

Biological Function for 6-Methyladenine Residues in the DNA of *Escherichia coli* K12

M. G. MARINUS AND N. RONALD MORRIS

*Department of Pharmacology
Rutgers Medical School
College of Medicine and Dentistry of New Jersey
Piscataway, N.J. 08854, U.S.A.*

(Received 15 October 1973, and in revised form 10 January 1974)

A strain of *Escherichia coli* K12 mutant at the *dam-3*† site contains 0.08 mole % 6-methyl adenine as compared to 0.50 mole % in the wild type, and the residual DNA methylation is not due to the K12 modification methylase specified by the *hsp* genes. The *dam-3* mutant is more sensitive to ultraviolet irradiation and to mitomycin C than the wild type and also shows a higher mutability. DNA isolated from the *dam-3* mutant contains single-stranded breaks that are amplified in *dam-3 polA12* and *dam-3 lig-7* double mutants. A function of *dam*-specified 6-methyl adenine residues in DNA would, therefore, appear to be the protection of DNA from a nuclease(s) that causes the development of breaks. Combination of *dam-3* with *polA*, *recA*, *recB* and *recC* is lethal.

1. Introduction

The biological role of the major portion of the 6-methyladenine in the DNA of *Escherichia coli* is not known. There are approximately 4.4×10^4 6-MeA† residues in the *E. coli* K12 chromosome (Vanyushin *et al.*, 1968). A minute portion of this 6-MeA is known to play a part in the K12 DNA modification–restriction system. Modification–methylation of specific adenine moieties by the K12 modification methylase, a product of the host-specificity (*hsp*) gene complex, confers protection against endonucleolytic cleavage by the K12 restriction enzyme, endonuclease III (Boyer, 1971; Meselson *et al.*, 1972; Smith *et al.*, 1972). However, the fraction of the total 6-MeA that is involved in modification–restriction must be minute, since no measurable difference in the 6-MeA content of DNA from *hsp*⁺ and *hsp*⁻ strains has been detected (Gough & Lederberg, 1966; Arber, 1968; Mamalak & Boyer, 1970). As a first step toward determining the function(s) of the majority of methylated bases in DNA, Marinus & Morris (1973) isolated mutants of *E. coli* K12 with reduced amounts of 6-MeA in DNA. The mutations causing adenine undermethylation mapped in a gene (*dam*) located at minute 65 on the genetic map (Marinus, 1973), well separated from the *hsp* genes that are responsible for host specificity (Taylor & Trotter, 1972). The *dam* mutations are not lethal, i.e. none of the *dam* strains is significantly temperature sensitive for growth; however, because of residual adenine methylation it was not clear whether 6-MeA has an indispensable biological function(s). In this

† Abbreviations used: *dam*, DNA adenine methylase; 6-MeA, N⁶-methyladenine; 5-MeC, 5-methylcytosine; *hsp*, host specificity.

paper evidence is presented that a function of *dam*-specified 6-MeA residues is to protect DNA from a nuclease(s) that causes the development of single-stranded breaks. Data are presented which show that endonuclease III is not instrumental in the production of these breaks.

2. Materials and Methods

(a) Bacterial strains and bacteriophages

The bacterial strains used in this work are listed in Table 1. They were constructed by bacteriophage P1vir transduction using the following parental strains: N108 (*recCts1*) and N138 (*recBts1*) from J. I. Tomizawa; N203 (*recAts*) from J. I. Tomizawa *via* B. Low; MM383 (*polA12*) from M. Monk *via* B. Low; and KS253 (*lig-7*) from I. R. Lehman. Strains GM1, 2, 3 and 4 and M94 were described previously (Marinus & Morris, 1973). *λvir* was donated by S. Champe and P1vir was obtained from E. A. Adelberg.

(b) Media

The minimal medium used was M9 salts (Adams, 1959) supplemented with 0.2% glucose, 10^{-5} M-thymidine and growth factors at predetermined optimal concentrations (Adelberg & Burns, 1960). K medium (Weigle *et al.*, 1959) is M9 medium supplemented with 1% Casamino acids. Complete medium was Difco brain-heart infusion broth (20 g/l), solidified when required with 1.6% Difco agar. Lambda agar consists of 10 g Tryptone, 5 g yeast extract, 5 g NaCl, 8.5 g agar and water to 1 l. The pH was adjusted to 7.2 with NaOH. Lambda soft agar consists of 0.6% Difco agar.

(c) Efficiency of plating of lambda

Acceptor strains were grown to stationary phase in K-maltose at 37°C. The cultures were centrifuged, resuspended in twice the original volume of 10^{-2} M-MgSO₄, shaken for 1 h at 37°C, and stored at 4°C. To 0.1 ml of phage suspension was added 0.2 ml of the strain to be infected, the mixture incubated 20 min at 37°C, after which it was incorporated into soft agar and poured on lambda plates. The plates were incubated overnight at 37°C.

