

Host Specificity of DNA produced by *Escherichia coli*

XIV.† The Role of Nucleotide Methylation in *in vivo* B-specific Modification

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N-6-methyladenine is the only methylated base detected in bacteriophage fd DNA. Its frequency is (1) host dependent: fd grown on a strain providing B-specific modification to DNA carries twice as many methyl moieties in its DNA as phage grown on a non-modifying host; and (2) dependent on the number of sites with affinity for B-specific restriction: the DNA of a B-restriction insensitive double mutant of fd is only half as much methylated after growth on R as its wild type parent.

These results permit one to correlate methylation of specifically located adenines with B-specific modification. Quantitative measurements suggest that one *N*-6-methyladenine is carried per B-host specificity site on the modified, single stranded fd DNA. Wild-type fd has two such sites. The remaining methyl groups are probably unrelated to B-modification. The biological function assigned to methylation brought about by B-specific modification is the protection of the DNA from its destruction by restriction endonuclease R-B.

1. Introduction

A hypothesis explaining the chemical nature of DNA modification by *Escherichia coli* as nucleotide methylation is based on the observations that *S*-adenosylmethionine or methionine, a precursor of *S*-adenosylmethionine, are required for modification *in vivo* (Klein & Sauerbier, 1965; Arber, 1965) and that *S*-adenosylmethionine is required for B-specific modification *in vitro* (Kühnlein, Linn & Arber, 1969). *S*-adenosylmethionine is the methyl donor in enzymic methylation of nucleic acids (Gold & Hurwitz, 1963, 1964a; Fujimoto, Srinivasan & Borek, 1965). Direct evidence for this hypothesis was sought by comparing the frequency of methylated bases in modified and unmodified DNA, but no significant results were obtained with either phage λ DNA or bacterial DNA (Ledinko, 1964; Klein, 1965; Gough & Lederberg, 1966; Lederberg, 1966; Smith, Wood & Arber, cited by Arber, 1968; Mamelak & Boyer, 1970), indicating that only a minor fraction, if any, of the methylated bases in these DNA's are involved in strain-specific modification.

In the experiments presented here, we determined the methylated bases contained in the DNA of phage fd. The low over-all methylation of fd DNA made it possible to

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(d) Analysis of the *fd* DNA

(i) Method (1)

The DNA was hydrolysed with HClO_4 according to Wyatt (1951). The hydrolysate was diluted with an equal volume of water, centrifuged to remove the carbon, and then 30 μl . of the supernatant containing 25 g each of carrier *N*-6-methyladenine and 5-methylcytosine were placed near the corner of a sheet of Whatman no. 1 paper and chromatographed in isopropanol-water (65:35, v/v)-2 *N*-HCl (Wyatt, 1951). The area containing cytosine, 5-methylcytosine and *N*-6-methyladenine was chromatographed in a second dimension in *n*-butanol-water-33% aqueous ammonia (86:13:1, by vol.). Cytosine, 5-methylcytosine and *N*-6-methyladenine separate in that order of increasing R_F value. The 5-methylcytosine and *N*-6-methyladenine spots, located in ultraviolet light, as well as the inorganic phosphate spot, were cut out and counted on the paper in a scintillation counter. The counting efficiencies of ^{14}C and ^{32}P , used to calculate the frequency of methylated bases of the DNA, were determined by counting standards of L-[methyl- ^{14}C]methionine and [^{32}P]orthophosphate under the same conditions.

(ii) Method (2)

The DNA together with 0.5 mg carrier salmon sperm DNA was adjusted to 0.15 M with ammonium acetate and precipitated with 2 vol. of ethanol. The precipitate was washed with 70% ethanol, 0.02 M-ammonium acetate and dried. The DNA was redissolved in 0.12 ml. of 0.02 M-Tris·HCl (pH 7.6), 0.002 M-magnesium acetate, containing 40 μg pancreatic DNase, and incubated for 1 hr at 37°C. Then the pH was adjusted to 8.9 by addition of 5 μl . of 2 M-Tris. 5 μl . venom phosphodiesterase (1.5 mg/ml.; Worthington) was added and the incubation continued for 2 hr. 50- μl . fractions were placed near a corner of a sheet of Whatman 3 MM paper and run on electrophoresis in 0.1 M-ammonium formate buffer (pH 3.5) to separate the 4 major nucleotides (*N*-6-methyldeoxyadenylic acid and 5-methyldeoxycytidylic acid migrate with deoxyadenylic acid and deoxycytidylic acid, respectively). The areas containing deoxycytidylic, deoxyguenylic and deoxythymidylic acid were located with ultraviolet light, cut out and counted in a scintillation counter. A strip containing the combined deoxyadenylic and *N*-6-methyldeoxyadenylic acid spot was cut out. 5 *N*-HCl was run down the strip to cover the spot and the paper was dried at room temperature. This treatment hydrolysed the nucleotides to the corresponding purines. 30 μg *N*-6-methyladenine was placed over the adenine spot and the two bases were separated by chromatography in *n*-butanol-water-33% aqueous ammonia as under method (1). The areas containing *N*-6-methyladenine and inorganic phosphate were cut out for radioactive measurements.

