass rod and washed four times with 1 ml of 75% ethanol and once again with 1 ml of 100% ethanol. Finally, DNA was left to dry and resuspended in 50–100 µl of water. By diluting 1:200 again with water, its yield was measured considering that 1 OD at 256 nm corresponded to 50 µg. The purity of the recovered DNA was estimated on the basis of the ratio of the ODs at 260 and 280 nm (ranging between 1.7 and 1.9), the spectral properties and the absence of U in the HPLC hydrolysate.[33]

DNA hydrolysis. A 30-µg aliquot of purified DNA was introduced into a glass vial containing 300 µl of 88% formic acid (75% final concentration). The vial was closed with flame and placed in a dry block at 160°C for 1 hr.[33] The hydrolysed residues were dried, suspended in 30 µl of 20 mM trifluoroacetic acid at pH 2.2 and run at 13,000 rev./min for 5–10 min in an Eppendorf centrifuge.

HPLC analysis. A 20-µl aliquot of hydrolysed DNA material was percolated through the 131051 L ODS-55 Biorad Bio-Sil HPLC column (250 x 4 mm) in reverse phase condition (solid-liquid distribution chromatography).[35] This type of analysis provided two kinds of information:[33] the first concerned the amount of each separated base (in terms of ODs detected in arbitrary units at 256 nm); the second concerned the level of radioactivity incorporated by a base (expressed in cts./min). The Beckman System Gold instrument was used. This was combined with the 125 model part (distributing the solvent), the 166 model detector, the software IBM PS/255SX and the 7725 model Rhyodyne injector. All solutions were filtered through a nylon membrane, with pores of 0.22 µm, and degassed before use. Because of their small volume, the samples were harvested at 12,000 rev./min for 12 min. In elution, the buffer A (0.05 M KH_2PO_4 at pH 4.4) and the buffer B (0.05 M KH_2PO_4 at pH 4.4 plus 20% methanol) constituted the movable phase. The flow rate was 0.8 ml/min and the general conditions were the following: the system remained in the zero per cent buffer B for 10 min; after 20 min, the system reached 100% buffer B; after 5 min, the system returned to the initial condition. The detector was adjusted at 254 nm. Fractions of 0.4 ml were collected every 30 sec. They were dried totally, resuspended in 50 µl of water and tested for radioactivity. To each sample 1 ml scintillator (Pico Fluor 40) was added. The radioactivity of the fractions was read for 3 min in a Packard Tri-Carb TR β-counter.

Phage infection. For titration of *phage*λ and determination of the corresponding pfu, the KL16 strain of *E. coli* was used. Five ml of a saturated culture (with about 1.10^{10} cells/ml) was centrifuged at 5,000 rev./min for 10 min. The sedimented bacteria were resuspended in 2.5 ml of a 10 mM $MgSO_4$ solution. One hundred microliters of this suspension were mixed with 4–8 µl of a phage suspension diluted 1:1,000,000 with 10 mM $MgSO_4$. The phage suspension had as original host, the GC57 strain of *E. coli*. To renew it, bacteria were grown in LB at 30°C. Five ml of their suspension was centrifuged at 5,000 rev./min for 10 min and resuspended in 2.5 ml of 10 mM $MgSO_4$. Hence, 50 µl of bacterial suspension (1.10^9 cells/ml) were mixed with 5 µl of the phage suspension to be renewed. This was previously diluted 1:1,000 with the buffer λ (10 mM Tris-HCl at pH 8 and 10 mM $MgSO_4$). After an incubation of 5 min at 30°C, 2 ml of LB with 10 mM $MgSO_4$ were added to the mix. The incubation continued for 6 hrs at 37°C. At the end of this time, 0.1 ml chloroform was added. The mixing lasted 15 min, in ice, followed by a centrifugation at 13,000 rev./min for 10 min. Half milliliter of the supernatant was transferred into an Eppendorf tube. A drop of chloroform was added. The phage stock solution was stored at 4°C in the dark. The bacterial culture infected with phage was maintained at room temperature for 5 min. Three ml of 7 g/liter "LB soft agar" (still melted) and 30 µl of 10 mM $MgSO_4$ solution were added. Following a vigorous shaking, the mix was poured in a *Petri* dish containing the solidified LB. One hundred µl of bacterial suspension, taken at various times from the inoculum, were mixed with equal portions of the phage suspension previously diluted 1:1,000,000. After an incubation of about 12 hrs, at 37°C, the number of formed colonies was recorded (considering that one of them originated from a single bacterium). The maximal density of phage population corresponded to $1.3.10^{11}$ pfu/ml.