(d) Ultraviolet irradiation and mitomycin C treatment

Cultures to be irradiated were grown either to logarithmic or stationary phase in complete medium at 37°C. The cells were diluted in cold M9 salts to a concentration of

TABLE I
Genotypes of strains used

Strain	Sex	Genotype
GM28	F ⁻	Prototrophic
GM29	F ⁻	<i>dam-1</i>
GM33	F ⁻	<i>dam-3</i>
GM44	F ⁻	<i>thr-1 leu-6 proA2 metB1 lacY1 galK2 ara-14 tsx-33 thi-1 thyA21 thyR14 sup-37</i>
GM45	F ⁻	As GM44 but <i>dam-3</i>
GM50	F ⁻	As GM44 but <i>dam-3 hsp-1</i> (<i>r_k⁻ m_k⁻</i>)
GM54	F ⁻	<i>dam-3 polA12 lacZ thy rha</i>
GM55	F ⁻	As GM44 but <i>dam-3 uvrA6 metB⁺ argE3</i>
GM56	F ⁻	As GM44 but <i>dam-3 recBts1 thyA⁺</i>
GM58	F ⁻	As GM44 but <i>dam-3 recCts1 thyA⁺</i>
GM64	F ⁻	<i>dam-3 lig-7 lacY A482</i>
GM71	F ⁻	<i>dam-3 recAts leu-6 gal-6 tsx^r lacY1</i> or <i>Z4 tonA2? sup-59?</i>

5×10^6 to 10×10^6 /ml and 3-ml portions irradiated in 10-cm Petri dishes, 43 cm from a General Electric germicidal lamp. The irradiated cultures were diluted in M9 salts and plated in brain-heart infusion agar. The plates were incubated overnight in darkness at 37°C.

Cultures to be treated with mitomycin C were grown to log phase at 37°C, centrifuged and resuspended in M9 salts. Mitomycin C (5 µg/ml) was added and the culture incubated with shaking at 37°C in the dark. At various times, samples were withdrawn, diluted in M9 salts and plated on brain-heart infusion agar to determine survival. The plates were incubated overnight in darkness at 37°C.

(e) *Reversion of genetic markers*

Cultures were grown to stationary phase in M9 medium at 37°C, centrifuged and resuspended in 0.1 the original volume. Portions of 0.1 ml were plated on selective medium and the revertants scored after 2 to 3 days incubation at 37°C.

(f) *Labeling conditions and lysis procedure*

Bacteria were grown in M9 medium or K medium for 3 generations with 0.1 µM-[³H]-thymidine (0.2 Ci/mmol) or 10 µM-[¹⁴C]thymidine (0.02 Ci/mmol) at various temperatures as described in the text. For *thyA*⁺ strains, 100 µg deoxyadenosine/ml was included in the medium. Cultures were harvested and washed once with cold M9 salts and the cells lysed at 0°C as described by Vapnek & Rupp (1970). The cells were resuspended in 1.0 ml 0.05 M-Tris (pH 7.8)-10% sucrose; 0.2 ml lysozyme solution (5 mg/ml in 0.25 M-Tris, pH 8.0) and then 0.4 ml 0.25 M-EDTA (pH 8.0) were added. After 5 min incubation at 0°C, 0.4 ml of a 2% solution of Sarkosyl (sodium lauryl sarcosinate) was added to lyse the spheroplasts. For one set of experiments, the lysate was digested with 500 µg ribonuclease/ml for 30 min at 37°C, followed by 200 µg pronase/ml for 4 to 5 h at 37°C.

(g) *Sedimentation of DNA in neutral and alkaline sucrose gradients*

Samples of 0.1 ml, as described in section (f) above, were layered on 5-ml linear 5% to 20% neutral or alkaline sucrose gradients and centrifuged at 30,000 revs/min at 20°C for 75 min in a Spinco SW39 rotor. Alkaline gradients contained 0.3 M-NaOH, 0.7 M-NaCl and 0.001 M-EDTA. Neutral gradients contained 0.01 M-Tris·HCl (pH 7.8), 0.001 M-EDTA and 1.0 M-NaCl. The gradients were fractionated by pumping from the bottom of the tube. Fractions (6 drops) were collected directly onto Whatman 3 MM discs, which were processed as described previously (Marinus & Adelberg, 1970). Recovery of input DNA was greater than 90%. The equation $S_1/S_2 = (M_1/M_2)^{0.38}$ and a molecular weight of 16×10^6 for single-stranded λ DNA were used to determine the molecular weight of DNA from different fractions (Studier, 1965).

(h) *In vivo assay of methyl group incorporation into DNA*

Methyl group incorporation into DNA of bacterial cultures was determined as described elsewhere (Marinus & Morris, 1973).

(i) *Reagents*

[³H]thymidine (50 Ci/mmol) and [¹⁴C]thymidine (50 mCi/mmol) were obtained from New England Nuclear. Sarkosyl NL97 was purchased from Geigy; ribonuclease and pronase from Worthington Biochemicals Corp.

3. Results

(a) *Residual adenine methylation in vivo in dam strains*

Strains of *E. coli* K12 mutant at the *dam-3* site have only 0.08 mole % 6-MeA compared to 0.5 mole % 6-MeA in the wild type (Table 2). It was possible that the residual adenine methylation observed *in vivo* was due, in part, to the K12 DNA modification methylase, a product of the *hsp* genes. To test this possibility, the

TABLE 2
In vivo adenine methylation in wild type and mutant strains

Strain	Genotype	Cts/min incorporated into DNA				Mol % 6-MeA
		Observed		Adjusted		
		6-MeA	5-MeC	6-MeA	5-MeC	
GM44	Wild	20,046	11,792	20,046	11,792	0.50
GM45	<i>dam-3</i>	3360	12,216	3244	11,792	0.08
GM50	<i>dam-3 hsp-1</i>	1539	5537	3277	11,792	0.08