3. Results

(a) Identification of the methylated bases in *fd* DNA

Phage *fd*·B, *fd*·0 and *fd*·K were grown on host strains B, 0 and K, respectively, in medium supplemented with L-[methyl- ^{14}C]methionine. Free DNA in the lysates was degraded with pancreatic DNase and the phage purified. The phage DNA was then extracted and examined by two methods for the presence of ^{14}C -labelled methyl groups.

In method (1), the DNA was hydrolysed with HClO_4 , and the bases were separated by two-dimensional paper chromatography (see Materials and Methods), together with markers of 5-methylcytosine and *N*-6-methyladenine. All the radioactivity was found to migrate together with *N*-6-methyladenine.

In method (2), the DNA was treated with pancreatic DNase and venom phosphodiesterase, and the resulting 5'-nucleotides were separated by electrophoresis at pH 3.5. Only the area of deoxyadenylic acid and *N*-6-methyl-deoxy-adenylic acid (the two compounds do not separate) was radioactive. The nucleotides of this region were hydrolysed with HCl and the adenine and the *N*-6-methyladenine were separated by

chromatography. Again, the radioactivity comigrated with a marker of *N*-6-methyladenine.

We conclude that adenine is the only base of fd DNA to receive methyl groups from methionine during growth in any of the host strains used. In particular, no 5-methylcytosine could be detected.

(b) *Extent of methylation of unmodified and B-modified fd DNA*

Phage fd was grown on various host strains in medium containing *L*-[methyl-¹⁴C]methionine and [³²P]orthophosphate. The DNA was analysed as described above, except that counting of the ³²P spots of the chromatograms permitted one to measure the amount of DNA, i.e. the total DNA in method (1) and the adenine nucleotides in method (2). The number of *N*-6-methyladenines was calculated from these determinations by assuming that the specific activity of ³²P in fd DNA was the same as that of the exogenous ³²P and that the specific activity of ¹⁴C in *N*-6-methyladenine was the same as that in the exogenous ¹⁴C in methionine. It was further assumed that one DNA molecule contains 6600 nucleotides (see Marvin & Hahn, 1969) and that it has an adenine content of 23.5% (our own determination, in agreement with the value of 24.4% published by Hoffmann-Berling, Marvin & Dürwald, 1963).

The results presented in Table 1 (for method (1)) and in Table 2 (for method (2)) show that B-modified fd DNA is consistently more methylated than unmodified fd DNA. The average number of *N*-6-methyladenines per fd-B DNA molecule was 3.8 (with individual determinations ranging between 4.9 and 2.7), while it was only 1.8 (with individual determinations between 2.0 and 1.7) for fd-0 DNA. We attribute the higher *N*-6-methyladenine content for fd-B DNA to B-specific modification.

Phage fd grown on strain K carries the same number of methylated moieties as the

TABLE 1
N-6-methyladenine content of the DNA of fd-B, fd-0, and fd-K as determined by method (1)

Experiment	Source of DNA	Analysis	Total DNA (nmoles)	6-MetAde (pmoles)	6-MetAde residues/6600 nucleotides
I	fd-B	a	16.5	9.15	3.66
		b	15.9	7.95	3.30
II	fd-B	a	16.7	12.3	4.86
		b	19.7	14.3	4.80
	fd-0	a	21.0	6.2	1.95
		b	30.4	9.3	2.02
	fd-K	a	15.3	4.1	1.73

Phage fd was grown in Tris medium supplemented with [³²P]orthophosphate (0.06 μ Ci/ml. in experiment I and 0.17 μ Ci/ml. in experiment II) and *L*-[methyl-¹⁴C]methionine (batch 32, 29.1 μ Ci/ μ mole), treated with DNase, and purified. The DNA was extracted and analysed according to method (1) by base hydrolysis and 2-dimensional chromatography. The amount of *N*-6-methyladenine (6-MetAde) was determined from the radioactivity (¹⁴C) of the corresponding area of the chromatogram and the total DNA from the radioactivity (³²P) of the inorganic phosphate spot at the origin.

