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DNA REPAIR AND MUTAGENESIS OF BACTERIOPHAGE T7

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Lori Ann Dodson

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This thesis is dedicated
to all the people that have helped me
through the years, especially my beloved
parents, sisters, and Warren.

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ABSTRACT

A DNA replication-packaging system of bacteriophage T7 has been developed as a vehicle for studying mutagenesis. With this system, the complete life-cycle of T7 can be performed in vitro and the biological consequences of in vitro manipulations determined by examining viable phage particles. The close resemblance between this system and the in vivo condition was demonstrated in part by in vivo and in vitro host cell reactivation studies of T7 damaged by alkylating agents. Moreover, these studies provided good evidence for in vitro repair of at least some of the lethal damage inflicted by these chemicals and demonstrated significant levels of in vitro DNA synthesis even when alkylated DNA was provided as template. The actual demonstration of mutagenesis was accomplished by including O⁶-methyldeoxyguanosine triphosphate as a precursor during the DNA synthesis reaction and measuring either reversion of an amber mutation or generation of temperature-sensitive mutants in phage produced by in vitro packaging. Until now, only indirect evidence supported the proposal that alkylating agents are mutagenic because of their ability to react at the oxygen sites in DNA, particularly the O-6 position of guanine. The aforementioned mutagenesis result provided the first direct evidence for the mutagenic role of O⁶-methylguanine and also demonstrated the high fidelity of in vitro replication of T7 under standard conditions. Measurements of reversion were made possible by modifying the encapsulation protocol so that very high DNA packaging efficiencies were routinely obtained. A novel application of the in vitro T7 system is to examine molecular mechanisms of host functions induced by DNA damage,

and some experiments directed towards this goal were performed. Inducible reactivation of T7 phage damaged by alkylating agents or UV light was demonstrated and the phenomena were characterized with respect to the induction kinetics and dependence on host functions.

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CHAPTER I

INTRODUCTION

An understanding of cellular mechanisms responsible for maintaining genetic integrity is essential to our understanding of fundamental biological processes such as mutagenesis and aging, as well as cancer and other diseases. Highly accurate DNA replication is a multicomponent process involving at least three discrimination steps that reduce errors. This normally accurate synthesis of DNA can be perturbed by chemicals and radiation that introduce lesions into the genetic material, thereby altering the template for replication. Alterations in replication proteins and substrates may also diminish fidelity. In the case of UV radiation damage, and perhaps many other types of damage as well, a majority of the mutations arise from the processing of DNA damage by cellular DNA synthesis and repair systems. Even though extensive information concerning these cellular processing systems has accumulated in recent years, many molecular details remain unknown. To facilitate our understanding of the genetics and biochemistry of mutagenesis, the efforts of several laboratories have been directed towards in vitro studies of DNA replication and repair. The subject of this thesis is the development and characterization of an in vitro DNA replication-packaging system that makes it possible to use bacteriophage T7 as a vehicle for studying various aspects of mutagenesis. Potential applications of this system include the study of molecular mechanisms of host responses induced by DNA damage.

Fidelity of DNA Synthesis

Measurements of "spontaneous" mutation frequencies suggest that the average frequency of base pair substitutions is in the range of 10^{-7} to 10^{-11} misincorporations per base pair replicated (for review, see Cox, 1976). This high accuracy of DNA synthesis results from at least three discrimination steps that reduce errors (for review, see Loeb and Kunkel, 1982). The first and second discrimination steps occur at the growing point of the DNA chain. The replication machinery initially reduces errors by discriminating against insertion of an incorrect nucleotide or by preferential selection of correct nucleotides (base selection). The second discrimination step occurs after nucleotide insertion. Mistakes are removed by the action of a 3'→5' exonuclease at the time of or immediately after incorporation (proofreading). The specificity of base selection and proofreading results from the DNA polymerase, accessory replication proteins, the nature of the mismatch, and the surrounding nucleotide sequence. A postsynthetic correction of mismatches (mismatch repair) provides a third opportunity to reduce errors. In bacteria, newly synthesized DNA is recognized by a delay in methylation at the N-6 position of adenine in GATC sequences. This may ensure that noncomplementary base pairs are excised only from the daughter strand.

The fidelity of DNA replication can be diminished by variations in the nucleotide substrates. During polymerization reactions in vitro, large imbalances in the relative ratios of the deoxynucleotide precursors favor potentially incorrect substitutions. Certain modified deoxynucleoside triphosphates can be incorporated by DNA polymerases, and many early studies of fidelity and mutagenesis employed the base analogues

aminopurine and bromodeoxyuridine. More recently, the deoxynucleotide precursor pool has been identified as a significant *in vivo* target of modification by chemical mutagens (Topal and Baker, 1982). Residues alkylated at positions involved in Watson-Crick hydrogen bonding (such as O⁶-methyldeoxyguanosine triphosphate) and some other base analogues are thought to be mutagenic due to the "ambiguous" nature of their base pairing during incorporation or replication (Singer and Kroger, 1979). Alterations in the template itself by chemical mutagens and radiation also enhances misincorporation when the template is copied by DNA polymerases. However, for some types of damage, cellular processing of lesions by DNA repair systems appears to be required for the fixation of mutations and the production of altered genetic information.

Cellular Responses to DNA Damage

DNA itself is quite sensitive to damage by harmful radiations and chemical agents in the environment. The stability of DNA and the precision of its replication are preserved in part by enzymes that continually repair genetic lesions (for review, see Hanawalt et al., 1979; Lindahl, 1982). Excision repair is one of the fundamental DNA repair processes, effective in removing bulky lesions such as pyrimidine dimers introduced by UV irradiation. In E. coli, the initial step is incision of a phosphodiester bond by the concerted action of at least the uvrA, uvrB, and uvrC gene products, and the cleavage may occur adjacent to, or on both sides of, the dimer. Alternatively, in Micrococcus luteus and phage T4-infected E. coli, a pyrimidine dimer-DNA glycosylase cleaves initially the 5' glycosyl bond within a pyrimidine dimer in DNA. Several additional glycosylases specific for other types of nonconventional residues have

also been identified and these enzymes are widely, if not universally, distributed.

Many conditions that damage DNA or inhibit DNA replication induce an emergency response in E. coli that is referred to as the SOS cascade (for review, see Witkin, 1976; Little and Mount, 1982). The cellular responses induced include enhanced capacity for DNA repair and mutagenesis, altered responses to phage infection, prophage induction, and inhibition of cell division. The primary event believed to trigger the coordinated expression of these functions ("SOS response") is activation of RecA protein as a protease which specifically inactivates the LexA repressor. Genes repressed by LexA (referred to as din genes for damage-inducible) are then expressed at much higher levels. Currently identified din genes include the nucleotide excision repair genes uvrA and uvrB, two genes mapped at the umuC locus (Elledge and Walker, 1983) that are required for mutagenesis by a variety of agents, and also the recA and lexA genes themselves.

The adaptive response (Samson and Cairns, 1977) is a second kind of induced response to genetic damage, quite distinct from the SOS response. After prolonged exposure of E. coli to low concentrations of an alkylating agent, the bacteria are extremely resistant to the killing and mutagenic effects of a subsequent exposure to the drug at a concentration hundreds of times greater. The enhanced survival and decreased mutagenesis are due to the induction of enzymes that specifically repair particular kinds of damaged bases (Schendel and Robins, 1978; Evensen and Seeberg, 1982; Karran et al., 1982).

T7 as a Model In Vitro System

Our understanding of the molecular events that constitute biological processes such as DNA replication and recombination has been enhanced significantly by the development of in vitro systems. Bacteriophage T7 provides an ideal system with which to study DNA metabolism, in part because of the large background of information concerning the genetics (for review, see Studier, 1972; Hausmann, 1977) and biochemistry (for review, see Richardson et al., 1979) of this phage and the thorough characterization of the T7 molecule (Rosenberg et al., 1979; Dunn and Studier, 1981). More importantly, however, is the ability to complete multiple rounds of chromosome duplication in an in vitro DNA synthesis reaction (Hinkle and Richardson, 1974; Masker and Richardson, 1976) and, in a separate reaction, to package DNA into phage heads to form viable phage (Kuemmerle and Masker, 1977; Masker and Serwer, 1982). Thus, in vitro packaging can be employed as a probe for examining the biological activity and genetic make-up of T7 DNA subjected to prior in vitro manipulations. As part of this thesis, the coupled DNA replication-packaging system was developed as a vehicle for studying mutagenesis.

The initial experiments (Chapter II) were designed to characterize the response of the in vitro DNA synthesis and packaging reactions to DNA containing alkylation damage. We found that the survival of intact phage after exposure to methyl or ethyl methane sulfonate (MMS and EMS, respectively) closely resembled the biological activity of DNA pretreated with these alkylating agents and subsequently packaged into phage heads. We also observed at least partial repair of DNA damage immediately prior to or during encapsulation and significant levels of DNA synthesis when

alkylated DNA was provided as template. These studies supported the proposal that the in vitro system accurately reflects the in vivo condition and provided a basis for our in vitro measurements of mutagenesis by O⁶-methylguanine (m⁶G).

The actual demonstration of mutagenesis with the in vitro T7 system was accomplished by including m⁶dGTP as a precursor during the DNA synthesis reaction (Chapter III). The m⁶G lesion is especially interesting because it is repaired in a novel fashion by an inducible methyltransferase (Karran et al., 1979; Olsson and Lindahl, 1980; Karran et al., 1982) and has been postulated as a mutagenic lesion based on indirect evidence obtained with several alkylating agents (Coulondre and Miller, 1977; Abbot and Saffhill, 1979). Mutagenesis was demonstrated using two distinct assays: (1) reversion of a specific amber mutation in an essential gene and (2) generation of forward temperature-sensitive mutants. In addition, these experiments established that the fidelity of in vitro DNA replication of T7 is nearly the same in vitro as it is in vivo. Problems with recombination during the DNA synthesis reaction were circumvented by genetic means, and improvements in the efficiency of packaging DNA enabled us to use reversion as an assay.

A potential application of this in vitro T7 system is to examine molecular mechanisms of host SOS responses induced by DNA damage. Preliminary in vivo experiments directed towards this goal are described in Chapter IV. We demonstrated inducible reactivation of T7 phage damaged by MMS or UV light and characterized the phenomenon with respect to induction kinetics and the dependence on host functions.

CHAPTER II

IN VITRO HOST CELL REACTIVATION OF ALKYLATED BACTERIOPHAGE T7 DNA BY REPAIR-DEFICIENT STRAINS OF E. COLI

Summary

An in vitro system capable of packaging T7 DNA into phage heads to form viable phage particles has been used to monitor the biological consequences of DNA damaged by alkylating agents and an in vitro DNA replication system has been used to examine the ability of alkylated T7 DNA to serve as template for DNA synthesis. The survival of phage resulting from in vitro packaging of DNA pre-exposed to various concentrations of MMS or EMS closely paralleled the in vivo situation in which intact phage were exposed to the alkylating agents. Host factors responsible for survival of alkylated T7 have been examined by using wild type strains of E. coli and mutants deficient in DNA polymerase I (polA) or 3-methyladenine-DNA glycosylase (tag). For both the in vivo and in vitro situations, a deficiency in 3-methyladenine-DNA glycosylase dramatically reduced phage survival relative to wild type whereas a deficiency in DNA polymerase I had an intermediate effect. Furthermore, when using the tag mutant as an indicator strain, phage survival was enhanced when alkylated DNA was packaged with extracts prepared from a wild type strain in place of the tag mutant or by complementing a tag extract with an uninfected tag extract, indicating in vitro repair during packaging.

Introduction

An integrative scheme for repair of E. coli and phage DNA containing damaged bases has recently been proposed (Warner et al., 1980). One general pattern is that DNA containing damaged bases is first acted upon by a DNA glycosylase that generates apyrimidinic or apurinic (AP) sites. E. coli contains at least three AP endonucleases with at least two mechanisms of incision that may then remove the AP sites prior to repair by a DNA polymerase and DNA ligase. Alternatively, the AP sites may be repaired by direct insertion of the missing base (Livneh et al., 1979).

Certain bacteriophage and viruses offer a convenient avenue for elucidating the various repair pathways of the host, since they rely heavily (or exclusively) upon host repair functions for dealing with DNA damages. In the past, bacteriophage T7 served as the biological model with which to study the toxic action of alkylating agents on DNA (Lawley et al., 1969; Verly and Brakier, 1969; Venitt and Shooter, 1972; Verly et al., 1974; Karska-Wysocki, et al., 1976). Only recently (Lane et al., 1979), however, has the role of host-mediated repair in T7 phage viability been examined.