Cultures were grown in M9 medium with 30 µg adenine/ml and 10 µg [¹⁴C]methyl-L-methionine/ml (11 mCi/mmol). DNA was extracted, purified, hydrolyzed to free bases and the amount of label in 5-MeC and 6-MeA determined as described previously (Marinus & Morris, 1973). The mol % was calculated as follows: the mol % of 6-MeA in wild type DNA was taken from the literature to be 0.50 (Vanyushin *et al.*, 1968). *dam* mutant DNA is known to be fully methylated with respect to 5-MeC; therefore, the cts/min in 5-MeC can be used as an internal standard. The counts in 6-MeA were adjusted to this internal standard by multiplying them by the ratio of wild type cts/min in 5-MeC to mutant cts/min in 5-MeC. The adjusted count was then converted to mol % 6-MeA in DNA by the equation:

$$\frac{\text{adjusted 6-MeA cts/min in mutant}}{\text{adjusted 6-MeA cts/min in wild type}} = \frac{x \text{ mol \%}}{0.5 \text{ mol \%}}$$

extent of *in vivo* adenine methylation was determined in strains GM45 (*dam-3 hsp*⁺) and its derivative GM50 (*dam-3 hsp-1*). The results in Table 2 show that there was no detectable difference in adenine methylation between the two strains. The residual activity could be due to either an unidentified adenine methylase or to "leakiness" of the *dam-3* mutation.

(b) *Abnormal restriction by dam strains*

The original *dam* strains isolated after nitrosoguanidine treatment, GM2, 3 and 4, restrict unmodified λ less efficiently than the wild type by a factor of 10⁻¹ to 10⁻³ (Marinus & Morris, 1973). When the *dam-1* and *dam-3* mutations were transduced by phage P1 into several different genetic backgrounds, restriction of unmodified λ was only 0.5 to 0.1 that of wild type. The data for one set of transductants is shown in Table 3. In agreement with the previous data (Marinus & Morris, 1973), no difference was found between *dam* and wild type strains in their ability to modify λ DNA (data not shown). The decreased ability to restrict unmodified λ by strains GM2, 3 and 4 is presumably due to other extraneous mutations acting in concert with the *dam* mutation.

TABLE 3
Efficiency of plating of λ. O and λ. K on wild type and dam mutants

	GM28 (wild)	GM29 (<i>dam-1</i>)	GM33 (<i>dam-3</i>)	M94 (<i>hsp</i> ⁻)
λ. O (M94)	3.8 × 10 ⁻⁴	7.5 × 10 ⁻³	3.8 × 10 ⁻³	1.0
λ. K (GM28)	1.0	1.0	1.0	1.0

Phage lysates were prepared from GM28 and M94 and the efficiency of plating on wild type and mutant strains determined as described in Materials and Methods.

(c) *Cultural characteristics of dam strains*

Strains GM28 (wild), GM29 (*dam-1*) and GM33 (*dam-3*) were produced by phage P1 transduction of W3110 *trpS*, advantage being taken of the fact that *dam* and *trpS* are 90% co-transducible (Marinus, 1973). These strains have the same doubling time in broth (36 min) or minimal medium (65 min) and they incorporate radioactive thymidine into DNA and leucine into protein at similar rates (data not shown). For a given optical density, however, the viable count of GM29 (*dam-1*) and GM33 (*dam-3*) was always two to three times less than that of GM28 (wild). This suggests that either the *dam* cells have a greater mass than the wild type or that the cells are of similar size but a proportion of the *dam* population is inviable. Direct microscopic observation showed that the *dam* cells were two to four times longer than the otherwise isogenic wild type, suggesting that the difference in size could account for the observed change in the ratio of optical density to viable count.

(d) *Increased ultraviolet irradiation and mitomycin C sensitivity and mutability in dam strains*

Like the *dam* mutants, certain u.v. irradiation-sensitive strains of *E. coli* show an abnormal optical density to viable count ratio (Capaldo-Kimball & Barbour, 1971). There also appears to be a change in the extent of DNA methylation after u.v. irradiation of *E. coli* (Whitfield & Billen, 1972); and Schein *et al.* (1972) have suggested that the absence of methylated bases in *Micrococcus radiodurans* DNA may be related to the u.v. irradiation-resistance of this strain. These facts prompted us to examine the u.v. irradiation sensitivity of the *dam-3* strain as well as other metabolic and phenotypic changes associated with u.v. irradiation-sensitive strains of *E. coli*.

The results in Figures 1 and 2 show that GM33 (*dam-3*) is more sensitive to u.v. irradiation and mitomycin C than GM28 (wild). The survival of AB2470 (*recB21*) is shown in Figure 1 for comparison. The rate of u.v.-induced DNA degradation is the same in wild type and *dam-3* strains (data not shown). Moreover, wild type and

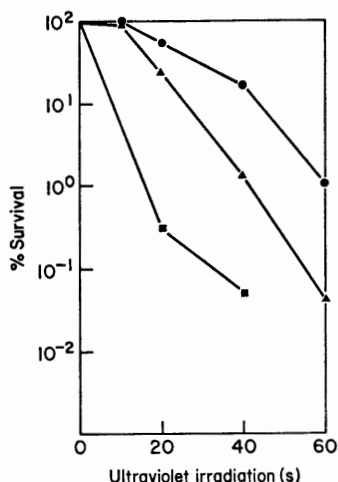


FIG. 1. Survival of GM28 (wild), GM33 (*dam-3*) and AB2470 (*recB21*) following exposure to u.v. irradiation. Stationary phase cells were diluted in cold M9 salts, irradiated for the times shown and survival measured as described in Materials and Methods. —●—●—, GM28; —▲—▲—, GM33; —■—■—, AB2470.