RESULTS

Proliferation-dependent DNA Yield. In M9, the CGC of *E. coli* MRE 600, as that of *E. coli* KL 16, was represented by a sharp sigmoidal curve developing in about 5 hrs, since the average length of its *lag*, *logarithmic* and *stationary* stages lasted 1, 3 and 1 hrs, respectively (Fig. 1A). In LB, the CGC of the *E. coli* MRE 600, also as that of *E. coli* KL 16, was decelerated lasting about 10 hrs (Fig. 1B). At the end of proliferation, while the density of the MRE 600 strain reached 3.5 OD_{600nm}/ml in M9 and 1.3 OD_{600nm}/ml in LB, that of the KL 16 strain reached 2 OD_{600nm}/ml in M9 and 1.2 OD_{600nm}/ml in LB. The yield of DNA extracted in mid-CGC of the MRE 600 strain corresponded to $1.4.10^{-5}$ nmoles/1.10^9 bacteria if their culture was maintained in M9 and to 2.10^{-4} nmoles/1.10^9 bacteria if their culture was maintained in LB.

Separation of unmethylated and methylated bases from a DNA hydrolysate. When percolated through HPLC, control bases, mixed in equimolar proportions (4 nmoles) in 20 µl of buffer A, were eluted in the following time-order: C, m^5C, G, T, A and m^6A (Fig. 2A). This order corresponded to that of elution of bases present in the DNA hydrolysate obtained from *E. coli* MRE 600 grown in M9. However, in this case, there was a striking difference in the molar proportions of the eluted unmethylated vs. methylated bases: C, G, T and A essentially resulted in equimolar proportions, as expected; m^5C and m^6A, compared to C and A (Fig. 2B), oscillated around molar values of the order of 1 and 2%, respectively, in agreement with the literature.[1]

Proliferation-dependent development of DNA synthesis and methylation. Figure 2C showed the HPLC profile of a DNA hydrolysate prepared in mid-CGC of *E. coli* MRE 600 kept in LB and treated with [^{14}C]Met also in LB. Such a profile revealed that, as for mammals,[9] C was the only base not incorporating the labelled carbon coming from [^{14}C]Met, since this carbon did not enter the pyrimidine ring. By contrast, m^5C was labelled to a notable extent, comparable to that of m^6A. The radioactivity found in the two modified bases meant that post-synthetic DNA modification took place, as for mammals,[9,26] by virtue of transfer, via AdoMet, of the whole radioactive methyl group from [^{14}C]Met to the DNA Cs and As. In addition, in Figure 2C, the radioactivity found in the A, G and T bases revealed that, once again, as for mammals,[9,26] DNA synthesis occurred because there was, via C_1-chain oxidation, an insertion of the labelled carbon coming from the methyl group of [^{14}C]Met into the purine heterocycle of A and G and into the -CH_3 of T.

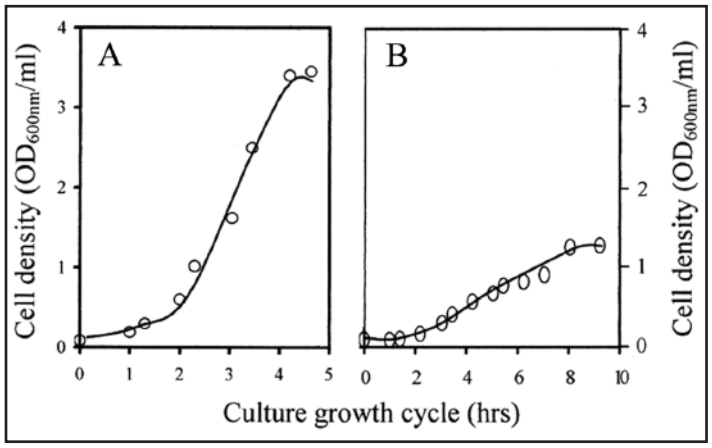

Figure 1. The CGC development of the MRE 600 strain of *E. coli* depended on the type of cultural medium used. The bacterial proliferation, detected from the time of inoculum, assumed a sigmoidal shape in 5 hrs in M9 (A) and in10 hrs in LB (B). The values represent the mean of several experiments.