T7 is well-suited for studies of DNA metabolism because of the thorough genetic analysis and biochemical characterization of both T7 (for review, see Studier, 1969; Studier, 1972; Hausman, 1977) and its host, E. coli (for review, see Kornberg, 1980). A unique advantage is that biological activity of T7 DNA can be employed as a sensitive endpoint to the measurements. A cell-free system for packaging exogenous T7 DNA into phage heads to form viable T7 phage has been developed (Kerr and Sadowski, 1974; Kuemmerle and Masker, 1977). As a result, purified T7

DNA can be manipulated in vitro and the biological consequences monitored. An attractive feature of this packaging system is that, at least for the case of UV damage, viability of phage particles formed in vitro from UV-irradiated DNA parallels the survival of UV-irradiated T7 phage (Kuemmerle and Masker, 1977). These results raise the question as to whether the in vitro packaging system might also be used to monitor the biological consequences of chemically-damaged T7 DNA. Here we report the response of the packaging system to DNA alkylated by exposure to MMS or EMS, chemicals which introduce DNA lesions of distinct spectra. Also, host factors responsible for repair have been investigated by in vivo and in vitro host cell reactivation (HCR) studies. Bacterial indicators included wild type, polA, and tag-2. We found that this in vitro packaging system closely mimics the in vivo situation with respect to alkylation damage. Furthermore, during the packaging reaction we observed in vitro repair of DNA damaged by exposure to MMS or EMS.

Materials and Methods

Bacterial strains and bacteriophage T7. Strains of E. coli used in this study are shown in Table 1. Bacteriophage T7 were from the collection of F. W. Studier and included amber mutants T7am29 (gene 3), T7am28 (gene 5), and T7am147 (gene 6). In the text, T7 mutants are designated by subscript notation indicating gene only. For example, T7_{3am5am6am} refers to the triple amber mutant am29, am28, and am147.

Media, chemicals, and reagents. L-broth (Miller, 1972) was routinely supplemented with 10 µg of thymine per ml. T7 diluent (Studier, 1969) and low-salt T7 diluent (Kerr and Sadowski, 1974) have been previously

Table 1. Strains of Bacteria

Strain	Relevant Geneotype	Comments	Source
W3110	wt		P. Hanawalt
HMS146	<u>recB21</u>	<u>recB</u> mutant of strain W3110	W. Masker
AB2497	wt	<u>thy</u> mutant of strain AB1157 (<u>supE</u>)	A. Ganesan
BK2106	<u>tag-2</u>	<u>xth</u> ⁺ derivative of strain BK2012 (<u>tag xth</u>) (see Evensen and Seeberg, 1982)	E. Seeberg
KLC124	wt		J. W. Chase
KLC154	<u>polA1</u>	<u>polA</u> transductant of strain KLC124	J. W. Chase

described. Phosphate buffer consists of 0.15 M NaNO_3 , 0.20 M Na_2HPO_4 (pH 7.0 at 0.20 M). Sources of materials for media and reaction mixtures have been described (Masker, 1977; Masker et al., 1978). MMS and EMS were obtained from Eastman. [^{14}C]MMS (59 mCi/mmol) was purchased from Amersham. DNA was prepared as described by Richardson (1966). Amounts of DNA are expressed as nucleotide phosphorus equivalents or as T7 genome equivalents (where 1 nmol of DNA is taken to equal 7.5×10^9 intact chromosomes of T7 phage).

Alkylation of phage and DNA. T7 phage were alkylated as follows: intact phage at a concentration of 4.4×10^{11} PFU/ml was alkylated for 2 h at 37°C in phosphate buffer containing various concentrations of MMS or EMS. After treatment, 10 μl samples were diluted 1000-fold into ice-cold T7 diluent. Phage survival was determined by making appropriate dilutions in T7 diluent and plating on the indicator strain. Controls were carried out in the same way but without alkylating agents.

DNA was alkylated as follows: purified DNA at a concentration of 0.92 mM was incubated for 2 h at 37°C in phosphate buffer containing various concentrations of MMS or EMS. After treatment, 40 μl were diluted with 105 μl ice-cold 28 mM Tris (pH 7.5 at 0.05 M). Controls were carried out in the same way but without alkylating agents.

Quantitation of lesions. ^3H -labeled T7 DNA or phage were incubated with various concentrations of MMS or EMS as previously described. After treatment, single-strand breaks and alkaline-labile sites (including apurinic sites) were calculated from molecular weight determinations (Charlesby, 1954) after sedimentation in alkaline sucrose gradients.

Total alkylation of T7 DNA after treatment of purified DNA or phage was calculated from the radioactivity bound to DNA after incubation with [^{14}C]MMS and the specific radioactivity of the alkylating agent.

Reaction conditions for in vitro packaging of DNA. Extracts used for packaging were prepared as previously described (Kuemmerle and Masker, 1977), except that when strain BK2106 (tag) was used infection with T7_{3,5,6} was allowed to proceed for 22 min. The preparation of extracts from uninfected bacteria deficient in exonuclease V (recB) (Masker, 1977) and details of the reaction conditions for packaging of DNA (Kuemmerle and Masker, 1977) have been previously described. Extracts used for packaging that were prepared from T7_{3am5am6am}-infected strain BK2106 (tag) were complemented by adding 3 μl portions of extract prepared from uninfected strain HMS146. (recB) to 20 μl portions of the packaging extract. For most experiments, the values shown for the numbers of infective centers are the averages of four separate determinations, each plated in duplicate.

Reaction conditions for in vitro synthesis of DNA. The preparation of extracts from T7-infected bacteria and the reaction conditions for in vitro DNA synthesis have been previously described (Hinkle and Richardson, 1974; Masker et al., 1978).

Results

Host cell reactivation. E. coli mutants deficient in 3-methyl-adenine-DNA glycosylase (tag) have recently been isolated and characterized (Karran, 1980). The enzymatic release of 3-methyladenine is greatly

impaired in these mutant strains. This is presumably responsible for the observation that Hcr of bacteriophage λ damaged by exposure to MMS is lower in a tag mutant than in a polA strain. We have used a tag mutant to study HCR of bacteriophage T7. Phage incubated with various concentrations of MMS were plated on several host strains. As seen in Figure 1A, survival was lowest for all doses when the indicator strain was BK2106 (tag) (decreased about 1000-fold below wild type at the highest dose). The polA mutant was intermediate between the tag and wild type strains. These data are consistent with the results obtained for HCR of λ and clearly demonstrate that 3-methyladenine is a lethal lesion for T7 phage and argue against the possibility that T7 maintains a glycosylase of its own. Figure 1B shows a similar experiment, except that purified T7 DNA was exposed to MMS before being packaged into phage heads. Analogous to the in vivo situation, survival of phage resulting from packaging of damaged DNA was lowest when the tag mutant was used both to prepare the extract used for packaging and as the indicator strain; values for the polA strain were intermediate.

The spectrum of lesions in DNA after exposure to EMS is distinct from that generated with MMS (Bannon and Verly, 1972; Lawley and Shah, 1972; Verly et al., 1974; Lawley et al., 1975). Also, EMS is less toxic (Verly et al., 1974) and presumably more mutagenic for phage (Loveless, 1959). Thus, we were interested in contrasting the effects of EMS with those obtained for MMS. Figure 2 shows an experiment analogous to that described in Figure 1, except that EMS was the alkylating agent. On a molar basis, treatment of intact phage or purified DNA with EMS was less toxic than similar treatment with MMS. Again, the survival of alkylated

Figure 1. Comparison of survival of MMS-treated T7 phage and T7 DNA packaged in vitro. (A) Suspensions of T7 phage were alkylated with various concentrations of MMS as described in Materials and Methods. After treatment, the phage were diluted and plated on the indicated strains. Each data point represents the average of at least two separate experiments; average experimental error in $\log S/S_0$ was less than ± 0.17 . (B) T7 DNA was alkylated with various concentrations of MMS as described in Materials and Methods and subsequently packaged in vitro using extracts prepared from T7 γ am γ am γ am-infected cultures of the indicated strain. Phage resulting from in vitro packaging of damaged DNA were plated on the same strain used to prepare the extracts for packaging. The data shown are the results of a typical experiment. Packaging efficiencies varied from an average of 7×10^{-4} for strain W3110 (wt) to 3×10^{-6} for strain BK2106 (tag). Data are presented as $\log S/S_0$ (where S is phage titer after alkylation and S_0 is phage titer for the non-alkylated control) as a function of MMS concentration. Different symbols indicate the bacterial strains used for titrating phage: (○) W3110 (wt); (△) KLC124 (wt); (▲) KLC154 (polA); (□) AB2497 (wt); (■) BK2106 (tag).

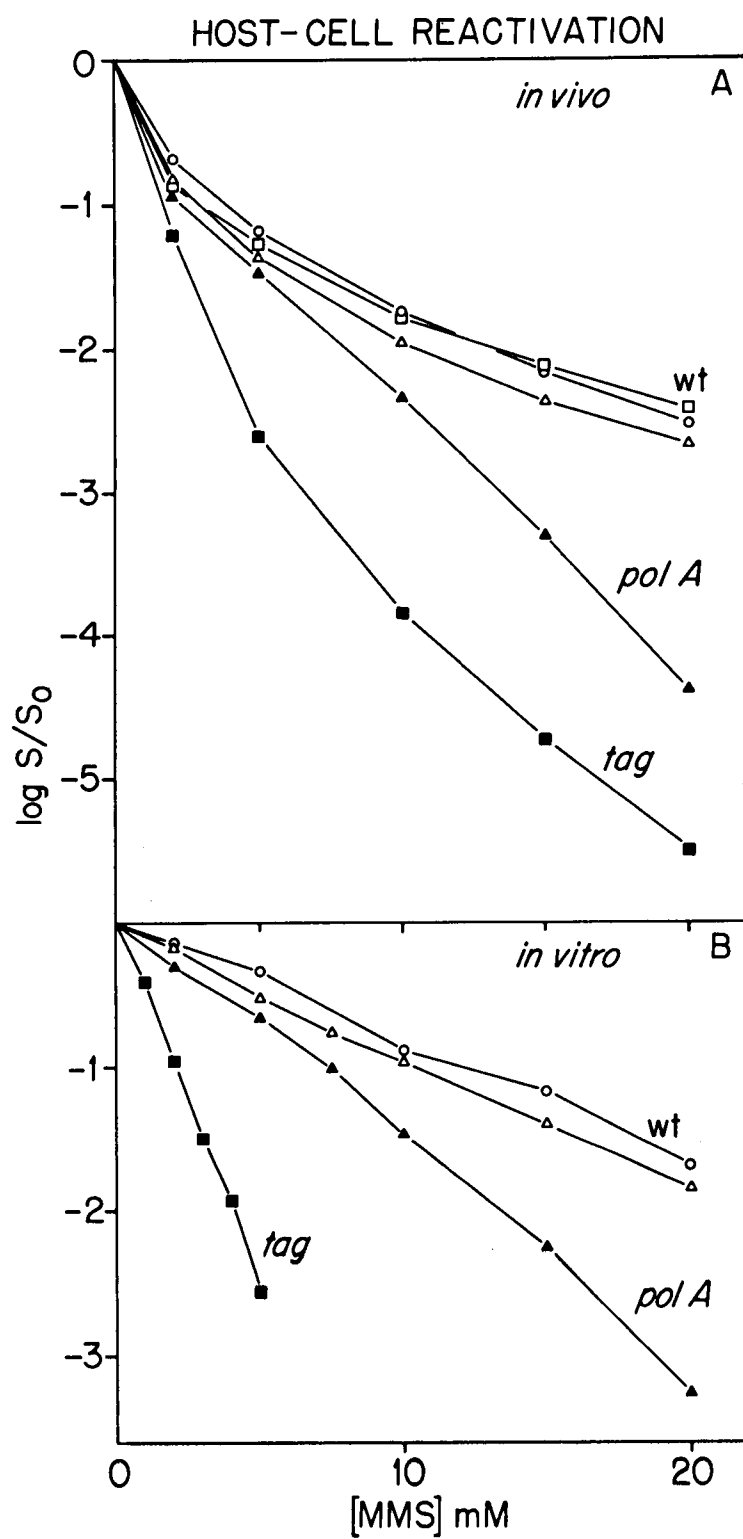


Figure 1.