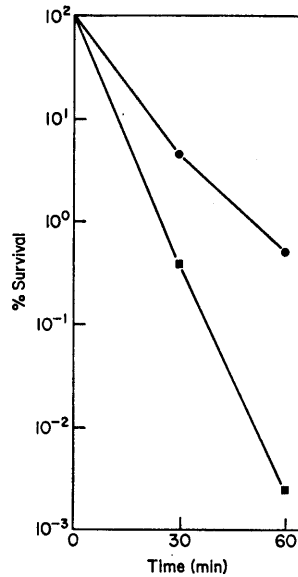


FIG. 2. Survival of GM28 (wild) and GM33 (*dam-3*) following exposure to mitomycin C. Log phase cells in M9 salts plus 5 μg mitomycin C/ml were incubated in the dark with shaking at 37°C. At the times indicated, portions of the culture were removed and viability determined as described in Materials and Methods. —●—●—, GM28; —■—■—, GM33.

dam-3 cells are equally proficient in host cell reactivation of a u.v.-irradiated λ phage, the DNA of which is 6-MeA deficient to the same extent as *dam-3* host DNA (Marinus & Morris, unpublished data). The u.v. irradiation sensitivity of *dam* bacteria, therefore, is due to some cytoplasmic component and is not associated with 6-MeA deficient DNA *per se*.

An increase in mutability is observed in *E. coli* under conditions (e.g. u.v. irradiation) promoting breakage and repair of DNA (Witkin, 1969). That there may be increased DNA breakage and repair in *dam-3* bacteria is suggested by the data in Table 4. The Table shows that the rate of reversion of identical genetic markers in strains GM44 (wild) and GM45 (*dam-3*) is four to 45 times higher in the *dam-3* background depending on the marker selected.

Certain u.v. irradiation-sensitive strains of *E. coli* are more susceptible to thymine-less death than wild type strains (Sedgwick & Bridges, 1971; Siegel, 1973). This is

TABLE 4
Reversion frequencies ($\times 10^{-9}$) of identical markers in *dam-3* and wild type strains

	<i>leu-6</i>	<i>proA2</i>	<i>lacY1</i>	<i>strA</i>	<i>rif</i>
GM44 (wild)	3.67	0.16	4.5	0.16	4.0
GM45 (<i>dam-3</i>)	26.3	2.67	161.0	5.0	182.4
GM45	7.2	16.7	35.7	31.0	45.6
GM44					

Stationary phase cultures of GM44 and GM45 were centrifuged, concentrated and portions plated on selective media. After 2 to 3 days incubation at 37°C the colonies were scored. *strA* and *rif* denote mutation from sensitivity to resistance.

not true of the *dam* mutants, the loss of viability in the absence of thymine of GM44 (*thy*⁻ *dam*⁺) and GM45 (*thy*⁻ *dam*-3) occurs at the same rate (data not shown). *rec*⁻ strains of *E. coli* are recombination deficient in addition to being sensitive to u.v. irradiation (Clark & Margulies, 1965). The *dam*-3 mutation, however, has no effect on recombination proficiency in conjugation or transduction-mediated recombination.

(e) *Molecular weight of DNA from dam strains*

Many of the phenotypic differences between *dam* and wild type strains could be explained by the presence of a "lesion" in the DNA of the *dam* strains. To investigate this possibility, the DNA of exponentially growing cultures of GM1 (wild) and GM45 (*dam*-3) was uniformly labeled with [¹⁴C]thymidine and [³H]thymidine, respectively. The cells were harvested, pooled, lysed and portions of the lysate sedimented through alkaline sucrose gradients (Fig. 3). The sedimentation profile of wild type [¹⁴C]DNA shows a uniform distribution with a single peak. The sedimentation profile of [³H]DNA, however, is much broader due to an increase of more slowly sedimenting material. Results similar to those in Figure 3 were obtained if the strains were lysed separately and the DNA co-sedimented. The difference in sedimentation profiles of DNA from wild type and *dam* bacteria was variable from experiment to experiment and dependent on the growth medium and genetic background of the strains. The difference was most pronounced in polyauxotrophic *dam* strains growing in minimal medium but was almost undetectable in otherwise prototrophic *dam* strains growing in rich medium. The broader sedimentation profiles were detected in the original *dam* isolates, GM2, 3 and 4, obtained by nitrosoguanidine mutagenesis and in *dam* recombinants prepared by phage P1 transduction. The broader sedimentation profiles were not found in *dam*⁺ transductants arising from the same cross. It thus appears that the broader sedimentation distribution of DNA