The quantitative analysis of the data emerging from the experiments made at 1 to 9 hrs from inoculum (in the same conditions of Fig. 2C) showed that the synthetic and methyltransferase pathways of DNA depend upon the bacterial CGC in a differential fashion. (i) The specific labelling accounting for synthesis of A, G and T (Fig. 3B) appeared to be much lower than that accounting for construction of m^5C and m^6A (Fig. 4A and B). This meant that the methyltransferase pathway develops faster than the synthetic one. (ii) The specific labelling of T was always lower than that of A and G (Fig. 3B), since—one thought—the exit of the C_1-chain, oriented towards synthesis of A and G, is highly facilitated in comparison with the one oriented towards synthesis of T. (iii) The specific labelling of m^5C was much lower than that of m^6A (Fig. 4A and B), probably because the *dam* methyltransferase enzyme system works faster than the *dcm* methyltransferase enzyme system. (iv) In harmony with the development of the specific labelling of all bases taken together (Fig. 3A) and of the specific labelling of single A, G and T (Fig. 3B), the specific labelling of m^6A gradually decreased during the CGC (Fig. 4A), while that of m^5C was minimal after the inoculum and increased, with a sharp peak, in mid-CGC (Fig. 4B). Finally, during the CGC, the time-dependent gradual decrease of the synthetic rate of the single A, G and T bases (Fig. 3B) was inversely proportional to the S-shaped increase of the bacterial population density (Fig. 1B). In this respect, the rates of construction of m^5C and m^6A along the DNA heteroduplex appeared to diverge: on the one hand, the rate of the A methylation slowly decreased against the increase of the bacterial density; on the other, the rate of the C methylation reached a maximal value in mid-CGC and then it decreased. In any case, the *phage* λ was always able to lead the bacteria, adapted to the growth in LB, to a lysis.

