Methylation Pattern of Lambda Deoxyribonucleic Acid

CECILIA HIDALGO¹ AND HOWARD A. NASH

Laboratory of Neurochemistry, National Institute of Mental Health, Bethesda, Maryland 20014

Received for publication 14 July 1972

Deoxyribonucleic acid (DNA) extracted from phage λ grown on *Escherichia coli* K-12 strain W4032 had 113 ± 10 5-methylcytosine residues and 215 ± 20 6-methyladenine residues per genome, as determined by three independent methods. These methylated nucleotides were distributed equally among the two strands of λ DNA. Shearing of double-stranded DNA to half-length fragments revealed a slight deficiency of 5-methyl cytosine in the 55% guanine plus cytosine half. Shearing the DNA to fragments of smaller length showed that the distribution of methylated nucleotides along the double helix was uniform with the exception of an undermethylated fragment arising from the center of the λ DNA molecule. The implication of these results for the function of methylated nucleotides in the λ DNA molecule is discussed.

Methylated bases have been found to be present in deoxyribonucleic acid (DNA) from bacteria, phages, and animal and plant cells. A small fraction of the methyl groups present in bacterial and phage DNA have been implicated in host-controlled restriction and modification (1). No biological role has yet been found for the rest of the methylated bases present in the DNA molecule. In an attempt to infer the role of these other methylated bases, we determined the distribution of methylated nucleotides in the λ DNA molecule. DNA from phage λ was chosen because its genetics have been extensively studied, the physical localization of most of its genes on the DNA molecule is known, and techniques exist for the physical resolution of different genetic regions as well as the separation of single strands of the phage genome (6, 10).

We found that λ DNA extracted from phage grown in a K-12 derivative of *Escherichia coli* contains both 5-methylcytosine (5MC) and 6-methylaminopurine (6MAP), in agreement with previous studies (5, 9). In addition, we have demonstrated that both strands have the same methyl content, and, along the double helix, only a small fragment corresponding to a region in the center of the linear genetic map shows a different degree of methylation from the rest.

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* K-12 strain W4032 (HfrC, lac Δ [complete], pro⁻, metA, T6^R,

described by Cook and Lederberg [3]), was obtained from R. Perlman. *E. coli* K-12 C600 was obtained from M. Gottesman. The lambda phage strains used were λ cl857 and λ b2cl857.

Media and reagents. Modified TPA medium contained, per liter: NaCl, 0.5 g; KCl, 8.0 g; NH₄Cl, 1.1 g; tris(hydroxymethyl)aminomethane (Tris), 12.1 g; KH₂PO₄, 0.46 g; sodium pyruvate, 0.8 g; 19 L-amino acids, 150 mg each; L-methionine, 10 mg; 0.16 M Na₂SO₄, 1.0 ml; and adenine, 10 mg. The final pH was 7.4, adjusted with HCl. Tris-Mg buffer was 0.015 M Tris-hydrochloride and 0.01 M MgSO₄, pH 7.4.

Electrophoretically pure pancreatic deoxyribonuclease was obtained from Sigma Chemical Co. Snake venom phosphodiesterase was obtained from Worthington Biochemical Corp. [³H-methyl]-methionine and [¹⁴C-methyl]-methionine were obtained from New England Nuclear Corp., Amersham-Searle, or Schwarz BioResearch, Inc. Specific activities of [³H-methyl]-methionine ranged from 2.6 to 7.6 Ci/mmol. [¹⁴C-methyl]-methionine specific activities ranged from 12.8 to 60 mCi/mmol. Carrier-free ³²P was obtained from New England Nuclear Corp.

DNA preparation. To obtain λ DNA labeled either with [¹⁴C-methyl]-methionine or with ³²P and [³H-methyl]-methionine, λ phage was grown on W4032 in modified TPA medium. Cells were allowed to grow in modified TPA medium at 37°C to a concentration of 3×10^8 cells/ml, centrifuged, and resuspended in 0.1 volume of Tris-Mg buffer. The phage was added at a multiplicity of infection of 1 to 3, and adsorption was allowed to proceed for 15 min at 37°C. At this point, the original volume was restored by adding fresh modified TPA medium with the radioactive isotopes, and the culture was further incubated for 2.5 hr at 37°C with shaking. Chloroform was added to 1% volume, and, after shaking for 10 more min, the bacterial debris was removed by centrifugation and the clear lysate was