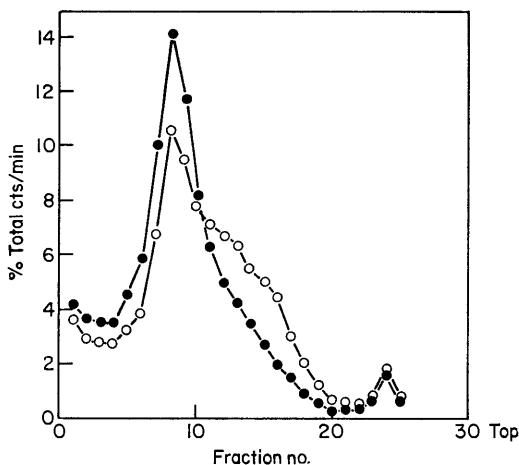


FIG. 3. Zone sedimentation in alkaline sucrose of [¹⁴C]DNA from GM1 (wild) and [³H]DNA from GM45 (*dam*-3). After 3 generations of growth in radioactive M9 medium, the cells were harvested, pooled and lysed and portions sedimented in 5% to 20% alkaline sucrose gradients. The gradient was centrifuged in an SW39 rotor at 30,000 revs/min for 75 min at 20°C. Total ³H cts/min, 185,444; total ¹⁴C cts/min, 20,166. —○—○—, [³H]DNA from GM45; —●—●—, [¹⁴C]DNA from GM1.

is a specific consequence of the *dam* mutations, and can be interpreted to mean that there are more "breaks" in the chromosome of *dam* bacteria than wild type. (Throughout this paper the word break will be used to refer to a "lesion" in methyladenine-deficient DNA, which results in a sedimentation rate in alkaline sucrose slower than that of wild type DNA.)

The breaks in DNA of *dam* strains may be the result of nuclease action, and it was possible that endonuclease III, the restriction enzyme, was involved in this process. The sedimentation profiles of DNA from strains GM45 (*dam-3 hsp⁺*) and GM50 (*dam-3 hsp-1*) were, however, identical showing that this endonuclease is not responsible for introducing breaks in DNA.

(f) *Sedimentation profiles of DNA from GM54 (dam-3 polA12) and GM64 (dam-3 lig-7)*

Since there are breaks in the DNA of *dam* mutants, and because these strains grow normally, it was possible that the DNA is continually being broken and subsequently repaired by repair enzymes to ensure viability. Consequently, it might be expected that a *dam* strain that is unable to repair DNA damage would be inviable. To test this possibility, a strain (GM54) carrying *dam-3* and *polA12* was constructed by P1 transduction. The latter mutation specifies a temperature-sensitive DNA polymerase I (Monk & Kinross, 1972). This strain, GM54, is temperature sensitive for growth, whereas the *dam-3 polA⁺* or *polA12 dam⁺* parental strains are not. Shifting an exponentially growing culture of GM54 from 30°C to 43°C induces filamentation and decreases the rate of [³H]thymidine incorporation compared to the same culture growing at 30°C (data not shown).

The DNA of a *dam*, repair-deficient strain would be expected to be of lower average

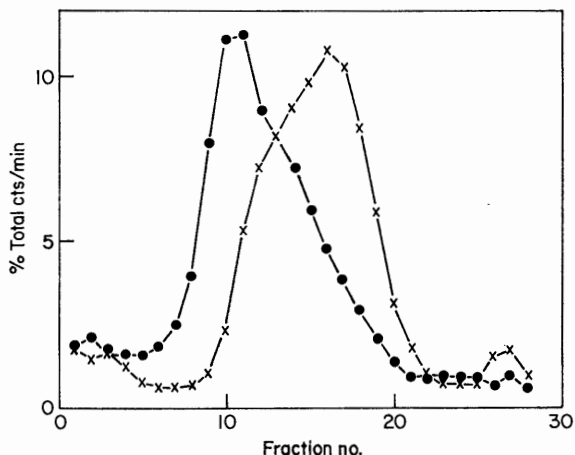


FIG. 4. Sedimentation in alkaline sucrose of [¹⁴C]DNA from GM54 (*dam-3 polA12*) grown at 30°C and [³H]DNA from GM54 grown at 42°C. A logarithmic phase culture of GM54 growing in K medium at 30°C was split and half incubated at 30°C with [¹⁴C]thymidine and the other half at 42°C with [³H]thymidine. After 2 h incubation the cells were harvested, pooled, lysed and portions sedimented in alkaline sucrose gradients. The gradient was centrifuged in an SW39 rotor at 30,000 revs/min for 75 min at 20°C. Total ³H cts/min, 109,211; total ¹⁴C cts/min, 10,159. —x—x—, [³H]DNA from GM54 grown at 42°C for 2 h; —●—●—, [¹⁴C]DNA from GM54 grown at 30°C for 2 h.

molecular weight than DNA from a *dam*, repair-proficient strain. A culture of GM54 growing exponentially at 30°C was split and one half incubated at 43°C with [³H]-thymidine and the other at 30°C with [¹⁴C]thymidine. After two hours of incubation the cells were harvested, pooled, lysed and co-sedimented through a 5% to 20% alkaline sucrose gradient (Fig. 4). The results show that the [¹⁴C]DNA sediments faster than the [³H]DNA. In separate experiments using λ DNA as a marker, the fast sedimenting DNA has an average molecular weight of around 200×10^6 and the slow sedimenting DNA about 60×10^6 . The DNA of a GM54 culture incubated for four hours at 43°C gave an identical distribution to that shown for the [³H]DNA in Figure 4. This suggests that the DNA may reach a limit size.