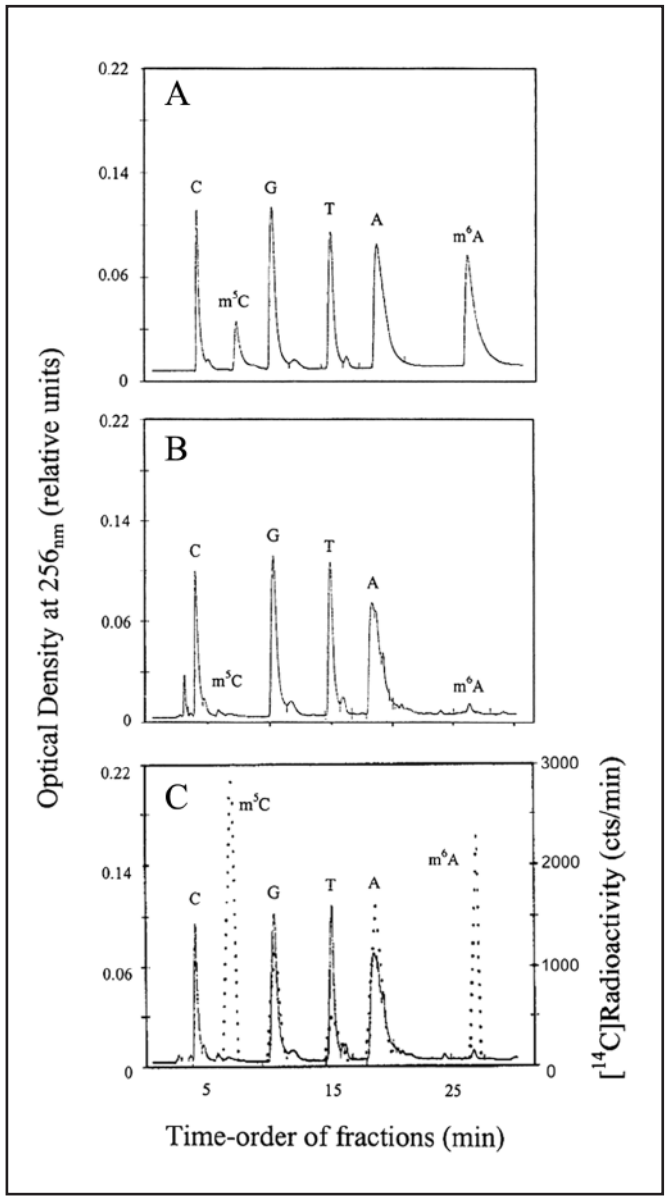

Figure 2. The DNA hydrolysates from the MRE 600 strain of *E. coli* were analyzed through HPLC. (A) The first control chromatography used free bases from a standard solution. (B) The second control chromatography separated the bases present in 30 µg of unlabelled DNA hydrolysate extracted from a 2-ml suspension of 1.10^8–1.10^{10} bacteria maintained in M9 and taken in their mid-CGC. (C) The experimental chromatography separated the bases present in 30 µg of ^{14}C-labelled DNA hydrolysate obtained from a 2-ml suspension of 1.10^8–1.10^{10} bacteria maintained in LB and taken in their mid-CGC. The left ordinate shows the ODs of bases detected in arbitrary units by a computerized instrument (continuous line); the right ordinate shows their radioactivity (dotted line). The experiment was repeated several times.

DISCUSSION

The experiments here described demonstrated that the two pathways, leading to DNA synthesis and methylation, originated in the bacterial cell by exploiting methionine as an universal donor of a carbon atom and of a methyl group (Fig. 5). This anabolic model was crucial in the evolution of the genome functions. It was preserved by the eukaryotic cell, although at the level of the DNA modifying enzyme bifurcation one of the two branches, the one yielding m^6A, was suppressed in mammals.[9,25] Actually, in both bacterial (Fig. 2C) and mammalian[9,32,33] cells, the carbon coming from the $-CH_3$ of methionine proved to arrive via formic acid into the $-CH_3$ of T and into the aromatic heterocycle of A and G (to be incorporated by the semiconservatively newly replicating DNA sequences), whilst the whole $-CH_3$ of methionine proved to construct on these sequences, via AdoMet, m^5C and m^6A in bacteria (Fig. 2C) and m^5C in mammals.[9,32,33]

In this framework, the question concerning the speed of the reactions in the pathway leading to DNA synthesis vs. that of the reactions in the pathway leading to post-synthetic DNA modification assumed great interest. Figure 3b showed that, during the *E. coli* CGC, the rate of formation of T was always lower than that of A and G, i.e., the time required by the carbon from the $-CH_3$ of methionine

