Proc. Nat. Acad. Sci. USA
Vol. 69, No. 1, pp. 187–190, January 1972

Methylation of Cytosine Residues in DNA Controlled by a Drug Resistance Factor

(host-induced modification/R factors/N^6-methyladenine/5-methylcytosine)

STANLEY HATTMAN, EDWARD GOLD, AND ALAN PLOTNIK*

Department of Biology, University of Rochester, Rochester, New York 14627

Communicated by S. E. Luria, November 8, 1971

ABSTRACT **The proportion of 5-methylcytosine (5MeCyt) and 6-methylaminopurine (N^6-methyladenine, 6MeAde) in bacteriophage P22 DNA was analyzed as a function of the host-specificity the phage carried. In the DNA of P22 grown in strains harboring the modifying drug-resistance-transfer-factor N-3, the 5MeCyt content was at least twice that after growth in strains lacking the factor. In contrast, the 6MeAde level of P22 DNA was unaffected by the presence or absence of the factor. The 6MeAde and 5MeCyt levels were unaffected by factors 222 and N-1, which do not modify phage DNA.**

The 5MeCyt/6MeAde ratio was only slightly higher in the DNA of *Salmonella* strains that had received the N-3 factor. After transfer of the N-3 factor to *Escherichia coli* strain B, which normally lacks 5MeCyt, a high content of 5MeCyt is observed. We conclude that the N-3 factor controls a DNA methylase specific for cytosine residues. If the N-3 host specificity is imparted by cytosine methylation, this would be the first instance where a biological role for 5MeCyt has been elucidated.

The amount of 6-methylaminopurine (N^6-methyladenine, 6MeAde) in bacteriophage P22 DNA is affected by the host cells in which the virus is grown (1). P22 grown in cells unable to confer LT-host specificity (2-4) contained roughly half as much 6MeAde (1), an indication that loss of ability to methylate certain adenines on the viral DNA leads to loss of the LT-specificity. These data contrast with those on other bacteriophage modification systems, in which methylation also appears to be involved, but where no gross changes in 6MeAde have been correlated with changes in host specificity (5–9).

Besides the LT modification, P22 DNA can carry specificities determined by drug-resistance-transfer-factors (10–14). The N-3 factor, which determines a specific restriction-modification system, as well as other R-factors that do not modify or restrict P22, do not affect the 6MeAde content of P22 DNA (1). We report here an N-3-directed increase in the 5-methylcytosine (5MeCyt) content of P22 DNA. Analysis of the methylated bases in host cell DNAs indicates that the bacterial DNA also is subject to the N-3-determined increase in 5MeCyt. We conclude that the N-3 drug-resistance factor controls a DNA methylase specific for cytosine.

MATERIALS AND METHODS

Phage and Bacterial Strains. P22 was obtained from Dr. K. Sanderson; a spontaneous clear-plaque-forming mutant was isolated and used for all the studies described. *Salmonella typhimurium* strains and their relevant properties are listed in Table 1; this table is reproduced in part from ref. 1. It is known that the N-3 factor modifies and restricts phages P22 and λ when harbored by strains of *Salmonella* and *Escherichia coli*, respectively (10, 12, 14). *E. coli* strain Bc251 $r_B^+m_B^+$-*mal*$^+\lambda^s$ (15) was obtained from Dr. J. Wiberg; *E. coli* B834 $r_B^-m_B^-$*met*$^-$*gal*$^-$ [derived from Bc251; (ref. 16)] was obtained from Dr. H. Revel; the R-factors were transferred to *E. coli* strains in our laboratory. The relative plating efficiencies of P22 grown and tested on various host cells is summarized in Table 2.

Media and Chemicals. [2-^{3}H]Adenine (Schwarz), [2-^{14}C]-cytosine (Schwarz), L-[methyl-^{3}H]methionine (Amersham/-Searle), 6-methylaminopurine (Calbiochem), and 5-methylcytosine (Mann) were obtained from the firms indicated in parentheses. Minimal media and broth were described (17).

Determination of 6MeAde Content. Growth and purification of labeled phage, isolation and hydrolysis of DNA, and paper chromatography were described (1, 17).