A double mutant strain, GM64 bearing *dam-3* and *lig-7*, was constructed by P1 transduction. The latter mutation specifies a temperature-sensitive DNA ligase (Pauling & Hamm, 1968), which results in a lethal phenotype at 43°C. The DNA from the double mutant grown at the restrictive temperature is of lower molecular weight than that from the same strain grown at the permissive temperature (Fig. 5(a)). Furthermore, the distribution and average molecular weight of the slowly sedimenting DNA is the same as that obtained from strain GM54 (*dam-3 polA12*) grown at the restrictive temperature (Fig. 4). That the reduction in molecular weight of DNA in

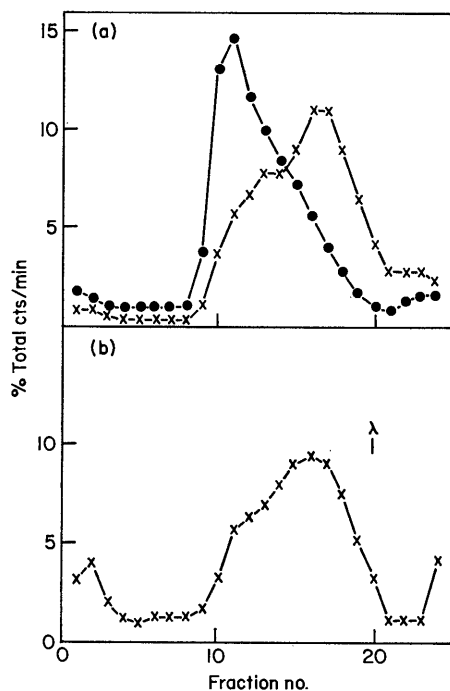


FIG. 5. Zone sedimentation in alkaline sucrose of DNA from GM64 (*dam-3 lig-7*).

(a) Co-sedimentation of DNA from GM64 grown in K medium at 30°C with [¹⁴C]thymidine and at 42°C for 2 h with [³H]thymidine. Total ³H cts/min, 30,431; total ¹⁴C cts/min, 25,908.

—x—x—, [³H]DNA; —●—●—, [¹⁴C]DNA.

(b) Co-sedimentation of DNA from GM64 and λ DNA. A culture of GM64 was grown in K medium with [³H]thymidine, centrifuged and washed to remove label. The culture was shifted to 42°C in non-radioactive medium and grown for 2 h. Total ³H cts/min, 6840. Gradients were centrifuged at 30,000 revs/min in an SW39 rotor for 75 min. —x—x—, [³H]DNA.

the *dam-3 lig-7* strain is not an artifact due to ^3H decay, was demonstrated by reversing the isotope labels (Fig. 6).

The DNA isolated from *polA12 dam+* and *lig-7 dam+* strains grown at 42°C for two hours in the presence of [^3H]thymidine was also examined on alkaline sucrose gradients. The molecular weight of such DNA was about 200×10^6 . The reduction in average molecular weight of DNA in the double mutants, therefore, is due specifically to the combination of *dam-3* with *lig-7* or *polA12* and not due to the action of the latter mutations alone.

It was possible that the reduction in molecular weight of DNA in *dam*, repair-deficient strains is a property of newly synthesized DNA, since in the above experiments the molecular weight of newly synthesized DNA has been estimated. To

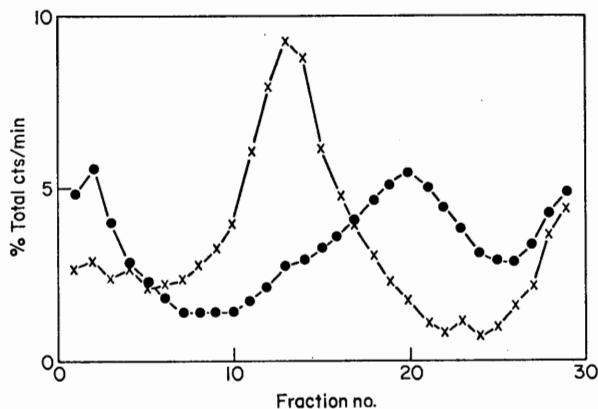


FIG. 6. Sedimentation in alkaline sucrose of [^{14}C]DNA from GM64 (*dam-3 lig-7*) grown at 42°C and [^3H]DNA from KS253 (*dam+ lig-7*) grown at 42°C . Logarithmic phase cultures of GM64 and KS253 growing in K medium at 30°C were shifted to 42°C and [^3H]thymidine added to the KS253 culture and [^{14}C]thymidine to the GM64 culture. After 2 h incubation the cells were harvested, pooled, lysed and portions sedimented in alkaline sucrose gradients. The gradient was centrifuged in an SW39 rotor at 30,000 revs/min for 60 min at 20°C . Total ^3H cts/min, 37,500; total ^{14}C cts/min, 7652. —●—●—, [^{14}C]DNA from GM64 grown at 42°C for 2 h; —×—×—, [^3H]DNA from KS253 grown at 42°C for 2 h.

investigate this possibility, strain GM64 (*dam-3 lig-7*) was grown to logarithmic phase with [^3H]thymidine at 30°C to uniformly label the DNA. The culture was centrifuged, washed free of label, and shifted to 42°C in non-radioactive medium. After two hours the culture was harvested and DNA from it subjected to analysis on alkaline sucrose gradients. The data in Figure 5(b) show that the labeled DNA has a sedimentation profile identical to that in Figure 5(a). The interruptions in DNA of *dam* cells are, therefore, not exclusively a property of newly synthesized DNA.

To determine if the slowly sedimenting DNA is produced by single or double-stranded breaks, the same lysate used to obtain data for Figure 5(a) was digested with ribonuclease and pronase, followed by sedimentation in neutral and alkaline sucrose. The data in Figure 7 show that the two DNA species co-sedimented in neutral sucrose, but some of the [^3H]DNA sediments slower than the [^{14}C]DNA in the alkaline gradient. The interruptions in DNA from *dam-3 lig-7* bacteria, therefore, appear to be single-stranded.