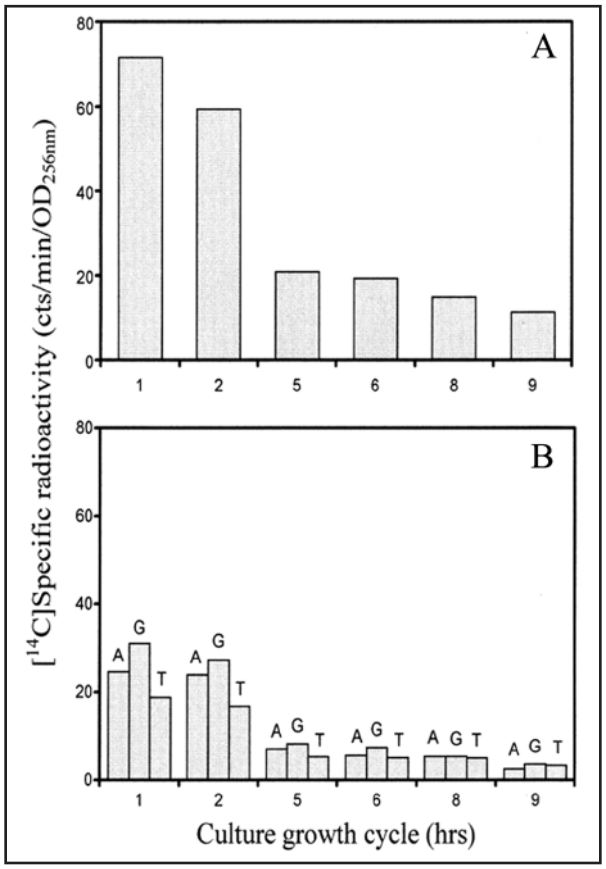

Figure 3. The kinetics of DNA synthesis in the MRE 600 strain of *E. coli* varied during its CGC. Parallel bacterial suspensions were maintained for 10 days in LB. Their volumes, used at the hrs indicated, were 10 ml (with 1.10^7 cells), 10 ml (with 1.10^7 cells), 3 ml (with 1.10^8 cells), 2.1 ml (with 1.10^{10} cells), 2.1 ml (with 1.10^{10} cells) and 2.1 ml (with1.10^{10} cells), respectively. All samples were similarly ^{14}C-labelled, while the specific radioactivity of the fractions eluted from the HPLC culumn was estimated as for Figure 2C. (A) The specific radioactivity shown by an entire 30-µg DNA hydrolysate before being chromatographed (incorporated from [^{14}C]Met) gradually decreased during proliferation. (B) A corresponding proliferation-dependent decrease characterized the specific radioactivities shown by the chromatographically separated single bases. The values represent the mean of three experiments.

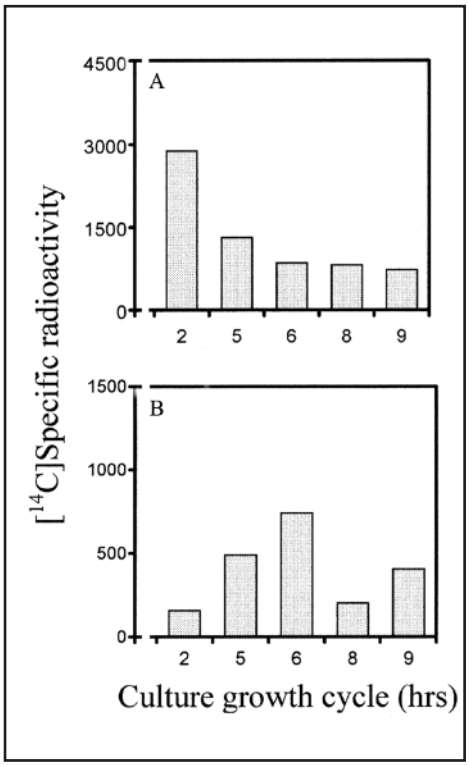

Figure 4. The kinetics of DNA methylation in the MRE 600 strain of *E. coli* varied during its CGC. The conditions were those of (Figs. 2c and 3b). (a) The specific radioactivity of m^{6}A, incorporated from [^{14}C]Met, gradually decreased as a function of the bacterial proliferation. (b) The specific radioactivity of m^{5}C, also incorporated from [^{14}C]Met, showed a maximal value roughly in the middle of bacterial proliferation. The ordinate shows the ratios of cts./min carried by m^{6}A or m^{5}C over 30 µg of DNA hydrolysate percolated through HPLC (the entity of the label of carbons 2 and 8 in the purine heterocycle of m^{6}A was irrelevant with respect to that of carbon in its methyl group, since in (Fig. 2c) the proportion of specific radioactivity shown by A was much lower than that shown by m^{6}A whereas the OD corresponding to the molar proportion of m^{6}A was much smaller when compared to that of A). The values represent the mean of three experiments.