Determination of 5MeCyt Content. Bacteria were grown to about 3×10^8 cells/ml at 37°C in minimal medium and infected with 5–10 phage/cell. After 5 min of adsorption, 5 ml

TABLE 1. *Salmonella strains and relevant properties*

Strain*	Host-specificity† phenotype	Drug resistance‡ markers on R-factor
LT2 wild type	$r_{LT}^+m_{LT}^+$	
LT2 (222)	$r_{LT}^+m_{LT}^+$	$Tc^rSm^rSu^rCm^r$
LT2 (N-1)	$r_{LT}^+m_{LT}^+$	$Tc^rSm^rSu^r$
LT2 (N-3)	$r_{LT}^+m_{LT}^+r_{N-3}^+m_{N-3}^+$	$Tc^rSm^rSu^r$
LT2 *pro* C90 *fer*	$r_{LT}^-m_{LT}^-$	
LT2 *pro* C90 *fer* (N-3)	$r_{LT}^-m_{LT}^-r_{N-3}^+m_{N-3}^+$	$Tc^rSm^rSu^r$
LT7 *leu* 119 *fer* Sm^r	$r_{LT}^-m_{LT}^-$	
LT7 *leu* 119 *fer* Sm^r (N-3)	$r_{LT}^-m_{LT}^-r_{N-3}^+m_{N-3}^+$	$Tc^rSm^rSu^r$

* The sources for these strains are given elsewhere (1). The $r_{LT}^-m_{LT}^-$ strains carrying the N-3 factor were prepared in this laboratory. Strains harboring the 222-factor have lost all the drug resistance markers except Tc^r; nevertheless, the strains still retain the ability to transfer the factor to other bacteria.

† The terminology for host specificity is that proposed earlier (4). Thus, for example, r_{LT} and m_{LT} refer to LT-specific restriction and modification, respectively.

‡ Tc = tetracycline; Sm = streptomycin; Su = sulfanilamide; Cm = chloramphenicol.

* Present address: Mt. Sinai School of Medicine, New York N.Y. 10029

TABLE 2. *Efficiency of plating of phage P22 grown and tested on various host strains* †*

Phage	Host strain $r_{LT}^+m_{LT}^+$	$r_{LT}^+m_{LT}^+$ (N-3)	$r_{LT}^-m_{LT}^-$	$r_{LT}^-m_{LT}^-$ (N-3)
P22·$r_{LT}^+m_{LT}^+$	1.0	9×10^{-4}	0.3–0.8	2.5×10^{-3}
P22·$r_{LT}^+m_{LT}^+$ (N-3)	1.0	1.0	0.7	1.0
P22·$r_{LT}^-m_{LT}^-$	2×10^{-5}	10^{-8}	1.0	5×10^{-4}
P22·$r_{LT}^-m_{LT}^-$ (N-3)	2×10^{-5}	2×10^{-5}	1.3	1.0

* Bacteria grown to saturation overnight in broth were used as indicators.

† Strains carrying the 222 or N-1 factor do not appreciably restrict/modify P22 phage (12).

was removed to flasks at 37°C supplemented with 1–2 μCi/ml [^{14}C]cytosine, 1 μg/ml of cytosine, and 50 μg/ml of thymidine. Lysis and further purification of the labeled phage DNA were as before (1, 17). After phenol extraction and alcohol precipitation, the DNA was hydrolyzed for 60 min at 100°C in 0.1 ml of 70% perchloric acid. The hydrolyzate was neutralized by the addition of H_2O and KOH; $KClO_4$ was removed by centrifugation. After several cycles of drying (under an air stream at 37°C), suspension (in 10% 2-propanol–10% acetic acid) and centrifugation, most of the $KClO_4$ was removed. Descending paper chromatography was performed in 2-propanol-concentrated HCl–H_2O 65:17:18. Authentic 5MeCyt and Cyt markers were included for each hydrolyzate. The 5MeCyt and Cyt regions were identified, cut out, and eluted into scintillation vials by successive washes with 0.1 N HCl (at least 99% recovery was attained, as measured by counting the label remaining in the paper). The eluates were dried and resuspended, and the 5MeCyt regions were chromatographed under the same conditions as above. Generally, about 1–2% of the original cytosine label overlapped into the 5MeCyt region; thus, after the second chromatographic run, there was less than 0.04% contamination of Cyt label in the 5MeCyt region. After rechromatography of 5MeCyt, the paper was cut into measured strips and the ^{14}C was eluted and counted; the 5MeCyt was again identified by means of its ultraviolet absorbance. The ^{14}C in cytosine was determined by directly counting the eluate from the first chromatographic run; in initial experiments, rechromatography of this material revealed ^{14}C only in cytosine.