Figure 2. Comparison of survival of EMS-treated T7 phage and T7 DNA packaged in vitro. (A) The procedure was the same as that described in Figure 1A, except that phage were alkylated with EMS. The average experimental error in $\log S/S_0$ was less than ± 0.20 . (B) The procedure was the same as that described in Figure 1B, except that EMS was the alkylating agent. Symbols are the same as those in Figure 1.

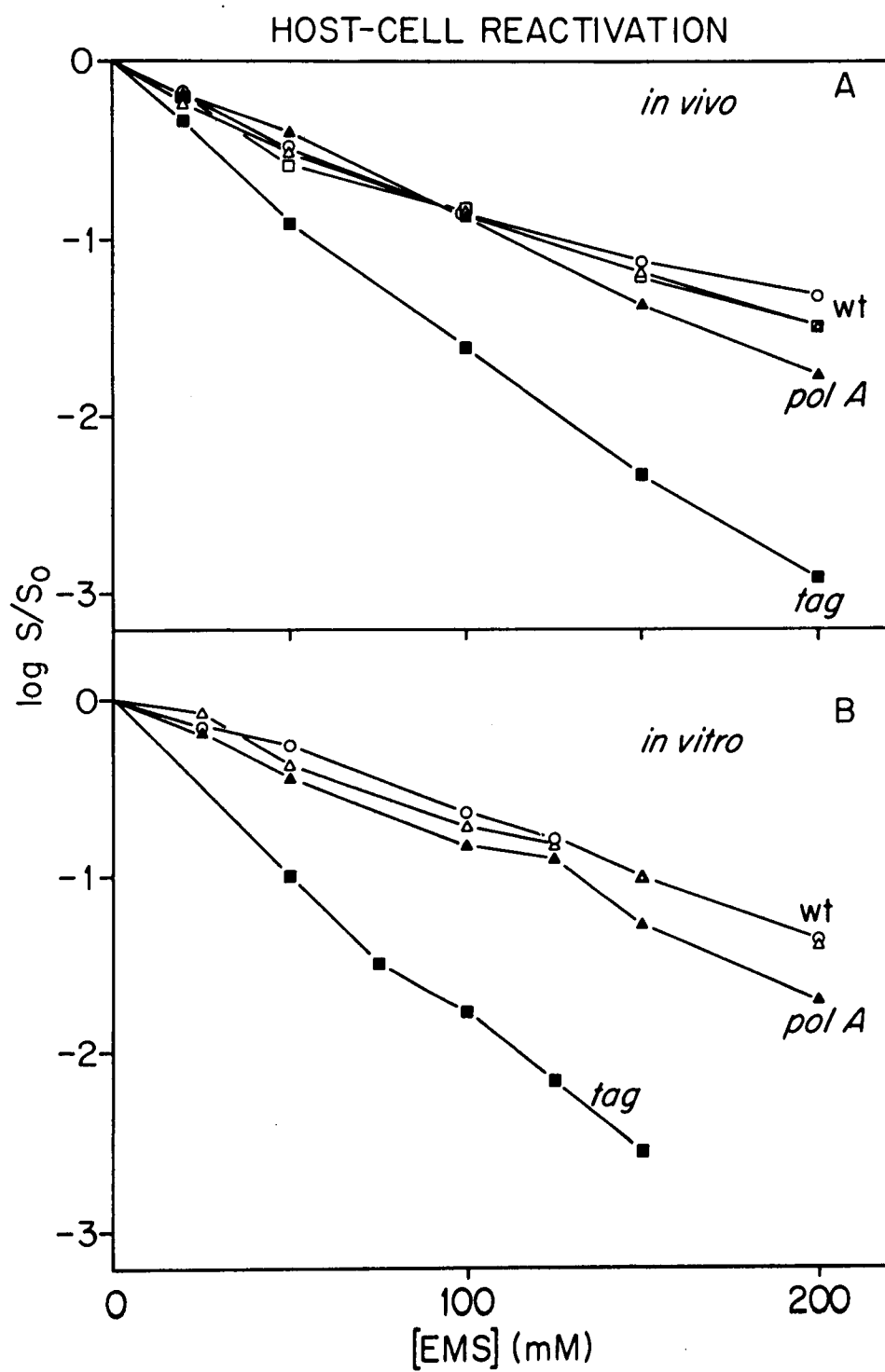


Figure 2.

phage (Figure 2A) was lowest when the tag mutant was the indicator strain. At the highest concentrations of EMS shown, phage survival was only slightly lower on the polA strain than wild type. The survival curves of viable phage resulting from in vitro packaging of DNA damaged by EMS (Figure 2B) were virtually identical to those obtained for the in vivo situation in which intact phage were treated. Thus, the T7 packaging system offers a sensitive means of monitoring DNA damage introduced by MMS or EMS.

In vitro repair during DNA packaging. The yield of viable phage after in vitro packaging of DNA is a linear function of DNA concentration (Kuemmerle and Masker, 1977). Thus, the reduction of viable phage seen in Figures 1B and 2B could be caused by elimination of suitable substrate due to selection against damaged DNA during packaging or by production of "wounded" phage by incorporation of predamaged DNA strands into newly formed T7 particles. UV sensitivity of irradiated DNA packaged into phage heads depends upon the repair capacity of the indicator strain on which the phage are titered rather than the strain used to prepare the packaging extract (Kuemmerle and Masker, 1977). Our earlier work suggests that these E. coli lysates are deficient in the repair of pyrimidine dimers and that the packaging proceeds normally irrespective of the presence of damage in the DNA. To determine whether this would also apply to DNA containing the wider variety of insults introduced by alkylating agents, DNA exposed to various concentrations of MMS or EMS was packaged using extracts prepared from either strain W3110 (wt) or BK2106 (tag), and phage survival was determined using both strains as indicators. For these chemicals, survival of phage resulting from packaging with an

extract prepared from wild type E. coli (Figure 3A and 3C) was not influenced by whether the indicator strain was glycosylase deficient. In fact, the extent of killing was similar to that seen for wild type in Figures 1B and 2B. The results of packaging treated DNA with an extract prepared from strain BK2106 (tag) are shown in Figure 3B and 3D. In this situation, where glycosylase deficiency presumably limited the repair capacity during the packaging reaction itself, survival was greatly influenced by the indicator strain. When wild type E. coli was used as an indicator strain in place of the tag mutant, survival was dramatically improved. Evidently, at least a portion of the damaged DNA was packaged into phage heads and not rejected at a prior selection step. Furthermore, these data indicate that damaged DNA was repaired during the packaging reaction employing a wild type extract.

In order to further investigate the possibility of in vitro DNA repair, we complemented a tag extract used for packaging with an extract prepared from an uninfected tag⁺ strain which should, by itself, not influence in vitro packaging. Figure 4 shows that survival was enhanced for both MMS- and EMS-damaged DNA when packaging was performed with complemented extracts, although under these conditions the repair of MMS-induced damage was not as complete as that found if wild type cells were used as the source of packaging extract.

In vitro DNA synthesis with alkylated DNA as template. The observation of in vitro repair of damaged DNA prompted inquiry as to the effects of alkylation damage on other aspects of in vitro DNA metabolism, especially DNA replication. T7 DNA can be synthesized in vitro with a system that mimics in vivo T7 DNA replication (Masker and Richardson,

Figure 3. The effect of packaging extract on in vitro host-cell reactivation. T7 DNA was alkylated with different concentrations of MMS (Figure 3A and B) or EMS (Figure 3C and D) and subsequently packaged in vitro by use of extracts prepared from T7_{3am5am6am}-infected cultures of strain W3110 (wt) (Figure 3A and C) or strain BK2106 (tag) (Figure 3B and D). Phage resulting from the packaging of damaged DNA were plated on both strains W3110 (wt) and BK2106 (tag). Symbols: Packaged with W3110 (wt) extract: plated on strains W3110 (wt) ($\square$) and BK2106 (tag) ($\bullet$); packaged with BK2106 (tag) extract: plated on strains W3110 (wt) ($\blacksquare$) and BK2106 (tag) ($\circ$).

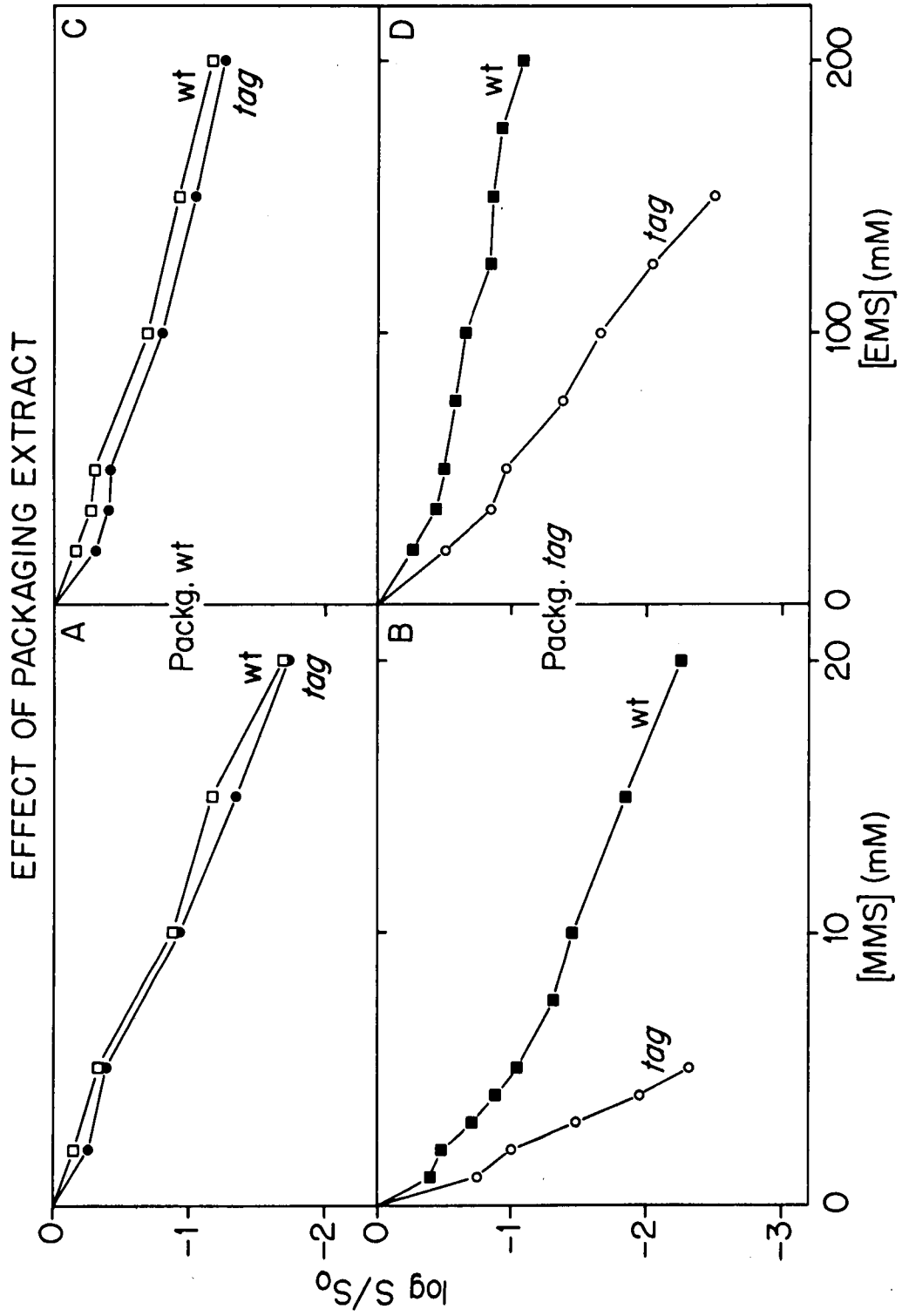


Figure 3.

Figure 4. Enhancement of phage survival by complementation during packaging. T7 DNA was treated with different concentrations of MMS (Figure 4A) or EMS (Figure 4B) and packaged in vitro using extracts prepared from T7 γ am γ am γ am-infected cultures of strain BK2106 (tag). The packaging reactions were carried out in duplicate and one of each of the duplicate reactions was complemented with an extract prepared from an uninfected tag⁺ strain as described in Materials and Methods. In both cases, the indicator strain was BK2106 (tag). The figure shows log S/S₀ as a function of chemical concentration as described in Figure 1. Symbols: (□) with uninfected extract, (●) no uninfected extract.