¹ Present address: Facultad de Química y Farmacia, Universidad de Chile, Vicuña Mackenna 20, Santiago, Chile.

treated with 10 g of polyethylene glycol/100 ml and 10 ml of 5 M NaCl/100 ml to precipitate the phage. After centrifugation for 15 min at $19,000 \times g$, the phage pellet was resuspended in 10 ml of Tris-Mg buffer, 7.76 g of CsCl was added, and the resulting solution was spun for 12 to 15 hr at 34,000 rev/min in a Beckman no. 40 rotor. The resulting band of phage was banded again in CsCl and then dialyzed in Tris-Mg buffer. The DNA was extracted three times with redistilled phenol, and it was extensively dialyzed against 0.1 M Na_2SO_4 or Tris-Mg buffer.

Shearing of λ DNA and mercury complexes. DNA solutions were usually sheared at a concentration of 25 $\mu\text{g}/\text{ml}$ in 0.1 M unbuffered Na_2SO_4 . A total volume of 22 ml was always used.

The shearing technique described by Skalka, Burgi, and Hershey (10) was followed. To shear the DNA to one-half, one-tenth, and one-fifteenth length fragments, a GT-21 Heller motor with controller unit was used. One-twentieth length fragments were obtained by shearing the DNA for 30 min at 20,000 rev/min in a Virtis-45 homogenizer.

The λ DNA halves were obtained by shearing for 60 min at 1,500 rev/min. One-tenth and one-fifteenth length fragments were produced by shearing at 4,000 rev/min for 4 hr or at 8,000 rev/min for 1 hr, respectively. All DNA solutions were sheared in a Virtis stainless-steel cup at 4 C. Fragment lengths were characterized by sedimentation in 1 M NaCl neutral 5 to 20% sucrose gradients with intact λ DNA as marker. The Burgi-Hershey formula (2) was used for estimation of molecular weights.

After shearing of the DNA, it was banded in $\text{Hg}-\text{Cs}_2\text{SO}_4$ density gradients according to Skalka et al. (10). Every time a new preparation of DNA was obtained, a trial centrifugation was made to determine an appropriate R_F . All R_F values used ranged from 0.20 to 0.30. The solutions contained, per ml, 5 to 50 μg of DNA, 0.05 ml of 0.1 M sodium borate, pH 9.0, 43 to 45% by weight of Cs_2SO_4 (Gallard-Schlesinger), and the amount of mercury specified above. The mercury was always added last. Volumes of 3.2 to 5.3 ml of solution were used. The tubes were overlaid with mineral oil and spun for 36 hr at 36,000 rev/min in a fixed-angle rotor, at 4 C. At the end of the run, fractions were collected dropwise from the bottom of the tube.

Separation of strands. The technique described by Hradecna and Szybalski (6) to separate the strands of λ DNA was followed, with the use of polyuridylic-polyguanylic acid [poly (UG)] obtained from Schwarz BioResearch, Inc. (lot 6801). The purified labeled phage was dialyzed overnight in 10^{-3} M sodium ethylenediaminetetraacetic acid, pH 8.0, at 4 C. The phage solution was then mixed with poly (UG) and sarkosyl, heat-denatured for 3 min at 95 C, chilled on ice, and banded in CsCl at a density of 1.72 g/cm³ in an SW39 rotor at 30,000 rev/min for 60 hr. Fractions were collected dropwise from the bottom of the tube.

Hydrolysis of DNA and thin-layer chromatography: bases. The purified DNA (ca. 50 μg) was hydrolyzed to free bases with 70% perchloric acid at 100 C for 1 hr. The hydrolysate was neutralized with KOH and centrifuged to remove the precipitated potassium

perchlorate and charred sugars. The resulting bases were separated by two-dimensional chromatography on commercial cellulose plates (Eastman Organic Chemicals) without fluorescent indicator. Unlabeled markers of 5MC and 6MAP were added. The first solvent system used was *n*-butanol-absolute methanol-concentrated ammonia-water (60:20:20:1); the second solvent was absolute methanol-concentrated hydrochloric acid-water (65:17:18). The spots were located under ultraviolet light, scraped into liquid scintillation vials, and counted directly in a Beckman liquid scintillation counter.