Determination of 5MeCyt/6MeAde Ratio. Bacteria were grown in minimal medium to about 3×10^8 cells/ml and infected, if desired, for 5 min at 37°C; 5 ml of cell suspension was removed to flasks containing [methyl-^{3}H]methionine (10 μCi/ml), methionine (4 μg/ml), and a mixture of adenine, uracil, and thymidine (40 μg/ml each). After 120 min of incubation, the phage from infected cells was purified and the DNA was isolated (1, 17); uninfected cells were washed by centrifugation and extracted with phenol–Sarkosyl (17). After alcohol precipitation, the nucleic acids were suspended in 10 mM Tris·HCl (pH 7.2), 5 mM NH_4Cl, 10 mM Mg(acetate)$_2$, and 150 mM NaCl, and treated for 2 hr with boiled pancreatic RNase (80 μg/ml). The digest was either neutralized and made 1.0 N in KOH (2–4 hr at 37°C), or precipitated with alcohol, washed, and then digested with alkali. The digest was acidified and washed several times by centrifugation in 5% cold trichloroacetic acid. The DNA was hydrolyzed in perchloric acid as above. The 5MeCyt and 6MeAde were well separated (R_fs about 0.28 and 0.60, respectively) by descending paper chromatography in *n*-butyl alcohol–NH_4OH–H_2O 86:1:13. The 5MeCyt and 6MeAde regions were identified (by means of the ultraviolet absorbance of cochromatographed authentic markers) cut out, eluted, and counted.

RESULTS

Restriction of phage P22 by independently controlled systems

In agreement with earlier reports (12), we observed that P22·$r_{LT}^+m_{LT}^+$ phage (carrying LT-host specificity) is severely restricted when it infects $r_{LT}^+m_{LT}^+$ cells containing the N-3 factor (see Table 2). It was of interest to determine whether the LT and N-3 systems were independently controlled. To test this, we transferred the N-3 factor into $r_{LT}^-m_{LT}^-$ cells and examined the modification/restriction properties of such a strain. As seen in Table 2, the $r_{LT}^-m_{LT}^-$ (N-3) host strongly restricts phages P22·$r_{LT}^+m_{LT}^+$ and P22·$r_{LT}^-m_{LT}^-$. In addition, phage P22·$r_{LT}^-m^-{}_{LT}$ (N-3) is resistant to N-3 restriction, while it remains susceptible to the LT restriction. These results indicate that the LT and N-3 restriction/modification systems are independent of one another.

Effect of R-factors on the extent of methylation of P22 DNA

We showed that the mutational loss of ability to confer LT-specificity ($m_{LT}^+ \rightarrow m_{LT}^-$) resulted in a decreased ability to methylate P22 DNA; in this case, about 40–50 fewer 6MeAde per DNA were present in P22·m_{LT}^- as compared to P22·m_{LT}^+ phage (1). When various R-factors were introduced into the m_{LT}^+ strain, no appreciable effect on the 6MeAde concentration was observed (1). It was conceivable that the N-3 factor affected so few sites that a gross change in 6MeAde content could not be detected. In view of the reduced concentration of 6MeAde in P22·m_{LT}^- phage, we considered it possible that a small increment in adenine methylation specified by N-3 might be detected if the phage were propagated in m_{LT}^- (N-3) cells; this possibility has now been excluded because no appreciable change in the concentration of 6MeAde is seen in P22 DNA after growth in these cells (Table 3, column *a*).

In view of these findings, we then turned our attention to the possibility that the N-3 specificity might be imparted by means of the methylation of specific cytosine residues. The proportion of 5MeCyt was determined in P22 DNA labeled after growth in various bacterial strains. The three most significant observations are as follows (Table 3):

(*a*) the $m_{LT}^+ \rightarrow m_{LT}^-$ mutation has no significant effect on the 5MeCyt content of P22 DNA (contrast this with the effect on 6MeAde);

(*b*) the presence of the N-3 factor increases the 5MeCyt content of P22 DNA 2- to 3-fold. It should be noted that the measurements of 5MeCyt content were made by two independent procedures; namely, the 5MeCyt/Cyt ([^{14}C]cytosine labeling) and 5MeCyt/6MeAde ([methyl-^{3}H]methionine labeling) ratios were determined;

(*c*) the presence of the N-1 or 222 factor does not influence the 5MeCyt concentration. Thus, it appears that the N-3 factor specifically affects the methylation of cytosine residues in P22 DNA.