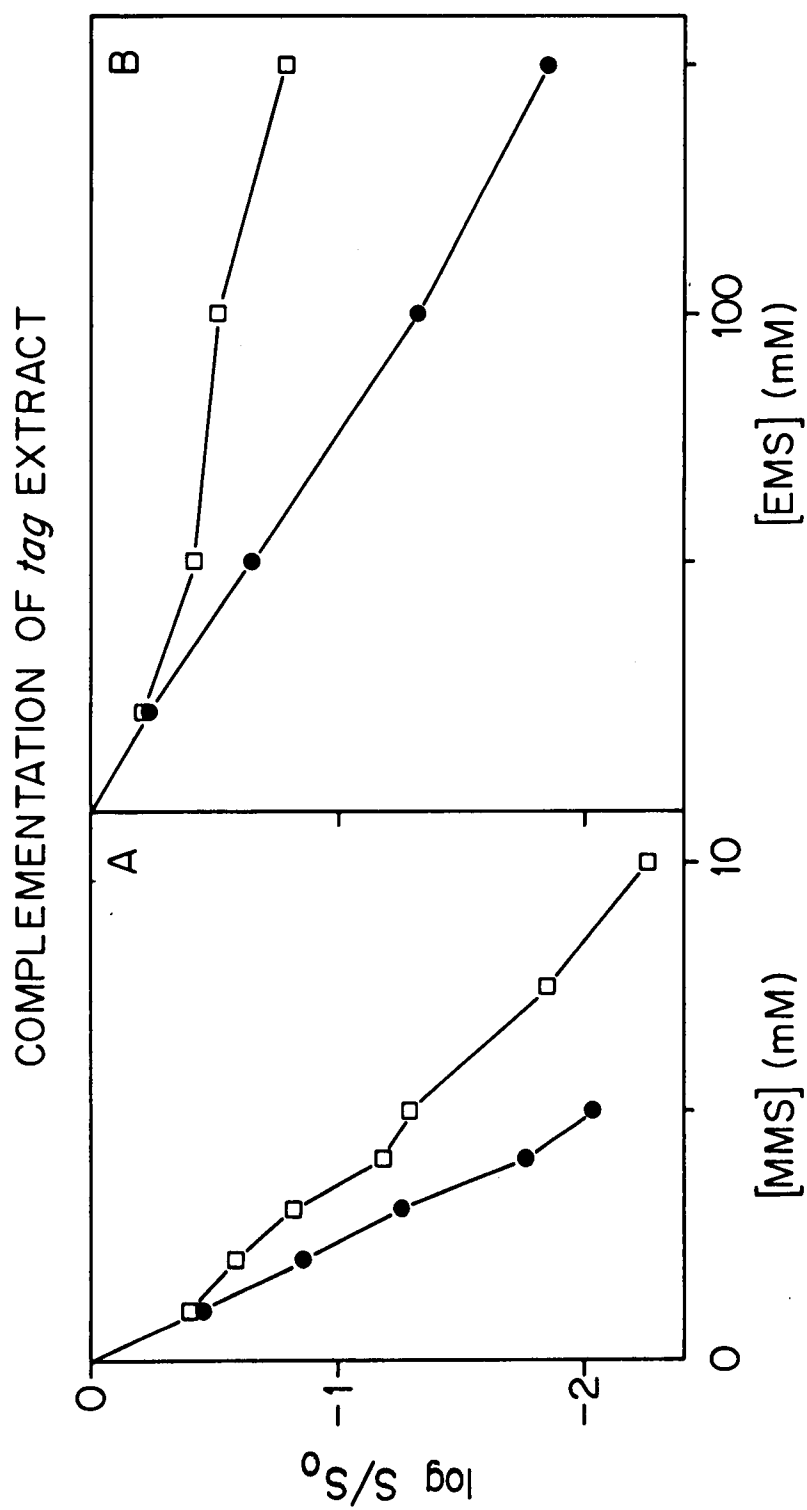


Figure 4.

1976a and 1976b) and the product of this reaction can be packaged into viable phage. Figure 5 shows the effect of alkylated DNA on in vitro DNA synthesis, plotted as a function of MMS or EMS concentration. DNA synthesis was markedly reduced for DNA exposed to low concentrations of MMS (Figure 5A), whereas high concentrations of EMS were required to see an effect. DNA synthesis was linear with time for all doses of MMS and EMS examined (selected indicated doses are shown in the insert). This was also true for DNA treated with concentrations of MMS sufficient to reduce the amount of synthesis below that observed for similar reactions without added DNA, thus suggesting that exogenously supplied T7 DNA is a preferred template over any contaminating endogenous DNA present in the extract. The possibility that DNA synthesis is affected by residual concentrations of MMS or EMS (present during the synthesis reaction at a concentration more than 100-fold lower than during DNA treatment) is unlikely since DNA exposed to MMS or EMS with no incubation (kept at 0°C) and then diluted into the DNA synthesis reaction gave results virtually identical to the non-alkylated control (data not shown). These results show that alkylation damage inflicts a marked reduction on in vitro DNA synthesis and suggests that either repair of alkylated bases is incomplete or that damages not corrected by this system cause reduction in both DNA synthesis and viability of packaged DNA.

Discussion

Restoration of biological activity to DNA rendered "inviabile" by external insults represents a stringent criterion for monitoring the integrity of DNA. Much of the motivation for the experiments outlined above derived from our interest in using in vitro packaging as a means

Figure 5. Effect of alkylated DNA on in vitro DNA synthesis. T7 DNA treated with various concentrations of MMS or EMS was added to extracts prepared from strain W3110 (wt) infected with T7₃am and incubated in the presence of [³²P]dATP at 30° under standard conditions for DNA synthesis as described in Materials and Methods. After 30 min. incubation, 100 µl samples of each reaction mixture were removed and acid precipitated. Radioactivity was determined and the calculated amount of DNA synthesized at indicated doses of MMS (Figure 5A) or EMS (Figure 5B) was compared to the non-alkylated control. DNA synthesis as a function of MMS or EMS dose is shown for separate experiments as indicated by the symbols. [³²P]dATP was present at 1.3 CPM/pmol (○), 1.9 CPM/pmol (■), and 2.7 CPM/pmol (▲). The insert shows the kinetics of DNA synthesis for DNA alkylated with selected indicated doses of MMS (left) or EMS (right). A control in which no exogenous DNA was added to the reaction is shown for comparison. Symbols: Left insert: (●) 0mM control, (□) 2 mM MMS, (◆) 15 mM MMS, (△) no exogenous DNA; Right insert: (●) 0mM control, (▼) 100 mM EMS, (◇) 200 mM EMS, (△) no exogenous DNA.

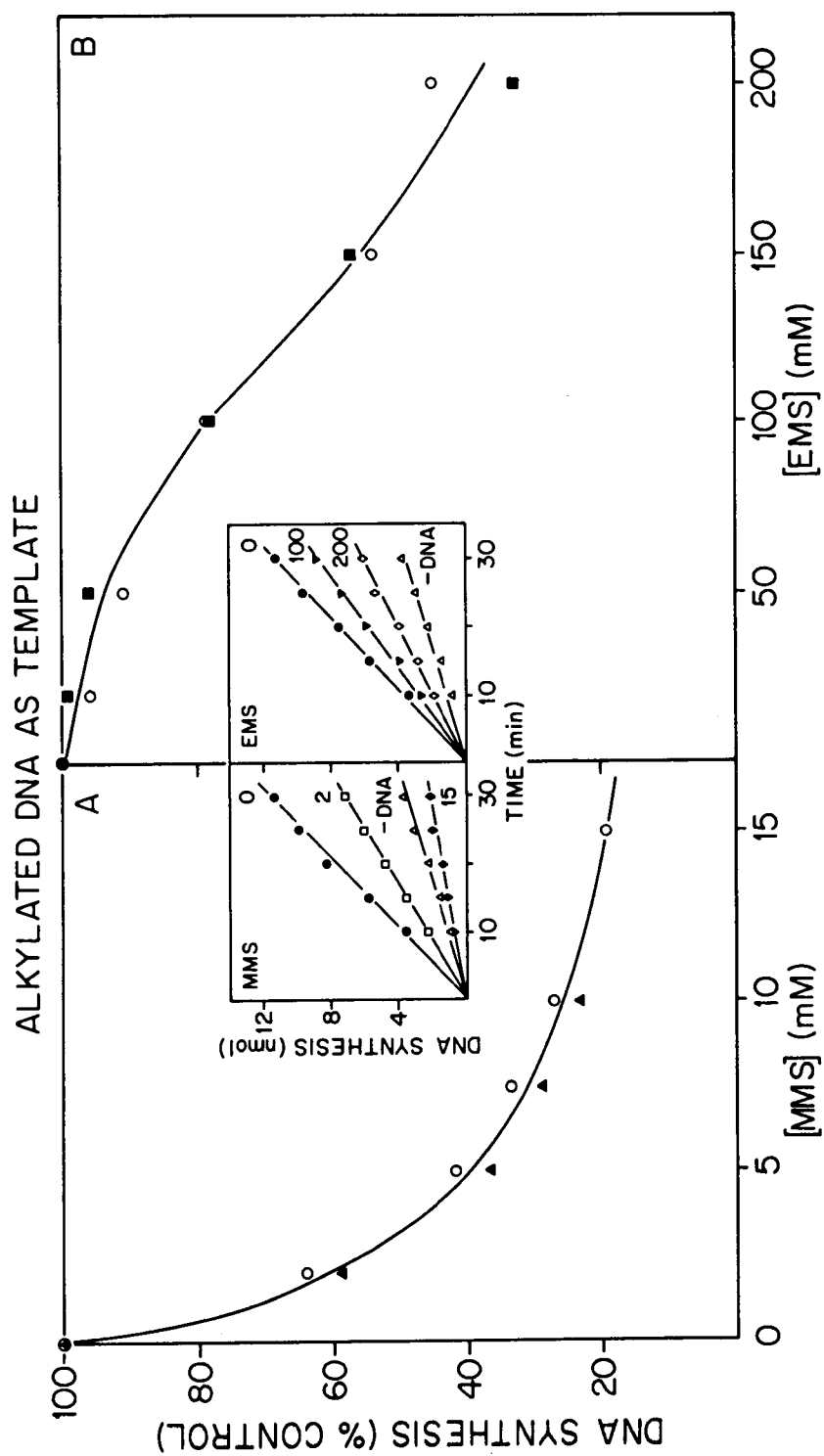


Figure 5.

for detecting physical damage and genetic alterations residing in T7 DNA that has undergone in vitro manipulations. Our data confirm and extend an earlier study (Kuemmerle and Masker, 1977) indicating that T7 DNA can be successfully packaged irrespective of the presence of UV-radiation damage (Kuemmerle and Masker, 1977) or alkylation damage due to exposure to MMS or EMS (Figures 1 and 2, pages 14 and 16). Moreover, these results provide good evidence for in vitro repair of at least some of the damage inflicted by these chemicals and document the effects of the host mutations polA1 and tag-2 on in vivo and in vitro host cell reactivation.

The critical role of 3-methyladenine-DNA glycosylase in DNA repair is emphasized by our studies. When compared to several wild type strains, the tag mutant showed a marked reduction in the ability to reactivate alkylated T7 phage. In fact, a comparison of our data (Figures 1 and 2) with that of Lane et al. (1979), who examined T7 host cell reactivation in xthA mutants (deficient in endonuclease VI), indicates that the effects of alkylation are much more acute in phage plated on the tag mutant than on those plated on an xth strain. This confirms that alternate incision endonucleases are available to act once an apurinic site is generated by 3-methyladenine-DNA glycosylase (Lindahl, 1979). Apparently, a similar situation persists with regard to the resynthesis step that presumably occurs subsequent to excision of the apurinic site. Our data agree with those of Lane et al. (1979) who saw that, relative to wild type, polA mutants show significantly less reactivation of MMS-treated T7 phage. However, Figure 2 reveals only minor differences between wild type and polA strains in their ability to support the growth of EMS-treated T7. Thus, the DNA synthetic activity of E. coli DNA polymerase I appears to

be dispensable under conditions such as UV-irradiation or EMS treatment where the number of lesions is low and killing is not extensive (Kuemmerle and Masker, 1977, Figure 2). We interpret this to mean that either another E. coli DNA polymerase or the phage DNA polymerase effectively substitutes for E. coli DNA polymerase I under the conditions employed in our experiments.