Mononucleotides. DNA solutions in amounts of 0.1 to 1 ml containing a total of 50 μg of DNA in Tris-Mg buffer, pH 7.4, were hydrolyzed to mononucleotides by consecutive treatment with pancreatic deoxyribonuclease and snake venom phosphodiesterase (8). A reaction mixture containing 20 μg of pancreatic deoxyribonuclease and DNA was incubated at 37 C for 30 min. The enzyme was then inactivated by heating to 95 C for 5 min. The digest was mixed with 0.1 volume of 1 M glycine buffer, pH 9.2, and 400 μg of snake venom phosphodiesterase, and was hydrolyzed to mononucleotides by incubating at 37 C for 30 min. The resulting mixture was adsorbed to activated charcoal, washed three times with distilled water, and eluted by washing three times with 50% ethanol-0.5% concentrated ammonia. The eluted solution was concentrated in vacuo to a final volume of 0.2 ml, and the mononucleotides were separated by two-dimensional chromatography on commercial PEI-cellulose plates (Eastman).

The first solvent used was 1 M LiCl, and the second was 0.5 N acetic acid. In between solvents, the plates were immersed in absolute methanol and shaken for 15 min to remove the LiCl. The spots were demonstrated by means of autoradiography with Kodak No-screen X-ray film. Nucleotide compositions were determined by scraping the radioactive spots and counting in a Beckman liquid scintillation counter.

RESULTS

Methyl content of whole λ DNA. The methylation of DNA obtained from λ cI857 phage grown on W4032 was studied by several different procedures. Purified [¹⁴C-methyl]-labeled DNA was hydrolyzed either to bases or to mononucleotides, and the extent of methylation was determined by assuming the specific activity of the methyl groups in the DNA to be equal to the specific activity of methionine in the growth medium. Table 1 shows the results of several determinations. Autoradiograms of thin-layer chromatograms of these hydrolysates demonstrated that 6MAP and 5MC are the only methyl-labeled compounds. Labeling the DNA with ³²P-orthophosphate followed by hydrolysis to mononucleotides also gave the same total content of 5MC and 6MAP. The determination of the methyl content by direct analysis of the ³²P-labeled mononucleotides did not require any assumption regarding the specific activity of the

TABLE 1. *Methylation of λ cI857 DNA grown on W4032*

DNA prepn	5MC/ λ genome ^a	6MAP/ λ genome ^a	Ratio, 5MC/6MAP
¹⁴ C-methyl bases	114 $\pm$ 10 (8)	213 $\pm$ 8 (8) ^b	0.54 $\pm$ 0.05
¹⁴ C-methyl mononucleotides	110 $\pm$ 7 (4)	218 $\pm$ 7 (4)	0.50 $\pm$ 0.04
³² P-mononucleotides	114 $\pm$ 10 (6)	215 $\pm$ 20 (6)	0.53 $\pm$ 0.07

^a Content of 5-methylcytosine (5MC) or 5-methylaminopurine (5MAP) per genome was calculated by using the molecular weight of λ as 32×10^6 and the molar absorptivity coefficient (based on phosphate) as 6.4×10^4 .

^b Numbers in parentheses represent number of determinations.

phosphate, since the counts obtained in the methylated mononucleotide spots were directly compared with the counts obtained in the four main mononucleotide spots. The fact that the same values for methyl content were obtained by ³²P labeling and ¹⁴C-methyl labeling validates the assumption made regarding the specific activity of the methyl groups.

The methyl contents for λ DNA described above are approximately twofold higher than those reported by Gough and Lederberg (5) for λ grown on K-12 strain C600. We have examined the methylation of λ DNA grown on C600 on low phosphate medium using orthophosphate labeling and hydrolysis to mononucleotides. From the average of two experiments, we found 5MC contents of 56 moles/genome and 6MAP contents of 132 moles/genome, in good agreement with the values reported by Gough and Lederberg. We have no explanation for the twofold difference in methylation for λ grown on W4032 and C600. The possibility of a different restriction-modification system between W4032 and C600 has been ruled out directly by comparing plating efficiencies of phage λ on each strain (data not shown). The difference in methylation might reflect the presence of the sex factor in W4032 or its absence in C600, or both, but we have no evidence on this point.