to enter the -CH_3 of T was longer when compared to the time required for its insertion into the aromatic heterocycle of A and G. This differential rate was in harmony with the fact that, in *HeLa* cells, even if the synthesis of T was, quantitatively, of the same order of that of A, a striking delay of its optimum with respect to that of synthesis of A and G occurred during the S-phase.[32,33] Such a correspondence was expected, since in both *E. coli* and *HeLa* cells the process leading to synthesis of T had to join in a pyrimidine ring carbamylphosphate and aspartate before accepting the -CH_3 (constructed with the carbon coming from formic acid) at the position 5 of U. This atom entered directly the A and G purine heterocycle. The longer time required by AdoMet to enter the nucleus justified the advantage of the bacterial cell over the mammalian cell in exploiting the methionine -CH_3: if in *E. coli* the specific building of A, G and T (Figs. 2C and 3B) was much lower than that of m^{5}C and m^{6}A (Figs. 3 and 4), in *HeLa* the specific building of A, G and T turned out to be either similar or only slightly lower than that of m^{5}C.[32,33]

In conclusion, the present investigation has answered to its main points. The first purpose was that of unequivocally demonstrating how the bacterial world used methionine to promote the pathways for synthesis and post-synthetic modification of DNA at the same time. This capability was inherited without major changes in evolution, with the exception that the reactions, forming the m^{6}A residue in the DNA, were maintained in lower eukaryotes[2,37] and plants[2,37] but completely suppressed in higher animal cells.[9,25] The second purpose was that of showing how methylation of the DNA C and A residues are uncoupled as a function of the bacterial CGC. This is a crucial issue in studies concerning the mechanisms and features of DNA synthesis and methylation in prokaryotes.

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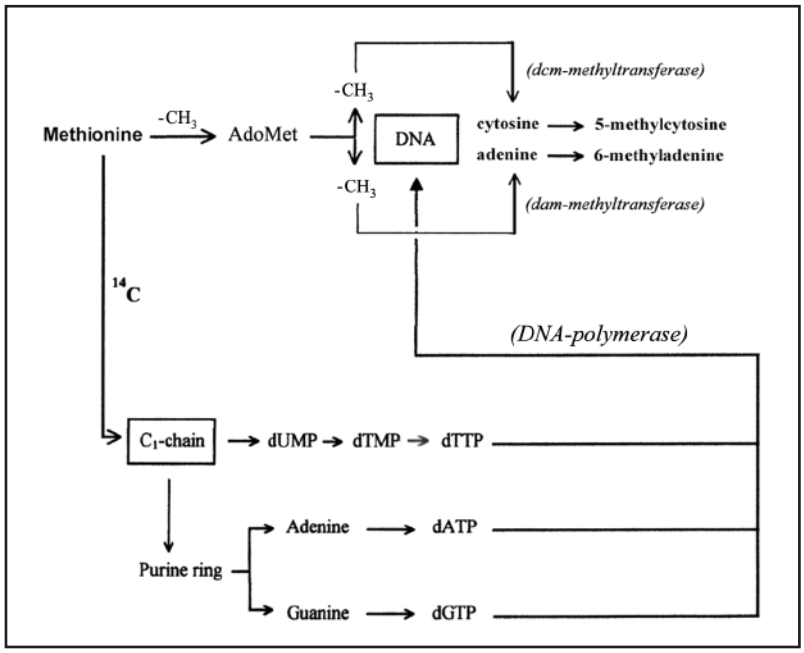

Figure 5. The pathways of synthesis and methylation of DNA proceed in parallel in bacteria. To accomplish DNA replication, the DNA polymerase system exploits the dATP, dGTP and dTTP deoxyribonucleoside triphosphates resulting from the C_1-chain oxidation which uses, via formic acid, the carbon coming from the -CH_3 of methionine to build the -CH_3 of T and the purine heterocycle of A and G (this carbon cannot enter the pyrimidine ring of C). In addition, the bacterial cell, via AdoMet, exhibits two parallel pathways for DNA post-synthetic modification: on the one hand, the *dcm*-methyltransferase system (including the methylation function exerted by type II RM endonucleases) constructs m^5C along the semiconservatively newly replicating DNA chains; on the other, the *dam*-methyltransferase system (including the methylation function exerted by type I, II and III RM endonucleases) along these chains constructs m^6A.

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