At equivalent concentrations of MMS, the survival of treated DNA packaged into phage heads was greater than that of treated whole phage. It is unlikely that the greater lethality observed for phage was due to a greater number of lesions introduced per genome. Prior studies with alkylating agents have shown similar yields of damaged bases for treated phage suspensions or isolated DNA using T2 (Brooks and Lawley, 1963; Lawley and Brooks, 1963) exposed to MMS or EMS and T4 (Brooks and Lawley, 1963; Lawley and Brooks, 1963) or T7 (Lawley et al., 1969) exposed to mustard gas. Furthermore, quantitation of damage introduced by MMS carried out as part of this study has shown that, irrespective of treatment of phage or DNA, the number of total lesions in the DNA were approximately equal and within the range expected from earlier studies (Verly et al., 1974). The greater lethality observed after MMS treatment of whole phage may reflect damaged phage proteins. Alternatively, the higher titers observed in vitro may be due to repair during packaging and that in the case of EMS-induced damage, the component of surviving phage resulting from repair was not fully resolved because of the low lethality. Unfortunately, due to the reduced efficiency of packaging observed with the tag mutant, the effect of 3-methyladenine-DNA glycosylase on survival could only be examined at exposures to low concentrations of MMS where

the contribution of repair is not readily discernible. It is, however, unlikely that the low efficiency of packaging with the tag mutant has a significant effect on our conclusions since controls indicate that our results do not fluctuate with packaging efficiency and, more importantly, we were able to complement extracts from phage-infected tag strains with extracts from uninfected cells so as to give HCR curves near to those found with extracts from phage-infected wild type cells.

Alkylation caused by exposure to MMS or EMS is far less toxic than depurination; one inactivation hit (63% lethality) appears to correspond to about 100 base alkylations (Verly et al., 1974). Consistent with this is the observation that, as seen in Figure 5, DNA synthesis was not markedly reduced when DNA containing 50-100 alkylations per genome was added as template to the in vitro reaction. Either removal of these lesions was extremely rapid or methyl and ethyl groups do not dramatically perturb the replication apparatus. Of course, rescue of partial genomes by in vitro recombination (Masker and Kuemmerle, 1980) may figure in this result.

While our experiments demonstrate effective in vitro repair of DNA damage and considerable replication of alkylated DNA template, the data say nothing about the fidelity of repair or replication on these templates. However, by coupling the DNA synthesis reaction with the packaging system (Masker et al., 1978), the entire process of T7 self-replication can be carried out in vitro; there is a high yield of viable phage, and newly replicated product DNA can be separated from the exogenous input DNA. Thus, chemical treatment of the template before or during DNA synthesis

and subsequent packaging of the product to form viable phage provides a way to investigate in vitro fixation of mutations.

CHAPTER III

MUTAGENESIS OF BACTERIOPHAGE T7 BY INCORPORATION OF O⁶-METHYLGUANINE DURING DNA SYNTHESIS

Summary

An in vitro system in which bacteriophage T7 DNA is replicated and efficiently packaged into procapsids to form viable phage has been used to examine mutagenesis. The fidelity of replication was assayed both by measuring reversion of an amber mutation in an essential gene and by generation of temperature-sensitive mutants among the phage produced in vitro. Under standard reaction conditions, the fidelity of DNA replication is about equal to that normally found in vivo. However, when O⁶-methyldeoxyguanosine triphosphate is included in the reaction, O⁶-methylguanosine is incorporated into newly synthesized DNA and the mutation frequencies increase 10- to 70-fold over the control. These experiments demonstrate in vitro mutagenesis with the T7 DNA replication-packaging system and provide the first direct evidence for the premutagenic role of O⁶-methylguanine.

Introduction

Studies on the fidelity of in vitro DNA synthesis have frequently been performed with synthetic DNA templates and purified DNA polymerases (Loeb and Kunkel, 1982). These measurements provide useful information, but represent a significant departure from the in vivo situation. Experiments employing natural DNA templates and well-defined enzyme systems have allowed valuable extensions of these studies (Loeb and Kunkel, 1982).

Even closer adherence to the *in vivo* situation is now possible using an *in vitro* system that replicates intact chromosomes of duplex T7 DNA with efficiency sufficient to replicate two to five times as much product DNA as the amount of exogenous template provided in the reactions (Hinkle and Richardson, 1974; Masker and Richardson, 1976). Also, biological activity of T7 DNA can be effectively monitored with an *in vitro* system able to encapsulate T7 DNA into proheads with good efficiency (Kuemmerle and Masker, 1977; Dodson and Masker, 1981). Thus, the ability to synthesize T7 DNA *in vitro* and to package that DNA into phage particles means that the entire process of T7 self-replication can be carried out *in vitro* (Masker et al., 1978). In this report, we examine mutagenesis with this *in vitro* system in which bacteriophage T7 DNA is replicated and then packaged into capsids to form viable phage. Using this system, we have examined the consequences of incorporating O⁶-methylguanine (m⁶G) into T7 DNA.

Only indirect evidence supports the original proposal (Loveless, 1969) that m⁶G is mutagenic because it may mispair with T and thus cause C→T transitions. For example, *in vivo* experiments with *E. coli* show that a majority of the mutations induced in a particular gene by methyl-nitronitrosoguanidine are of the GC→AT type (Coulondre and Miller, 1977), and *in vitro* measurements suggest that, during DNA synthesis (Gerchman and Ludlum, 1973) or transcription (Abbott and Saffhill, 1979), O⁶-methylguanine can base pair with T (or U). Furthermore, studies of *in vitro* DNA synthesis with prokaryotic DNA polymerases indicate that the presence of m⁶G in the template significantly favors incorporation of T rather than C in the product (E. T. Snow, R. S. Foote, and S. Mitra, unpublished

observation and E. Snow, Ph.D. dissertation, The University of Tennessee). Also, the mutagenicity of alkylating agents appears to correlate with their tendency to react at the oxygen sites in DNA, including the O⁶ of guanine (Lawley, 1974). Collectively these observations argue that direct alkylation of DNA can produce m⁶G residues that, if not corrected, might cause mutation by pairing with T. However, because all alkylating agents produce a number of lesions in DNA, among which O⁶-alkylguanine is often a minor product, it is important that the premutagenic role of O⁶-methylguanine be substantiated by more direct evidence.

The finding that the deoxyribonucleotide pool is alkylated to a greater extent than the DNA itself when cells are treated with N-methyl-N-nitrosourea prompted the proposal that mutation may result from incorporation of alkylated bases into DNA (Topal et al., 1980; Topal and Baker, 1982). Thus, if m⁶dGTP is a substrate for DNA synthesis, mutations might arise when m⁶G is incorporated opposite T in the template and subsequent action by O⁶-methylguanine methyltransferase (Foote et al., 1980; Olsson and Lindahl, 1980) produces a G•T mispair. Following this rationale, we included m⁶dGTP as a substrate in our in vitro T7 DNA synthesis-packaging system and monitored the mutagenic consequences of incorporation of m⁶G into newly replicated T7 DNA by assaying both reversion of an amber mutation and generation of forward temperature-sensitive mutations.

Materials and Methods

Bacterial strains and bacteriophage. The bacterial strains used in this study were: E. coli K12 strains W3110 (thy sup⁰) and 011' (thyA sup⁺) and Shigella sonnei strains Sh3-18 (sup⁺) and Shd₂371-48 (sup⁰)

(obtained from R. Hausmann). Phage mutants (from F. W. Studier) are designated by a subscript indicating the defective gene(s) and the type(s) of mutation (am = amber, ts = temperature sensitive, (-) = uncharacterized, and (+) = wild type); e.g. T7_{3am6am}. Phage mutants employed in this study and their location on the T7 genetic map are shown in Figure 6.

Media and chemicals. Sources of materials have been described (Masker and Richardson, 1976; Kuemmerle and Masker, 1977; Masker et al., 1978). The preparation of unlabeled and labeled m⁶dGTP is described elsewhere (Dodson et al., 1982; Foote et al., 1982) and was a generous gift from R. F. Foote and S. Mitra.

Reaction conditions for in vitro DNA synthesis and packaging of DNA.

Extracts for DNA synthesis were prepared by infecting strain W3110 at a multiplicity of two with T7_{3am6am} for 14.5 min at 37°C. The cells were concentrated, and lysed by being frozen, thawed, and treated with lysozyme as previously described (Hinkle and Richardson, 1974). The lysate was spun at 140,000 x g for 30 min. The resulting supernatant comprised 20% of the volume of the DNA synthesis reaction which included 30 nmol (nucleotide phosphorus) T7 DNA per ml in 30 mM TrisCl (pH 7.5), 20 mM MgCl₂, 10 mM 2-mercaptoethanol, 3 mM of each rNTP, and (except where noted) 3 mM of each dNTP (Hinkle and Richardson, 1974; Masker and Richardson, 1976). Reactions were incubated at 30°C. The extracts used for DNA packaging were prepared from strain W3110 infected at a multiplicity of three with T7 phage for 18.5 min at 30°C. The cells were concentrated 200-fold, frozen and thawed, and treated for 2 min at 30°C with 20 µg/ml lysozyme as previously described (Kuemmerle and Masker,

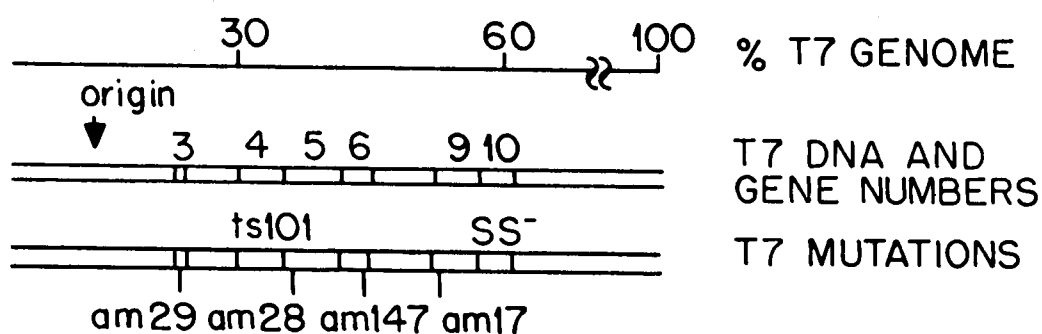


Figure 6. Organization of the T7 genome. Positions for T7 genes and mutations are from references Studier, 1972; Vetter et al., 1977; Campbell et al., 1978; Kruger and Schroeder, 1981. The *ts101* mutant is temperature sensitive for the gene 4 product. The *SS⁻* mutation (in gene 10) extends the T7 host range to include Shigella.

1977). The lysate was spun at $18,000 \times g$ for 20 min and the supernatant retained as a "packaging extract." To obtain more reproducible results these extracts were usually prepared with T7 Δ 5 Δ 6 Δ and supplemented with T7 procapsids separated from extracts of T7 Δ 5 Δ 6 Δ infected strain W3110 by centrifugation in CsCl gradients and stored in liquid N₂ until just before use (Masker and Serwer, 1982). Packaging efficiencies could also be improved by adding a small amount of extract from T7 Δ ts infected cells. These extracts were prepared in the same way as those used for DNA synthesis except that the infection was stopped after 10 min at 42°C. Controls, not shown, indicate that the addition of these extracts from T7 Δ ts infected cells did not produce measurable levels of in vitro recombination during packaging. A typical 0.03 ml in vitro packaging reaction contained 0.015 ml of a packaging extract from T7 Δ 5 Δ 6 Δ infected cells, 0.005 ml of a 6 μ g/ml preparation of T7 procapsid, and where indicated 0.001 ml of an extract from T7 Δ ts infected cells. The make up of the reaction mixture and reaction conditions have been previously described (Kuemmerle and Masker, 1977; Masker and Serwer, 1982).