Methyl content of λ DNA single strands. The proportion of methylated to total nucleotides was compared by using the separated strands of λ DNA doubly labeled with [³H-methyl]-methionine and ³²P phosphate. A typical experiment is illustrated in Fig. 1. The ratio of ³H to ³²P counts was calculated for each DNA-containing fraction; no significant differences were found between the r and l strands, indicating that the total content of methylated bases is the same for each strand (Table 2). To examine the content of each of the two methylated bases in each strand, DNA was labeled by growing λ cI857 on W4032 in TPA

FIG. 1. *Distribution of methylated base residues between the strands of λ DNA. Phage λ was doubly labeled with [³H-methyl]-methionine and ³²P. The two strands were separated by heat denaturing the DNA in the presence of poly (UG) followed by equilibrium density centrifugation in CsCl. The ratios between ³H and ³²P counts were determined for each DNA-containing fraction.*

medium with [¹⁴C-methyl]-methionine. The separated strands were hydrolyzed with perchloric acid and the bases were resolved by thin-layer chromatography. The ratio between 5MC and 6MAP obtained for each of the two separated strands was the same within experimental error (Table 3). We conclude that the two strands of λ DNA are equally methylated for both 5MC and 6MAP.

Methylation along the DNA molecule. Phage λ cI857 was grown on W4032 in TPA medium containing [¹⁴C-methyl]-methionine. The DNA was sheared to halves and the separated fragments were hydrolyzed to bases. Table 4 shows the average values for methylated base content obtained in three different experiments. The fractional content of 6MAP and 5MC per nucleotide (Table 4, columns 2 and 3) in each half is similar to that obtained for the intact DNA molecule.

To investigate in further detail any possible heterogeneity in methylation along the DNA molecule, smaller fragments were prepared by shearing doubly labeled [³H-methyl]- and ³²P-DNA to one-tenth, one-fifteenth, and one-twentieth molecular lengths and separating the fragments by banding them in Hg-Cs₂SO₄ density gradients. The methylation of each fragment was inferred from the ratios of ³H to ³²P counts in each DNA-containing fraction. Figure 2 illustrates a typical experiment of this kind. In all three types of gradients, with any of the three fragment lengths, the same general pattern for total nucleotides was obtained: a shoulder corresponding to a region of low guanine plus cytosine (GC) content and two major peaks.

Skalka et al. (10) have shown that, for λ DNA

sheared to one-tenth length fragments, the shoulder contains material of 37% GC content derived from the *b2* region. We have confirmed these results by shearing together λ cI857 and λ b2cI857 DNA to one-tenth length fragments followed by banding the fragments in Hg-Cs₂SO₄ gradients. The shoulder corresponding to a low GC composition shown in Fig. 2 was missing in the case of λ b2cI857 DNA (data not shown). By direct analysis of nucleotide composition of λ DNA sheared to one-tenth lengths, we found that the shoulder corresponds to 40% GC composition, and the two peaks to 46 and 57% GC, respectively. Figure 3 shows a gradient of λ DNA sheared to one-fifteenth fragment length. From Fig. 2 and 3, it can be seen that along the two major peaks the ³H to ³²P ratios are indistinguish-

able. However, the ³H to ³²P ratio decreased in the shoulder region. The same result was obtained for one-twentieth fragment lengths. Table 5 shows a summary of all results obtained by this method. The λ DNA one-fifteenth fragments show more dramatically the presence of an undermethylated region of DNA in the central part of the molecule. However, owing to the large variation in different gradients, it is not possible to calculate the exact length or the absolute extent of methylation of this region.

DISCUSSION

The major portion of the methylated nucleotides of λ DNA have no known role in either the lytic or lysogenic phases of the bacteriophage λ life cycle. The results presented above rule out several different models concerning the function of methyl groups in λ DNA.

After infection of *E. coli* by phage λ , the parental strands are found to have an asymmetric fate during replication; the l strand is found in circular monomers and the r strand in multigenome length molecules (7). Asymmetry in the fates of complementary strands is a predicted feature of the rolling circle model of DNA replication and may be a widespread feature in the physiology of viruses. The finding that both strands are equally methylated, both in terms of total content per strand and composition in each strand, implies that the bulk of methylated bases cannot be used as a recognition element to signal the r or l strand in directing asymmetric replication, or in other processes that distinguish between the two strands.