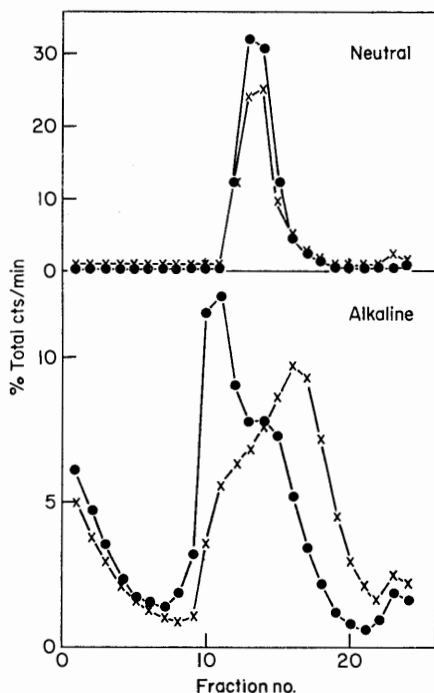


FIG. 7. Zone sedimentation of DNA from GM64 (*dam-3 lig-7*). The same lysate used to obtain the data in Fig. 5(a) was digested with 500 μ g ribonuclease/ml for 30 min at 37°C followed by 200 μ g pronase/ml for 4 h at 37°C. Portions of the lysate were sedimented through neutral and alkaline sucrose gradients. The gradients were centrifuged at 30,000 revs/min in an SW39 rotor for 75 min. Total cts/min were the same as for Fig. 5(a). —x—x—, [3 H]DNA; —●—●—, [14 C]DNA.

(g) *Combination of dam-3 with other genes involved in DNA repair*

Since *dam-3* in combination with *polA12* is lethal, we attempted to isolate strains carrying *dam-3* with mutations in other genes involved in DNA repair. Strain GM55 (*dam-3 uvrA6*) is viable, showing that the *uvrA* gene product is not involved in the repair of *dam* DNA.

Strains GM56 (*dam-3 recBts1*), GM58 (*dam-3 recCts1*) and GM71 (*dam-3 recAts*) were isolated and are temperature sensitive for growth, whereas *dam*⁺ *recBts1*, *dam*⁺ *recCts1* and *dam*⁺ *recAts* strains are not. Shifting an exponentially growing culture of these strains from 30°C to 42°C induced filamentation and decreased [3 H]thymidine uptake compared to the 30°C control culture (data not shown). The average molecular weight of DNA isolated from these strains grown at the permissive or non-permissive temperature for two hours was the same (about 200×10^6) in alkaline sucrose gradients (data not shown). The sedimentation distribution of this DNA, however, was broader than control DNA due to an increase in more slowly sedimenting material. The data were essentially the same as that shown in Figure 3 for *dam*⁺ and *dam*⁻ strains.

In section (f), evidence was presented that endonuclease III was not responsible for the observed breaks in the DNA of *dam* strains. Further evidence for this was obtained from a strain bearing *dam-3 recCts1* and *hsp-1*, which is temperature sensitive for growth, showing that the *hsp-1* mutation cannot suppress the temperature-sensitive phenotype.

(h) *Analysis of revertants*

Since *dam-3* in combination with *polA12* or *recBts1* or *recCts1* results in a temperature-sensitive phenotype, at least three types of revertants able to grow at the non-permissive temperature can be expected: (1) those that are *dam*⁺ or (2) *dam*⁻ *pol*⁺ and *dam*⁻ *rec*⁺ or (3) *dam*⁻ *pol*⁻ and *dam*⁻ *rec*⁻. Revertants of class (1) would be useful to show that *dam* is the structural gene for a DNA adenine methylase and class (3) revertants would be those in which nucleases acting on methyladenine-deficient DNA would be non-functional. Thus far 300 revertants able to grow at 43°C have been isolated from GM54 (*dam-3 polA12*), GM56 (*dam-3 recBts1*) and GM58 (*dam-3 recCts1*). All were class (2) revertants, indicating that the *dam-3* mutation is quite stable compared to the temperature-sensitive mutations.

4. Discussion

From the data presented, it appears that a probable function of *dam*-specified 6-MeA residues is to protect DNA from an endonuclease(s). This hypothesis would predict that mutants devoid of 6-MeA may not be viable. In fact, from 1500 screened nitrosoguanidine-treated survivors, only three *dam* strains were isolated and all contained substantial amounts of 6-MeA *in vivo* (Marinus & Morris, 1973). This residual activity is not due to the *hsp* modification methylase (Table 2). It could be due to either an as yet unidentified DNA adenine methylase or to the leakiness of the *dam* mutations. The original screening procedure for obtaining mutants was designed to isolate temperature-sensitive *dam* mutations. Since none of these was isolated, we are currently using other techniques to isolate such strains, which if isolated should demonstrate that *dam* is the structural gene for a DNA adenine methylase.

Although the *dam* and *hsp* DNA adenine methylases have similar biological functions, in that they both appear to protect DNA from specific nuclease attack, there is evidence that the gene products are biochemically distinct. *dam* strains modify DNA normally, suggesting that the recognition sites for the two enzymes are different. Furthermore, *hsp* mutants do not show any of the *dam* phenotypic traits, except loss of restriction, and contain normal amounts of 6-MeA in DNA within the limits of detection (Gough & Lederberg, 1966; Arber, 1968; Mamalak & Boyer, 1970). The data in Table 2 agree with these findings and show that the contribution of *hsp* 6-MeA to the total is minute, probably less than 2 to 3%. The findings that there are breaks in the DNA of GM50 (*dam-3 hsp-1*), and that *hsp-1* is unable to suppress the lethal phenotype of *dam-3 recCts1* cells, indicates that endonuclease III is not instrumental in the production of breaks in methyladenine-deficient DNA.