T7 mutagenesis system. Extracts prepared with phage mutants deficient in the gene 3 endonuclease and the gene 6 exonuclease (T7 Δ 5 Δ 6 Δ) were used to replicate T7 DNA. Under these reaction conditions, DNA synthesis in vitro requires the addition of exogenous dNTPs (normally provided in vitro by nucleolytic breakdown of host DNA). Controls performed as part of the present study show that DNA synthesis declines as the concentration of only one dNTP is lowered and falls to less than 5% of the maximum rate if one dNTP is omitted entirely (Figure 7). This

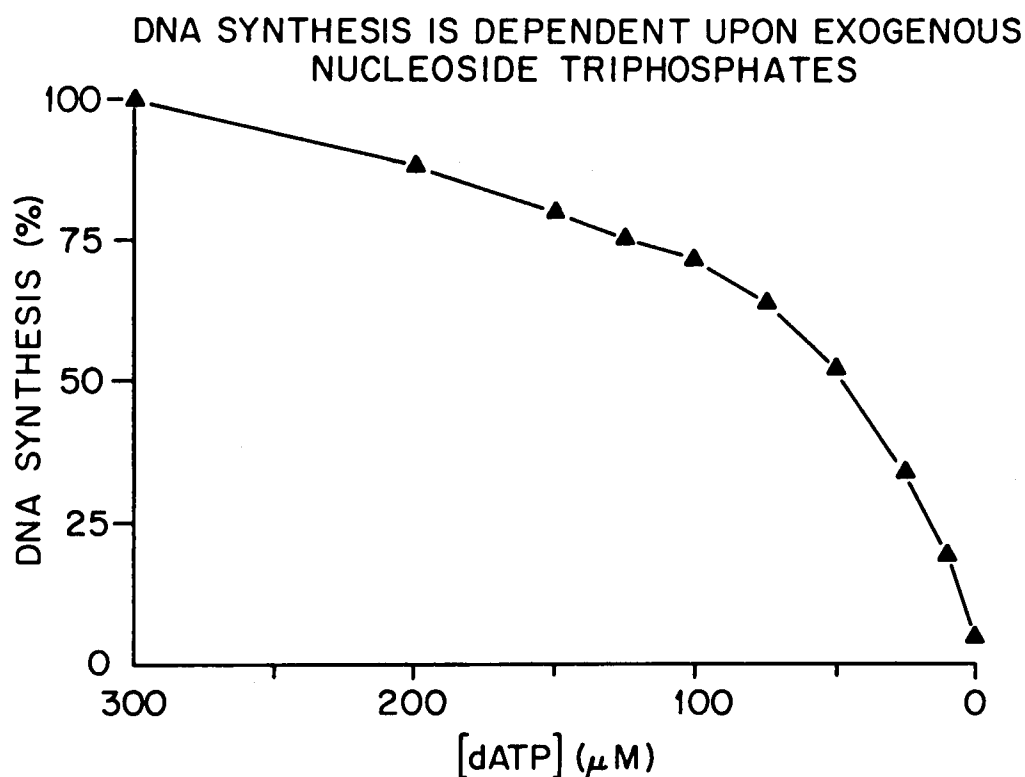


Figure 7. Dependence of DNA synthesis upon exogenous nucleoside triphosphates. T7 DNA was replicated *in vitro* in the presence of various concentrations of dATP at 30° under standard conditions for DNA synthesis as described in the text. The other three normal dNTPs were present at a concentration of 300 μM . After 30 min of incubation, 100 μl samples of each reaction mixture were acid-precipitated, and the amount of DNA synthesized was calculated (shown as nucleotide phosphorus equivalents). The data shown are an average of two experiments. The specific activity of [^{32}P]dCTP was 0.55 and 2.6 cpm/pmol.

dependence upon exogenous dNTP's is an important prerequisite for examining in vitro mutagenesis by modified nucleotide precursors.

It was previously shown that DNA replicated in vitro can be encapsulated in a second in vitro reaction so as to generate infective phage (Masker et al., 1978). To study mutagenesis it was necessary to modify this replication-packaging system so that a small number of mutant phage could be detected among a much larger population of nonmutant T7 produced in vitro and to eliminate possible artifacts due to in vitro recombination and contamination of the extracts with phage and/or DNA. This has now been accomplished by achieving higher DNA packaging efficiencies (as discussed above) and by appropriate genetic manipulations of the T7 used to produce extracts and DNA. Our protocol for determining reversion of an amber mutation is shown in Figure 8. We employed a (nonamber) mutant of T7 [designated "SS-" (Hausman et al., 1968)] that permits T7 growth on Shigella, to eliminate unwanted contributions from endogenous (SS⁺) phage and DNA present in the extracts. Since SS⁺ phage are unable to form plaques on strains of Shigella, the expression of any contaminating phage is blocked by plating on Shigella strains. Exogenous template DNA (6^{am}SS⁻) is replicated in vitro in the absence or presence of various concentrations of m⁶dGTP. Reversion of the gene 6 amber mutation was monitored by examining product phage on Shigella with or without an amber suppressor. The possibility of in vitro recombination during the DNA synthesis reaction was greatly reduced by preparing extracts from T7 phage deficient in gene 6. Furthermore, phage used in preparing extracts carry the same amber mutation in gene 6 as the exogenous DNA template, so that even if recombination did proceed at a low level, recombinants

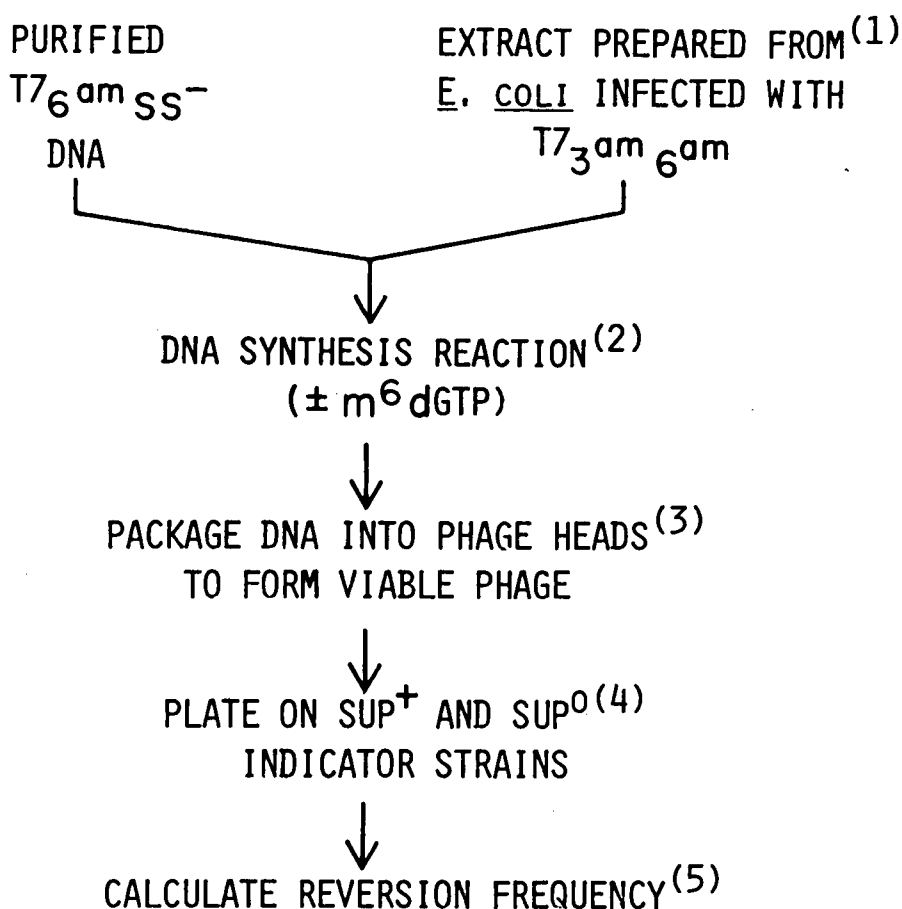


Figure 8. Strategy for the detection of in vitro mutagenesis. (1) DNA replication is blocked during the in vivo infection and recombination is reduced during the in vitro reaction by using T7 mutant in genes 3 and 6. (2) DNA synthesis is dependent upon exogenous nucleoside triphosphates. (3) Reactions containing partially purified T7 procapsids and extracts lacking unfilled phage heads are used to package DNA. The extracts used for DNA packaging are distinct from those used for DNA replication. (4) Only mutants in gene 10 of T7, referred to as "SS⁻", form plaques on *Shigella* hosts; expression of contaminating (SS⁺) phage is blocked. (5) Reversion of the gene 6 amber mutation (TAG) may occur as a result of m⁶G pairing with T in the template.

could not contribute to rescue of the amber mutation being monitored for reversion. Previous studies have shown that DNA packaging efficiency is enhanced when fast-sedimenting concatemer-like T7 DNA structures formed in the presence of the phage's gene 6 exonuclease are used as substrate (Masker et al., 1978). For this reason we included a small amount of extract from T7_{4ts} (6⁺) phage-infected cells in some of our reactions. The mutation in gene 4 blocks DNA replication during preparation of the extracts (Studier, 1972) and thus limits introduction of endogenous DNA into the reactions. Incubation at 44° blocks expression of any T7_{4ts}6⁺SS⁻ recombinants that may arise by recombination between the exogenous 6^{am}SS⁻ template DNA and any contaminating 4^{ts}6⁺DNA [requires a single crossover event between the gene 6 and gene 10 (SS⁻) mutations]. Generation of T7₆6⁺SS⁻ recombinants is very unlikely because a double crossover involving genes 4 and 6 would be required to occur under conditions highly unfavorable for recombination (Masker and Kuemmerle, 1980). As an additional control, we tested plating conditions permissive (30°) for all recombinants and found only an approximate doubling of the number of "revertants" counted on the suppressor-free strain of Shigella. Because this was true for all of our samples and the relative temperature sensitivity (30°/44°) is almost 10,000-fold for phage carrying the 4^{ts} mutation, it is very unlikely that recombination events introduce any artifact in our mutagenesis measurements. A protocol similar to the one just described was used to examine generation of forward temperature-sensitive T7 mutants. But, in that procedure wild type T7 DNA could be used, and, since very high packaging efficiencies were unnecessary, the extract from T7_{4ts} infected cells was not added.

Results

Reversion of an amber mutation by incorporation of m^6dGMP . To examine directly the accuracy of in vitro T7 DNA synthesis we compared the reversion frequency of the gene 6 amber mutation in phage resulting from the encapsulation of DNA before and after replication. Under standard conditions (Table 2, line 2), the amount of newly synthesized DNA was more than twice the amount of template DNA which was present at 1.5 nmoles in each reaction. Under those same conditions the biological activity (Sup^+) more than doubled compared to what was found due to the template alone when the reactions were held on ice (line 1). The phage yield measured on the suppressor-free indicator strain shows the in vitro replication approximately doubled the reversion frequency. For comparison, when a log-phase culture of 011' (Sup^+) was infected in vivo with T7 $_{\Delta 6}$ SS-phage at a multiplicity of infection of approximately one and grown under conditions so that the net yield of phage increased by a factor of five, we found an increase in the amber reversion frequency nearly the same as what we saw in vitro. Table 2 also shows the level of DNA synthesis, phage survival, and reversion frequency for samples containing various concentrations of dATP and m^6dGTP in the DNA synthesis reaction. The extent of DNA synthesis depends on the concentration of dATP, but for any particular concentration of dATP, increased concentrations of m^6dGTP did not reduce DNA synthesis significantly. The increase in reversion frequency was small for samples with decreased concentrations of dATP and no m^6dGTP , indicating that in our system the pool bias effect is not a major source of mutagenesis. Inclusion of m^6dGTP in the DNA synthesis reaction resulted in a marked increase in the reversion

Table 2. Reversion of an Amber Mutation^a

dATP Conc. (μ M)	m ⁶ dGTP Conc. (μ M)	DNA Synthesis (nmol)	Titer (PFU/ml)		Relative Reversion Frequency
			Sup ⁺ ($\times 10^5$)	Sup ^o ($\times 10^2$)	
300	0'control	0.4	180	1.1	0.4
300	---	3.9	410	6.0	1
10	---	1.1	230	9.7	2.8
50	---	2.2	380	9.5	1.6
100	---	2.9	350	7.6	1.5
50	[500 dGTP]	2.4	340	9.6	1.9
10	100	1.1	48	6.2	8.7
50	50	2.1	89	44	33
50	100	2.2	47	31	43
50	200	2.0	35	14	26
100	10	2.9	200	95	31
100	50	2.8	130	100	52
100	100	2.7	71	68	64
100	200	2.7	37	32	57
200	200	3.3	38	42	73

^aT7 DNA was replicated in vitro in the presence of various concentrations of dATP and m⁶dGTP at 30° under the conditions for DNA synthesis described in the text. The other three normal dNTPs and [³²P]dCTP (1.6 cpm/pmol) were present at a concentration of 300 μ M each unless otherwise indicated. After 0 min (line 1) or 30 min of incubation (remainder of table), 50 μ l samples of each reaction mixture were acid-precipitated, and the amount of DNA synthesized was calculated (shown as nucleotide phosphorous equivalents). After 0 min or 30 min of DNA synthesis, the DNA was diluted and then encapsulated during a 60 min incubation at 30° in 0.09 ml standard packaging reaction mixtures as described in the text. Resulting phage were incubated on strains of *Shigella* with or without an amber suppressor and reversion frequencies were calculated. Packaging efficiency (PFU per genome equivalent of DNA) was 0.4%. Reversion frequencies virtually identical to those calculated for the samples shown in lines 1 and 2 of this table were obtained in an experiment with a 3% packaging efficiency.

frequency as displayed in Figure 9. In fact, the absolute number of revertants increased by an order of magnitude when low concentrations of m^6dGTP were present during synthesis. For samples containing 100 μM dATP, the reversion frequency was linear in the range between 1 and 10 μM m^6dGTP (Figure 10). After adjusting for the loss of survival, 50- to 70-fold increases in reversion frequency relative to standard conditions were obtained for some of the samples containing m^6dGTP (Table 2). This compares with a 1.5- to 3-fold increase in reversion for samples with decreased concentration of dATP but without any m^6dGTP . For any particular m^6dGTP concentration, the reversion frequency was greater for higher dATP concentrations. This appears to be related to the higher level of DNA synthesis shown in Table 2 and may be due to some combination of the small pool bias effect in our system, reduced editing at higher replication rates (Loeb and Kunkel, 1982), and the higher probability of reversion as a direct consequence of more replication events at the site of the amber mutation.