The genes of λ are clustered in functional groups on the phage DNA, and these clusters are characterized by differing GC content (10). The head and tail genes make up the left arm of the phage chromosome, as it is usually written, and have a uniform GC content of 57%. Most of the right arm of the phage map is taken up with genes concerned with replication, recombination, and immunity; this region has a distribution of GC content that varies from 43 to 48%. The left arm

TABLE 2. Methylation of λ DNA single strands

Expt ^a	³ H to ³² P ratio	
	r strand	l strand
1	0.34 ± 0.02 (11) ^b	0.34 ± 0.01 (5)
2	0.44 ± 0.01 (12)	0.44 ± 0.02 (8)

^a Since the phage used to obtain the strands had different specific activities of ³²P at the times at which the experiments illustrated in the table were done, comparisons of ratios for l and r strand are valid only within each experiment.

^b The numbers in parentheses indicate the number of fractions averaged for each strand.

TABLE 3. Methylated 5-methylcytosine (5MC) and 6-methylaminopurine (6MAP) in each strand of λ DNA

Strand	Counts/min		Ratio
	5MC ^a	6MAP ^a	
r	283 ± 12	570 ± 25	0.50 ± 0.03
l	381 ± 15	683 ± 18	0.56 ± 0.03

^a Five determinations were made for each strand.

TABLE 4. Methylated bases in λ DNA halves^a

DNA half	6MAP/100 MN ^b	5MC/100 MN ^b	5MC/6MAP	6MAP/100 A ^c	5MC/100 C ^d
45% GC half	0.23 ± 0.02	0.12 ± 0.01	0.52 ± 0.06	0.83 ± 0.06	0.52 ± 0.02
55% GC half	0.21 ± 0.01	0.09 ± 0.01	0.43 ± 0.05	0.93 ± 0.05	0.34 ± 0.02

^a The results are averages of three different experiments.

^b Values are 100 × (moles of methylated nucleotide per mole of mononucleotide in each half).

^c Values are 100 × (moles of 6-methylaminopurine per mole of adenine in each half).

^d Values are 100 × (moles of 5-methylcytosine per mole of cytosine in each half).

FIG. 2. Distribution of methylated base residues among one-tenth length fragments of differing GC composition. Phage λ DNA doubly labeled with [^3H -methyl]-methionine and ^{32}P was sheared to one-tenth length fragments. The figure illustrates a typical $\text{Hg-C}_2\text{SO}_4$ equilibrium centrifugation gradient. The ratios between ^3H and ^{32}P counts were calculated for all fractions corresponding to regions I, II, and III. The average ratio values are given for each gradient zone.

FIG. 3. Distribution of methylated base residues among one-fifteenth length fragments of differing GC composition. Phage λ DNA doubly labeled with [^3H -methyl]-methionine and ^{32}P was sheared to one-fifteenth length fragments and banded in $\text{Hg-C}_2\text{SO}_4$ as described in the text. The ratios between ^3H and ^{32}P counts were calculated for all fractions corresponding to regions I, II, and III. The average ratio values are given for each gradient zone.

genes are typically transcribed and translated late after induction or infection of λ ; the right arm genes are expressed early. The data presented in Table 5 and Fig. 2 and 3 show that these regions cannot be distinguished from one another on the basis of methyl content, and thus rule out the possibility that early versus late transcription is regulated by recognition of sites identified by a major fraction of the methyl groups in λ .

An extreme hypothesis, i.e., that methylation is featureless and therefore plays no role in recogni-

TABLE 5. Methylation in λ DNA fragments

λ DNA sheared to	No. of determinations	Normalized ^3H to ^{32}P ratios in gradient region ^a		
		I	II	III
One-tenth	17	91 $\pm$ 6	100 $\pm$ 3	102 $\pm$ 5
One-fifteenth	7	76 $\pm$ 9	106 $\pm$ 10	103 $\pm$ 5
One-twentieth	6	90 $\pm$ 3	104 $\pm$ 3	100 $\pm$ 2