Effect of R-factors on the methylation of bacterial DNA

We extended the above 5MeCyt/6MeAde analysis to bacterial DNAs in the hope of further demonstrating specific N-3-mediated cytosine methylation, Summarized in Table 4 are the results of such experiments. The nonmodifying N-1 and 222 factors exert little influence on the 5MeCyt/6MeAde ratio in *Salmonella* DNA; however, a small increase (up to 20%) in this ratio was observed in the *Salmonella* strains harboring the N-3 factor. The magnitude of this change is considerably lower than that seen for P22 DNA; therefore, the relative proportion of Ade- and Cyt-methylation sites differs for phage and cell DNAs. (Inasmuch as absolute determinations of 5MeCyt and 6MeAde content were not made, we cannot calculate the number of methylated bases per cellular DNA molecule.) These findings indicated N-3 also influences the methylation of cytosine residues in bacterial DNAs; however, it was not clear whether this effect is controlled directly (by a new DNA methylase) or indirectly (by altering the specificity of the resident bacterial cytosine methylase). To test these possibilities, we transferred the N-3 factor to *E. coli* B strains, which are known to be devoid of 5MeCyt (5, 7, 9, 18–21), and examined the DNA for the presence of 5MeCyt. As seen in Table 4, the N-3 factor, but not the N-1 or 222 factors, directed extensive methylation of cytosine residues in cellular DNA. We interpret these observations as an indication that N-3 controls the formation of a DNA methylase specific for cytosine residues.

Incidental to these experiments, we confirmed the reports of others (10, 11) that phage λ is restricted/modified by the N-3 factor.

TABLE 3. *Analysis of methylated bases in P22 DNA*

Phage	(*a*)* Mole % 6MeAde	(*b*)* Mole % 5MeCyt*	(*c*) 5MeCyt/6MeAde† Calculated	(*c*) 5MeCyt/6MeAde† Observed
P22·$r_{LT}^+m_{LT}^+$	0.56‡	0.25	0.45	0.35
P22·$r_{LT}^+m_{LT}^+$ (N-3)	0.51‡	0.60	1.20	1.05
P22·$r_{LT}^+m_{LT}^+$ (222)	0.57‡	Not done	—	0.37
P22·$r_{LT}^+m_{LT}^+$ (N-1)	0.56‡	Not done	—	0.36
P22·$r_{LT}^-m_{LT}^-$ *pro* C90	0.30	0.30	1.0	0.58
P22·$r_{LT}^-m_{LT}^-$ *pro* C90 (N-3)	0.30	0.60	2.0	1.67
P22·$r_{LT}^-m_{LT}^-$ *leu* 119	0.31	Not done	—	Not done
P22·$r_{LT}^-m_{LT}^-$ *leu* 119 (N-3)	0.29	Not done	—	Not done

* Mole % 6MeAde and mole % 5MeCyt are based on radioactivity determination after paper chromatographic separation of the methylated base from its normal counterpart; the values are expressed in terms of 6MeAde per 100 adenine (6MeAde + Ade) residues and 5MeCyt per 100 cytosine (5MeCyt + Cyt) residues. The figures presented are the average of at least two independent preparations analyzed; the range of values was generally ±10% of the average value.

† The calculated values were obtained by dividing column (*b*)/(*a*), since the Cyt/Ade ratio of P22 DNA is roughly 1.0. The observed values were determined in [methyl-^{3}H]methionine labeling experiments.

‡ Values taken from ref. 1.

TABLE 4. *Analysis of 5MeCyt/6MeAde ratio in DNA derived from various bacterial strains*

Salmonella strains	5MeCyt/6MeAde
$r_{LT}^+m_{LT}^+$	0.55
$r_{LT}^+m_{LT}^+$ (N-3)	0.66
$r_{LT}^+m_{LT}^+$ (N-1)	0.52*
$r_{LT}^+m_{LT}^+$ (222)	0.56*
$r_{LT}^-m_{LT}^-$ *pro* C90	0.66
$r_{LT}^-m_{LT}^-$ *pro* C90 (N-3)	0.77
Escherichia coli B strains	5MeCyt/6MeAde
$r_B^+m_B^+$; $r_B^-m_B^-$	<0.01; <0.01
$r_B^-m_B^-$ (N-1)	<0.01*
$r_B^-m_B^-$ (222)	<0.01*
$r_B^-m_B^-$ (N-3)	0.66*
$r_B^+m_B^+$ (N-3)	0.68

* Based on a single determination.

DISCUSSION

We present evidence indicating that the N-3 factor controls a DNA methylase specific for cytosine. In preliminary *in vitro* experiments with crude extracts from *E. coli* B and B(N-3) we have observed methylation of cytosine residues in calf thymus and *E. coli* B DNAs with the B(N-3) extract only. The most important question is whether this methylation is of biological significance with respect to the restriction-modification phenomenon. Relevant to this question is that the nonmodifying N-1 and 222 factors do not control such an activity; only the modifying/restricting N-3 factor exhibits this property. The results reported here do not exclude the possibility that the N-3 factor also directs an (adenine) DNA-methylase that actually confers the N-3 host specificity. This possibility can be resolved by purification of the N-3 cytosine methylase enzyme, followed by an *in vitro* demonstration that it biologically modifies DNA (in a transfection system). These experiments are in progress. We should also point out that λ phage DNA is subject to N-3 modification/restriction. In preliminary experiments, we observed that λ·$r_B^-m_B^-$(N-3), but not λ·$r_B^-m_B^-$, contained 5MeCyt.