Generation of temperature-sensitive mutants. Because the frequency of mutagen-induced reversion often varies from site to site, we also monitored the conditional lethality of mutations in any essential T7 gene by measuring the generation of temperature-sensitive mutants. Wild type T7 DNA was replicated in the presence of various concentrations of dATP and m^6dGTP and packaged into phage heads to form viable phage (without the use of the T7_{4ts} extract). The initial screen for (reversible) temperature-sensitive mutants consisted of incubating the phage on lawns of suppressor-free indicator bacteria for a short time at the restrictive temperature (44°) followed by a long incubation at a permissive

Figure 9. Effect of m^6dGTP concentration on reversion frequency and survival. The procedure for DNA synthesis and packaging is described in Table 2. "Reversion Frequency" (upper frame) is the fraction of $amber^+$ SS^- phage per surviving phage. "Revertants" (lower frame) refers to the total number of $amber^+$ SS^- phage per plated mixture. Reversion frequency and survival are shown as a function of dATP and m^6dGTP concentrations in the synthesis reaction. Symbols: upper frame, reversion frequency: (Δ) 50 μM dATP, ($\blacktriangle$) 100 μM dATP; lower frame, number of phage as determined on indicator strains Sh3-18 (Sup^+) ($\bullet$) and ShD2371-48 (Sup^o) ($\circ$).

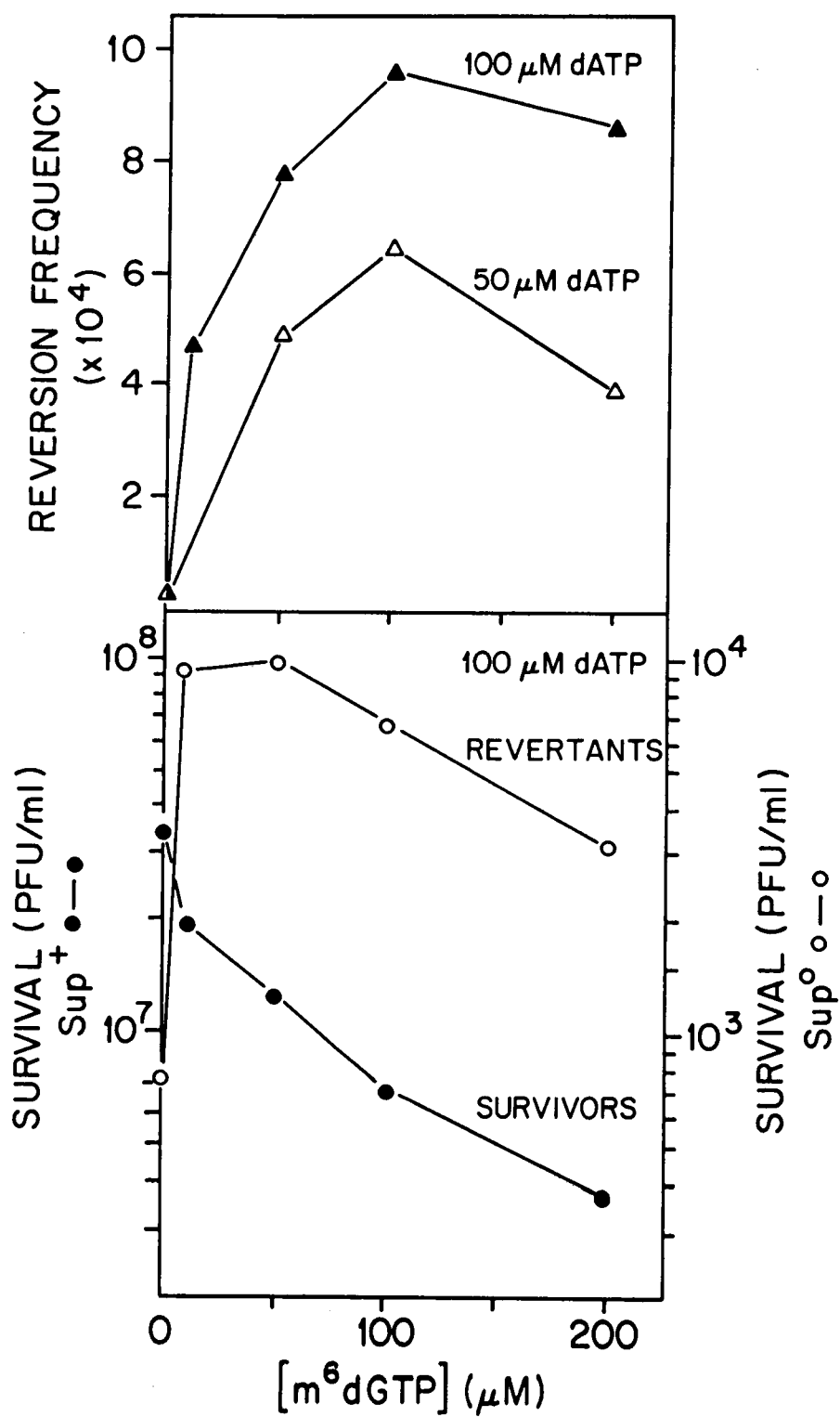


Figure 9.

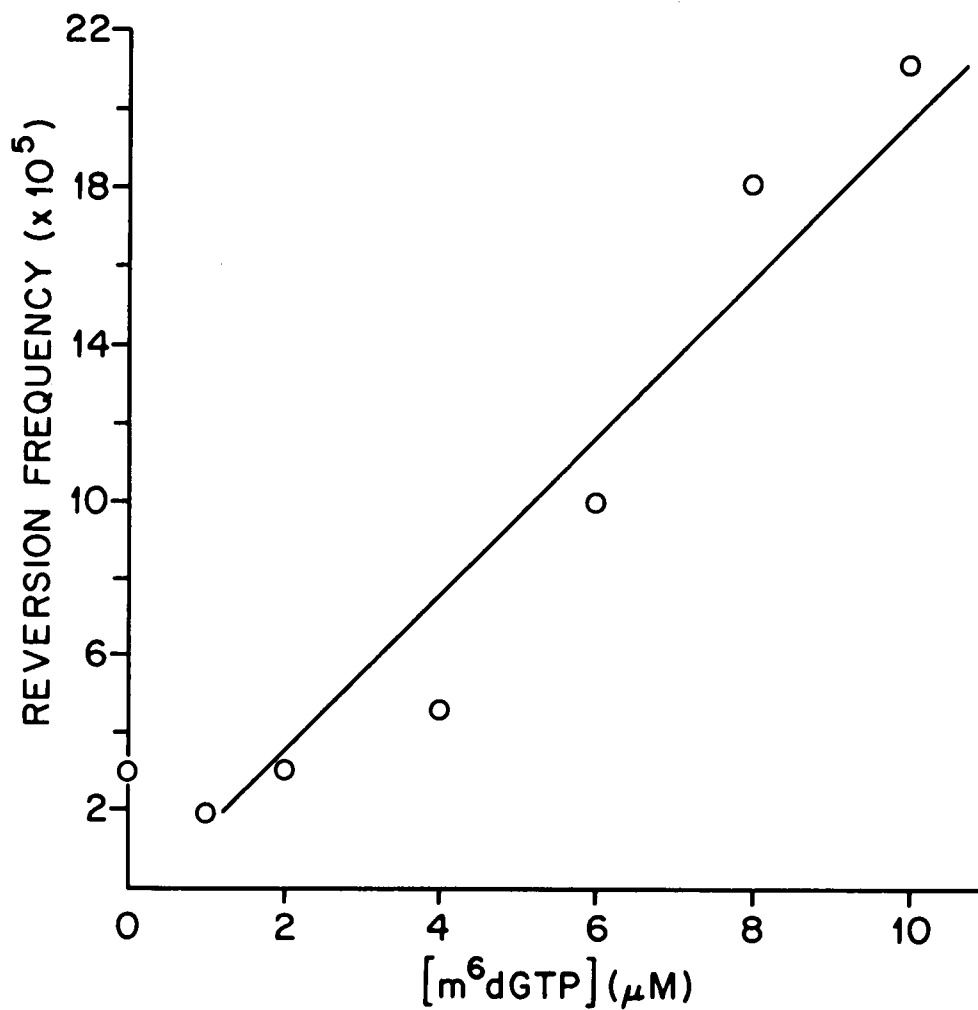


Figure 10. Effect of m^6dGTP concentration on reversion frequency. The procedure for DNA synthesis and packaging is described in Table 2. Reversion frequency is shown as a function of m^6dGTP concentration in the synthesis reaction. The concentration of dATP was $100 \mu M$.

temperature (30°). Phage that appear as plaques after the temperature shift were tested again and then titered for the final temperature-sensitivity determination. Phage were not classified as temperature-sensitive unless the titer was at least 1000-fold higher at the permissive temperature. As shown in Table 3 (lines 1 and 2), there was no increase in the number of temperature-sensitive mutants after replication under standard conditions. When m⁶dGTP was present, up to 1% of the viable phage were temperature-sensitive.

m⁶dGMP incorporation into T7 DNA. An experiment was performed to determine whether some of the m⁶dGTP introduced into the reactions was indeed incorporated into product DNA. Incorporation of m⁶dGMP into DNA is shown in Table 4 as a function of both m⁶dGTP concentration and the amount of time allowed for DNA synthesis. By using m⁶dGTP radiolabeled in a ring position ([8-³H]m⁶dGTP), we avoided possible underestimation of m⁶dGMP incorporation due to rapid in situ demethylation by methyltransferase (Foote et al., 1980; Olsson and Lindahl, 1980). The number of molecules of m⁶dGMP incorporated increased with time for reactions containing either 10 μM or 50 μM m⁶dGTP (Table 4). Moreover, incorporation was greater for reactions containing the higher m⁶dGTP concentration. By comparing these values with those shown in Table 2 (page 42), an increase in reversion frequency (relative to standard conditions) of approximately 30- and 50-fold corresponds, respectively, to about 10 and 80 m⁶dGMP molecules incorporated per genome equivalent of newly synthesized T7 DNA.

Table 3. Generation of Temperature-Sensitive Mutants^a

dATP Conc. (μ M)	m ⁶ dGTP Conc. (μ M)	DNA Synthesis (nmol)	Survival (%)	Number of Plaques Examined	Temperature-Sensitive Mutants	
					Number	%
300	0'control	0.3	25	8000	1	0.01
300	---	2.9	100	7200	1	0.01
100	---	2.0	108	6500	2	0.03
100	50	2.1	70	3500	17	0.49
100	100	1.9	50	2600	25	0.96
100	200	1.9	30	2700	13	0.48

^aThe procedure for DNA synthesis and packaging was the same as that described in Table 2, except that [³²P]dCTP was present at 0.8 cpm/pmol and 0.03 ml packaging reactions contained no extract prepared from T7_{4ts} phage. The reaction in line 1 was held at 0°C, all others were incubated 30 min at 30°C. As described in the text, temperature sensitivity of resulting phage was determined by a series of incubations at 44° and 30°. The packaging efficiency was 0.04%. "Survival" refers to the yield of phage produced by packaging a fraction of each sample and incubating at 30°. Survival is expressed relative to that obtained for the sample shown in line 2.