^a Ratios between ^3H and ^{32}P counts in all DNA-containing fractions on any given gradient were averaged, the gradient was divided in three regions from low to high GC content (the shoulder and the two main peaks illustrated on Fig. 2 and Fig. 3), and the mean ^3H to ^{32}P ratio for each region was divided by the total gradient average.

tion of specific DNA elements in λ , may also be ruled out from an examination of the fine detail of methylated base distribution. If methylation were not based on the interaction of methylating enzymes with specific DNA sites, one would expect that the methyl content of a region would reflect the density of the potential substrate nucleotides, adenine and cytosine. The density of methylated nucleotides with respect to potential precursor nucleotides may be calculated from the data in Table 4 by combining the density of methylated bases per nucleotide and the composition of each half. Such a calculation (Table 4, columns 5 and 6) reveals a similar density of 6MAP/adenine between the two halves but a very different density of 5MC/cytosine. This means that methylation of cytosine has proceeded by a mechanism that is responsive to dinucleotide or longer sequences. This finding is in agreement with earlier chemical studies showing that the distribution of methyl cytosines among the various pyrimidine tracts of *E. coli* DNA is nonrandom (4).

The results obtained by shearing the DNA to small fragment lengths reveal a more striking asymmetry than that discussed for halves. A small fragment, arising from the central portion of the λ genome, shows a decrease in methylation as compared to the total methylation of the rest of the DNA molecule, which is itself fairly constant. If we assume that the methylation of adenine and cytosine is only a function of the absolute amount of each base present, as done above for half molecules, we calculate that the ratio of methylated to total nucleotides would be expected to be 11% higher for a 40% GC fragment than for the entire molecule. Instead, we find that the 40% GC fragment is between 10 and 25% less methylated than the average molecule. In the other regions resolved by the C_2SO_4 -Hg gradient, the nonuniformity of methylation is much less dramatic.

Thus, the distribution of methylated nucleotides is not a simple reflection of either total nucleotides or the content of the potential acceptor nucleotides, adenine and cytosine. It is possible that this nonrandomness in methyl group distribution reflects the sum of many sequence-specific methylated sites—a few each for modification, transcriptional starts and stops, and replication signals. The data of earlier workers on modification and our data on distribution of methyl groups simply rule out that one of these functions accounts for the majority of methyl groups in λ DNA. On the other hand, a yet unknown single function for the bulk of the methylated nucleotides may exist.

ACKNOWLEDGMENTS

We thank J. Little for his help in teaching us his shearing techniques. We also thank J. Little, D. Neville, J. L. Rosner, and M. Shulman for their careful readings of the manuscript.

LITERATURE CITED

1. Arber, W., and S. Linn. 1969. DNA modification and restriction. *Annu. Rev. Biochem.* 38:467–500.
2. Burgi, E., and A. D. Hershey. 1963. Sedimentation rate as a measure of molecular weight of DNA. *Biophys. J.* 3:309–321.
3. Cook, A., and J. Lederberg. 1962. Recombination studies of lactose nonfermenting mutants of *Escherichia coli* K12. *Genetics* 47:1335–1353.
4. Doskočil, J., and Z. Šormová. 1965. The sequences of 5-methylcytosine in the DNA of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* 20:334–339.
5. Gough, M., and S. Lederberg. 1966. Methylated bases in the host-modified deoxyribonucleic acid of *Escherichia coli* and bacteriophage λ . *J. Bacteriol.* 91:1460–1468.
6. Hradecna, Z., and W. Szybalski. 1967. Fractionation of the complementary strands of coli phage λ DNA based on the asymmetric distribution of the Poly I, G-binding sites. *Virology* 32:633–643.
7. Ihler, G., and Y. Kawai. 1971. Alternate fates of the complementary strands of lambda DNA after infection of *Escherichia coli*. *J. Mol. Biol.* 61:311–328.
8. Kelly, T. J., Jr., and H. O. Smith. 1970. A restriction enzyme from *Hemophilus influenzae*. I. Purification and general properties. *J. Mol. Biol.* 51:379–391.
9. Ledinko, N. 1964. Occurrence of 5-methyldeoxycytidylate in the DNA of phage lambda. *J. Mol. Biol.* 9:834–835.
10. Skalka, A., E. Burgi, and A. D. Hershey. 1968. Segmental distribution of nucleotides in the DNA of bacteriophage lambda. *J. Mol. Biol.* 34:1–16.