TABLE 2
N-6-methyladenine content of the DNA of fd-B and fd-0 as determined by method (2)

Source of DNA	Analysis	Ade + 6-MetAde (nmoles)	6-MetAde (pmoles)	6-MetAde residues/6600 nucleotides
fd-B	a	3.00	5.22	2.70
	b	3.74	7.82	3.25
fd-0	a	2.52	2.69	1.66
	b	3.14	3.43	1.69

Phage fd was grown in Tris medium supplemented with [³²P]orthophosphate (0.17 μCi/ml.) and L-[methyl-¹⁴C]methionine (batch 37, 29.5 μCi/μmole). The DNA was extracted and digested according to method (2) with pancreatic DNase and venom phosphodiesterase to give the 5'-nucleotides. The nucleotides were separated by electrophoresis. The area containing the mixture of deoxyadenylic and N-6-methyl-deoxyadenylic acid was exposed to 5 N-HCl, in order to hydrolyse the nucleotides to the corresponding bases. Adenine and N-6-methyladenine (6-MetAde) were then separated by chromatography. The amount of 6-MetAde was determined by counting the radioactivity (¹⁴C) of the corresponding area and the total adenine nucleotide concentration by counting the radioactivity (³²P) of the inorganic spot at the origin of the chromatogram.

unmodified fd-0 DNA (Table 1). Assuming that K-specific modification is also obtained by methylation, it indicates that phage fd, which is not subject to K-restriction (Arber, 1966), has also no affinity for K-modification.

(c) Reduced methylation of DNA from a restriction-insensitive fd mutant

Double mutants of phage fd had been isolated which have entirely lost their sensitivity towards B-specific restriction (Arber & Kühnlein, 1967; Kühnlein & Arber, 1972). Wild type fd and one of these mutants (fd sB-1° sB-2°, strain 601) were grown on strain B and their DNA methylation measured according to method (2). Like non-modified fd-0 DNA, the mutant fd DNA, although grown on strain B, carried only half as much N-6-methyladenine as B-modified wild type phage DNA (Table 3). This result suggests that the mutated specificity sites on the DNA of strain 601 had not only lost their affinity for B-specific restriction, but also for B-specific modification.

TABLE 3
Comparison between the N-6-methyladenine content of fd sB-1° sB-2° B DNA and fd-B DNA

Source of DNA	Analysis	Ade + 6-MetAde (nmoles)	6-MetAde (pmoles)	6-MetAde residues/6600 nucleotides
fd-B	a	2.86	5.0	2.71
	b	2.78	4.9	2.73
fd sB-1° sB-2° B	a	15.2	14.4	1.47
	b	14.0	11.5	1.27

Wild type fd and its double mutant sB-1° sB-2° (strain 601) were grown on host strain B in Tris medium with [³²P]orthophosphate (1 μCi/ml.), L-[methyl-¹⁴C]methionine (batch 40; 25 μCi/μmole), 20 μg adenine/ml. and 20 μg uridine/ml. After treatment of the phage lysates with DNase and purification of the phage, the DNA was extracted and analysed according to method (2).

4. Discussion

The DNA of phage fd grown on the B-strain 2027 contains twice as much *N*-6-methyladenine as the DNA of phage grown on the non-modifying strain 993. This result was obtained in two independent determinations, i.e. by paper chromatography of HClO₄ hydrolysates of the DNA (method 1) and by electrophoretic analysis of deoxynucleotides from enzymic digests of the DNA (method 2). In the two experiments, the ratio of *N*-6-methyladenine carried by B-modified fd to that carried by non-modified fd was 2.4 (Table 1) and 1.8 (Table 2), respectively. The two host strains used had both been derived from *E. coli* K12: 2027 is a transduction hybrid carrying the genes for DNA restriction and modification from *E. coli* B, while 993 is a restriction- and modification-deficient mutant of K12. It is thus likely that the increased methylation of fd-B is related to its B-specific modification. Supporting this interpretation, when the double mutant fd *sB*-1° *sB*-2°, which is insensitive to B-specific restriction, was grown on B, it was found to contain only half as much *N*-6-methyladenine as wild type fd grown on the same strain (Table 3). We infer that fd *sB*-1° *sB*-2°-B does not carry B-specific methylation and that its residual methylation, as in fd-0, is unrelated to B-modification. In confirmation, replicative form prepared from fd *sB* *sB*-1° *sB*-2° on strain 0 accepts no methyl group from *S*-adenosylmethionine upon incubation with B-modification enzyme, but replicative form prepared from wild type fd does (Kühnlein & Arber, 1972).