That there are breaks in the DNA of *dam* strains would explain the observed phenotypic differences between *dam* and wild type strains. Since treatments such as u.v. irradiation that induce breakage and repair of DNA, lead to filamentation of the cells (Howard-Flanders, 1968), the finding that *dam* bacteria are longer than normal was not unexpected. The decreased restriction of unmodified λ DNA and the u.v. irradiation sensitivity of *dam-3* cells can be explained by assuming that the nucleases acting on 6-MeA-deficient DNA are also involved in repair of u.v. damage and in restriction of foreign DNA.

That the *recB,C* nuclease is involved in the repair of breaks in methyladenine-deficient DNA may explain the sensitivity to u.v. irradiation and decreased restriction

of unmodified DNA in *dam* cells. It is known that the *recB,C* nuclease is involved in both these processes (Simmon & Lederberg, 1972). On infection of *dam* bacteria with unmodified λ phage or exposure to u.v. irradiation, the *recB,C* enzyme may become saturated and restriction and repair not be as efficient as in a wild type strain.

If there is increased breakage and repair of DNA in *dam* strains, there should also occur an increased number of mistakes during DNA repair leading to an increase in mutation rate. The data in Table 4 verify this prediction. A further prediction is that *dam*, repair-deficient strains should not be viable. In sections (f) and (g), it was shown that *dam-3* in combination with *polA*, *recA*, *recB* and *recC* leads to inviability. In the *dam*, repair-proficient strains there would be constant breakage and repair of DNA such that, at any given time, only a few breaks would be detected (Fig. 3). In the absence of repair capability, breaks would not be repaired and DNA should sediment more slowly than control DNA on alkaline sucrose gradients. The data in section (f) clearly show that this is the case for *dam* strains lacking DNA polymerase I or DNA ligase.

The DNA isolated from *dam-3* bacteria contains breaks (Fig. 3), and these are amplified in *dam-3 polA12* and *dam-3 lig-7* strains (Figs 4 and 5). These breaks are not an artifact of radiation damage due to ^3H decay (Fig. 6). Because apparent breakage of DNA strands has been demonstrated only in alkaline sucrose, the possibility that alkali treatment introduces breaks selectively in methyladenine-deficient DNA cannot be excluded. We have found, however, that methyladenine-deficient λ DNA and fully methylated λ DNA co-sediment in sucrose gradients after alkali denaturation (Marinus & Morris, unpublished data). This reduces the likelihood, but does not exclude the possibility, that DNA from *dam* bacteria contains structures (e.g. ribonucleotides) which, upon alkali denaturation, would be hydrolyzed and thus reduce the molecular weight of DNA. That breaks in DNA actually exist *in vivo* would seem to be supported by the phenotype of *dam* strains and the fact that *polA* and *recA,B,C*, which are involved in the repair of single-strand DNA interruptions, when combined with *dam* render the cell inviable.

It is possible that there is a nuclease that introduces single-strand breaks specifically in methyladenine-deficient DNA. The identity of this hypothetical nuclease is not known, but endonuclease III can be excluded (sections (e) and (g)). It is equally possible that the nuclease introducing breaks in methyladenine-deficient DNA is non-specific and can also attack fully methylated DNA. In this case, the assumption can be made that single-strand interruptions are always present in DNA of *E. coli*, and are sealed at a slower rate in *dam* mutants than in wild type cells. For example, it has been established in *E. coli* that DNA replication is discontinuous; the primary product being small DNA chains that are joined to yield high molecular weight DNA (Sakabe & Okazaki, 1966). We have found that the size of such nascent DNA pieces and their rate of incorporation into high molecular weight DNA is the same in *dam-3* and *dam*⁺ bacteria (Marinus & Morris, unpublished data). Currently, we are attempting to identify the nuclease responsible for introducing breaks in methyladenine-deficient DNA.

The average molecular weight of DNA isolated from *dam-3 polA12* or *dam-3 lig-7* strains grown at the non-permissive temperature is 60×10^6 . Yet there are approximately 4.4×10^4 methyladenine residues per normally methylated chromosome, and if each of these were a site for nuclease attack the observed molecular weight of DNA in *dam-3* bacteria should be much less than 60×10^6 . The discrepancy between

expected and observed molecular weight of DNA may be: (1) that sequences of bases recognized by DNA adenine methylase are not identical to those of the hypothetical nuclease; (2) that the sequences are identical but most sensitive sites are protected by some feature of chromosomal structure; (3) that the 6-MeA residues are not distributed randomly throughout the chromosome; (4) that an equilibrium exists between breakage and residual repair; or (5) between breakage and net DNA synthesis. Experiments are in progress to discriminate between these possibilities.

We thank Michael Gottesman, I. Lehman, B. Low and J. I. Tomizawa for providing bacterial strains. This work was supported by grants to one of us (N. R. M.) from the U.S. Public Health Service (grant no. CA10665), and an Antoinette Adler Memorial grant for Cancer Research from the American Cancer Society (grant no. NP101A).

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