Finally, if the N-3 modification is achieved via methylation of certain cytosine residues, then this will be the first instance of a biological role for 5MeCyt in DNA. Higher organisms contain 5MeCyt, but not 6MeAde (22–32); however, no clear role for 5MeCyt has been demonstrated. We would favor the notion that 5MeCyt is essential for protection against nucleases designed to destroy foreign (viral?) DNAs.

This work was supported by a PHS grant AI-08738. E.G. and A.P. were prebaccalaureate students. We thank Ms. Eva Zehelein for her expert technical assistance, and gratefully acknowledge S. E. Luria's helpful criticism of the manuscript and suggestion to transfer the R-factors into *E. coli* B.

1. Hattman, S. (1971) *J. Virol.* **7**, 690–691.
2. Colson, C. & Colson, A. M. (1967) *Biochem. Biophys. Res. Commun.* **29**, 692–695.
3. Okada, M., Watanabe, T. & Miyake, T. (1968) *J. Gen. Microbiol.* **50**, 241–252.
4. Colson, A. M., Colson, C. & van Pel, A. (1969) *J. Gen. Microbiol.* **58**, 57–64.
5. Ledinko, N. (1964) *J. Mol. Biol.* **9**, 834–835.
6. Lederberg, S. (1966) *J. Mol. Biol.* **17**, 293–297.

7. Gough, M. & Lederberg, S. (1966) *J. Bacteriol.* **91,** 1460–1468.
8. Arber, W. (1965) *J. Mol. Biol.* **11,** 247–256.
9. Mamelak, L. & Boyer, H. (1970) *J. Bacteriol.* **104,** 57–72.
10. Watanabe, T., Nishida, H., Ogata, C., Arai, T. & Sato, S. (1964) *J. Bacteriol.* **88,** 716–726.
11. Arber, W. & Morse, M. L. (1965) *Genetics* **51,** 137–148.
12. Watanabe, T., Takano, T., Arai, T., Nishida, H. & Sato, S. (1966) *J. Bacteriol.* **92,** 477–486.
13. Bannister, D. & Glover, S. W. (1968) *Biochem. Biophys. Res. Commun.* **30,** 735–738.
14. Takano, T., Watanabe, T. & Fukasawa, T. (1968) *Virology* **34,** 290–302.
15. Arber, W. & Lataste-Dorolle, C. (1961) *Pathol. Microbiol.* **24,** 1012–1018.
16. Wood, W. B. (1966) *J. Mol. Biol.* **16,** 118–133.
17. Hattman, S. (1970) *Virology* **42,** 359–367.
18. Doskočil, J. & Šormová, Z. (1965) *Biochem. Biophys. Res. Commun.* **20,** 334–339.
19. Doskočil, J. & Šormová, Z. (1965) *Biochim. Biophys. Acta* **95,** 513–515.
20. Fujimoto, D., Srinivasan, P. R. & Borek, E. (1965) *Biochemistry* **4,** 2849–2855.
21. Lederberg, S. (1966) *J. Mol. Biol.* **17,** 293–297.
22. Wyatt, G. R. (1951) *Biochem. J.* **48,** 581–584.
23. Dunn, D. B. & Smith, J. D. (1958) *Biochem. J.* **68,** 627–636.
24. Brown, G. M. & Attardi, G. (1965) *Biochem. Biophys. Res. Commun.* **20,** 298–302.
25. Burdon, R. H. (1966) *Nature* **210,** 797–799.
26. Grippo, P., Iaccarino, M., Parisi, E. & Scarano, E. (1968) *J. Mol. Biol.* **36,** 195–208.
27. Burdon, R. & Adams, R. (1969) *Biochim. Biophys. Acta* **174,** 322–329.
28. Kalousek, F. & Morris, N. R. (1969) *J. Biol. Chem.* **244,** 1157–1163.
29. Kalousek, F. & Morris, N. R. (1969) *Science* **164,** 721–722.
30. Vanyushin, B. F., Tkacheva, S. G. & Belozersky, A. N. (1970) *Nature* **225,** 948–949.
31. Culp, L. A., Dore, E. & Brown, G. M. (1970) *Arch. Biochem. Biophys.* **136,** 73–79.
32. Craddock, V. M. (1970) *Biochim. Biophys. Acta* **240,** 376–383.