The accuracy in the determinations of the *N*-6-methyladenine content per fd DNA molecule depended on a number of factors. Values obtained by method 1 were somewhat higher than those obtained by method 2. Other sources of systematic errors might have been (1) the specific activities of the radioisotopes used as well as a possible difference of specific activities in endogenous *versus* exogenous label; (2) the counting efficiency of the radioisotopes; (3) the degree of completeness of DNA methylation; (4) a possible leakyness of the mutation responsible for the methionine auxotrophy of the host strains; and (5) a possible alternative methylation pathway. However, the fd-B phage used in these experiments were completely modified as defined by equal infectivity on strain B and strain O. In addition, Arber (1965) has demonstrated a strong requirement of methionine for modification, using strains with the methionine auxotrophy carried by the host strains used here. Therefore, the last three sources of error listed above were probably minor.

The number of *N*-6-methyladenine attributed to B-specific modification of fd-B DNA varied in the individual determinations between one and three per DNA molecule, with an average made over all measurements close to two. If wild type fd has two B-specificity sites and its double mutant fd *sB*-1° *sB*-2° has none, as suggested by a number of observations (Arber & Kühnlein, 1967; Kühnlein & Arber, 1972; Boyer, Seibienski, Slocum & Roulland-Dussoix, 1971; Boon & Zinder, 1971), it is reasonable to assume that the number of *N*-6-methyladenine produced on fd DNA in B-specific modification is two or a multiple of two, each specificity site receiving an identical number of methyl groups upon its modification. Our data would then suggest that each B-specificity site of the single stranded fd DNA becomes modified by methylation of one adenine nucleotide.

In vitro experiments show that only the double-stranded replicative form of fd DNA, but not the single-stranded viral DNA, is sensitive to B-specific modification (Kühnlein, Linn & Arber, 1969) and restriction (Linn & Arber, 1968). Upon *in vitro*

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modification fd replicative form receives four methyl groups (Kühnlein & Arber, 1972). If the viral strand has two, as suggested above, the other two *N*-6-methyladenines must be on the complementary strand. This distribution of methyl groups is in agreement with earlier observations that phage λ DNA is modified in both strands at each specificity site (Arber, Hattman & Dussoix, 1963; Keller, 1964; Kellenberger, Symonds & Arber, 1966; Meselson & Yuan, 1968).

Methylation of fd DNA unrelated to B-specific modification is low. By comparison, DNA of phage λ 0 is about 8 times and DNA of the bacterial strain 0 itself about 15 times higher methylated than fd DNA (J. D. Smith, W. B. Wood & W. Arber, unpublished results obtained with the methods used in this paper). In addition, bacterial and λ DNA contain *N*-6-methyladenine and 5-methylcytosine in a ratio of about 2 to 1, whereas only *N*-6-methyladenine was detected in fd DNA. The reason for the low methylation of fd DNA is unknown; it might be due to absence of acceptor sites for methylation by the previously described methylating enzymes of *E. coli* (Gold & Hurwitz, 1963, 1964a,b; Fujimoto *et al.*, 1965), or related to methyl transferase inhibitors triggered by the phage infection (Falaschi & Kornberg, 1965). If so, such inhibitors do not prevent B-specific methylation.

The *N*-6-methyladenines involved in B-specific modification are the first methylated moieties in DNA for which a biological function is known. Less direct evidence indicates that methylation is also responsible for DNA modification by *E. coli* K12 (Arber, 1965), by *Salmonella typhimurium* (Hattman, 1971) and by bacteriophage P1 (Arber, 1965; Hattman, 1970). The methyl groups protect the modified DNA from degradation by the restriction endonuclease, probably by blocking binding by the endonuclease (Yuan & Meselson, 1970). We note that methyl groups do not specifically mark a site on the DNA, permitting site recognition by the endonuclease. Rather, the methyl groups mask the nucleotide sequence of a specificity site, making it unrecognizable by the restriction endonuclease.

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Host Specificity

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