Table 4. Incorporation of m⁶dGMP^a

Conc. m ⁶ dGTP (μ M)	Time (min)	DNA Synthesis (nmol)	m ⁶ dGTP Incorp. (pmol)	Molecules m ⁶ dGMP Incorporated per Genome Equivalent of DNA
10	20	0.69	0.029	3
10	25	0.84	0.049	5
10	30	1.11	0.127	9
50	20	0.54	0.369	55
50	25	0.68	0.538	64
50	30	0.85	0.873	82

^aThe procedure for DNA synthesis was the the same as described in Table 2, except that O⁶-methyl [8-³H]dGTP was included in the reactions and 20 μ l samples were acid-precipitated at the indicated times. m⁶dGMP incorporation was determined by the amount of acid precipitable [³H]-radioactivity. The amount of newly synthesized DNA was determined in a duplicate reaction that included [³²P]dCTP.

Discussion

We have obtained high levels of mutagenesis with two distinct assays as a result of including m^6dGTP as a precursor for in vitro DNA replication. A possible explanation for the observed reversion of the amber 3'-ATC-5' / 5'-TAG-3' mutation is that m^6G is incorporated opposite T at the first and/or second position of the codon. Demethylation of the m^6G could then produce AT→GC transitions during subsequent rounds of DNA replication. S. Mitra has measured approximately 40 molecules of methyltransferase per cell equivalent of protein in our extracts used for DNA replication (data not shown). This is consistent with other measurements of the constitutive level of methyltransferase (Lindahl et al., 1982; Mitra et al., 1982). Thus, in a typical reaction the methyltransferase (which acts stoichiometrically and very rapidly) is present at about 3 molecules per genome of newly synthesized DNA. Our previous studies (Kuemmerle and Masker, 1977; Dodson and Masker, 1981) with this DNA packaging system have demonstrated that damaged DNA is not selected against during the encapsulation process. Also, at least some types of DNA damage can be repaired by the extracts used for packaging (Dodson and Masker, 1981). Thus, the methyltransferase present in the DNA replication reaction together with possible demethylation during subsequent packaging and growth on the indicator strain is probably sufficient (at least at low levels of m^6dGMP incorporation) to quickly convert the m^6G residues to G. Table 4 shows that at concentrations of 10 μM and 50 μM m^6dGTP , there were about 10 and 80 molecules, respectively, of m^6dGMP incorporated into DNA. Table 2 (page 42) shows that at 100 μM dATP and 10 μM m^6dGTP the reversion frequency was about 4.7×10^{-4} . If these mutations follow a

Poisson distribution and half occur at the first position of the amber codon, misinsertion at a particular A·T site occurs with a frequency of 2.3×10^{-4} . This leads to an estimate of 4.7 misinsertions per genome. At 50 μ M m^6dGTP , our measured reversion frequency suggests eight mutagenic events per genome. A possible source for the differences between these estimates and the data in Table 4 is that the higher values of incorporation (Table 4) include nonmutagenic $m^6G \cdot C$ pairs. Mismatch repair (Karran and Marinus, 1982), which in T7 does not discriminate between parental and daughter DNA strands (Bauer et al., 1981), could alter the apparent levels of incorporation and the yield of mutants. Also, incorporation of m^6dGMP (or the presence of m^6dGTP in the reaction) might lead to generation of partial genomes and more noninfective DNA molecules (Table 2 and Figure 9, page 44), especially if the number of m^6G incorporations exceeds the number of methyltransferase molecules in the extracts.

The data presented here demonstrate that mutagenesis can be examined in a relatively complete in vitro system that makes multiple copies of a duplex T7 DNA molecule and can also perform many of the steps of DNA repair and recombination. Our experiments also provide strong evidence for the premutagenic potential of O⁶-methylguanine.

CHAPTER IV

INDUCIBLE REACTIVATION OF BACTERIOPHAGE T7 DAMAGED BY METHYL METHANE SULFONATE OR UV LIGHT

Summary

We have examined the effects of host mutations affecting SOS mediated UV reactivation on the survival of bacteriophage T7 damaged by UV-light or methyl methane sulfonate (MMS). Survival of T7 alkylated with MMS was not affected by the presence of pKM101 nor by a umuC mutation in the host. The survival of UV-irradiated T7 was similar in umuC⁺ and umuC⁻ strains but was slightly enhanced by the presence of pKM101. When phage survival was determined on host cells preirradiated with a single inducing dose of UV light, these same strains permitted higher survival than what was seen with noninduced cells for both UV- and MMS-damaged phage. The extent of T7 reactivation was approximately proportional to the UV-inducing dose inflicted upon each bacterial strain and was dependent upon phage DNA damage. Enhanced survival of T7 after exposure to UV light or MMS was also observed after thermal induction of a dnaB mutant. Thus, lethal lesions introduced by UV light and MMS are apparently repaired more efficiently when host cells are induced for the "SOS" cascade and this inducible reactivation of T7 is umuC⁺-independent.

Introduction

In E. coli, a group of cellular functions is activated in response to new protein synthesis induced by conditions such as DNA damage that inhibit normal DNA replication (for review, see Witkin, 1976; Little and

Mount, 1982). These "SOS functions" include altered responses to phage infection that are referred to as Weigle reactivation and Weigle mutagenesis. Weigle reactivation (also called UV-reactivation) (Weigle, 1953) refers to the enhanced survival of phage that have suffered DNA damage when plaque-forming ability is determined on host cells induced for the SOS response. The accompanying increase in phage mutants is termed Weigle mutagenesis. Although the molecular details responsible for these phenomena are not yet understood, it has been demonstrated that the host recA, lexA, and umu functions are necessary for both Weigle reactivation and mutagenesis. In contrast to the pleiotropic effects and known regulatory roles of RecA and LexA in the SOS response, the umuC and umuD gene products appear to be specifically involved in Weigle reactivation and mutagenesis (Kato and Shinoura, 1977; Elledge and Walker, 1983), although the biochemical nature of these gene products is currently unknown. The function(s) of the muc genes carried on plasmid pKM101 (Perry and Walker, 1982) appears to be analogous to that of the chromosomal umuC and umuD genes (Elledge and Walker, 1983) since the UV-mutagenesis and inducible phage reactivation deficiencies exhibited by umuC and umuD strains are restored in bacterial strains that contain the plasmid (Walker and Dobson, 1979).

Although UV-irradiated lambda-infected E. coli has been the system of choice for most studies of inducible phage reactivation, additional insight into the mechanism(s) responsible for this phenomenon may be gleaned from studies with other bacteriophages and types of DNA damage other than UV. Some of these results are summarized in Table 5. For example the demonstration of Weigle reactivation in phages containing

Table 5. Inducible Phage Reactivation

Phage	Phage Damaging Agent	Bacterial Host Strain	Reactivation ^a	Reference
λ	UV	AB1157 (wt)	+	Defais et al., 1971; Defais et al., 1976
		<u>recA</u>	-	Defais et al., 1971
		<u>lexA</u>	-	Defais et al., 1971
		<u>umuC</u>	+/-	Walker and Dobson, 1979; Schendel and Defais, 1980
		<u>uvrA</u>	+/-	Defais et al., 1971; Defais et al., 1976; Rothman et al., 1979
		<u>uvrB</u>	+/-	Rothman et al., 1979
	MMS	GM3217 (<u>tif sfi</u>)	-	Janion, 1982
ϕ X174	UV	<u>E. coli C</u>	+	DasGupta and Poddar, 1975
		<u>uvrA</u>	+	DasGupta and Poddar, 1975
		<u>recA</u>	-	DasGupta and Poddar, 1975
T7	UV	AB1157 (wt)	+	McKee and Hart, 1975
		<u>recA</u>	+	McKee and Hart, 1975
		<u>uvrB</u>	-	McKee and Hart, 1975

^a+, reactivation; -, no reactivation; +/-, at least partial reactivation. The roles of host function in enhanced survival of damaged phage are summarized.

single-stranded DNA implies a mechanism distinct from recombination. Since various types of phage depend to a different extent on their host's DNA replication, repair, and recombination systems, comparison of reactivation of different bacteriophage and different types of damage may also provide clues as to the biochemical steps at which the proteins induced during the SOS cascade exert their influence. Previous reports have catalogued different effects of bacterial recA, uvrB, and polA mutations for Weigle reactivation of lambda and T7. This is of considerable interest since, unlike lambda, T7 is a virulent phage insensitive to SOS regulation of repression and because, unlike lambda, T7 maintains its own DNA replication system that is independent of host DNA replication enzymes and factors. But, like lambda and ϕ X174, T7 does rely almost exclusively on its host for DNA repair processes. Thus, questions arise as to whether reactivation of damaged T7 and lambda are both due to a single biochemical mechanism, what host factors influence the fate of the damaged phage, and, in particular, whether the product of the umu genes or the presence of the pKM101 plasmid would enhance the survival of UV-irradiated T7 as previously reported for lambda. Our interest in reactivation of DNA damage in T7 has also been prompted by our previous studies of non-induced repair modes in this phage and by the availability of in vitro systems for the replication and packaging of T7 DNA that allows measurements of in vitro mutagenesis (Kuemmerle and Masker, 1977; Masker et al., 1978; Dodson and Masker, 1981; Dodson et al., 1982). Our choice of damaging agents other than UV derives from the proposal (Radman et al., 1979) that UV mutagenesis and a substantial portion of MMS induced mutagenesis is mediated by SOS phenomena while EMS mutagenesis is mediated

by SOS phenomena while EMS mutagenesis is thought to be independent of thought to be independent of the SOS effect. In contrast, it has been reported (Janion, 1982) that reactivation is not observed for lambda and T7 phage treated with MMS. We have compared induced reactivation of intact T7 phage damaged with UV or MMS and grown on several E. coli mutants exposed to conditions that trigger the SOS response and present the results of our findings here.

Materials and Methods

Bacterial strains and bacteriophage T7. Strains of E. coli used in this study are shown in Table 6. Strain WM359 was constructed by transduction with P_{vir} (Miller, 1972) grown on strain GW2100 and selection for kanamycin-resistant recombinants. The presence of the pKM101 plasmid or the umuC mutation were routinely verified by testing resistance to the appropriate antibiotic and by measuring the frequency of reversion of the his mutation after exposure to MMS (Walker, 1977).

Wild type bacteriophage T7 were from the collection of F. W. Studier.

Media, chemicals, and reagents. L-broth (Miller, 1972) was routinely supplemented with 10 μg of thymine per ml. T7 diluent (Studier, 1969), phosphate buffer (Dodson and Masker, 1981), and sources of materials (Dodson and Masker, 1981) have been previously described. MMS was obtained from Kodak Laboratory Chemicals.

Alkylation of phage. T7 phage were alkylated as previously described (Dodson and Masker, 1981, and Chapter II, page 7).

Table 6. Bacterial Strains

Strain	Relevant Geneotype	Comments	Source
AB1157	WT ^a		P. Swenson
AB1157 pKM101			P. Swenson
GW2100	<u>umuC122::Tn5</u>	Tn5 insertion at the <u>umuC</u> locus of strain AB1157	G. Walker
TN207	<u>uvrA</u>	<u>uvrA</u> introduced into a <u>thr</u> derivative of strain W3110 by mating	D. Stallions
AB2499	<u>uvrB5</u>	A <u>uvrB</u> <u>thy</u> derivative of strain AB1157	A. Ganesan
AB2494	<u>lexA</u>	A <u>lexA</u> derivative of strain AB1157	C. Richardson
JC2926	<u>recA13</u>		A. Ganesan
WM295	<u>recA56</u>	UV-sensitive <u>thy</u> ⁺ recombinant of mating strain JC5088 (Hfr <u>recA56</u>) and strain W3110 (F ⁻ <u>thy</u>)	W. Masker
WM359	<u>uvrB5</u> <u>umuC122::Tn5</u>	<u>umuC</u> introduced into strain AB2499 by P1 transduction	This work
KLC474	<u>dnaB1029</u>		J. Chase

^aWT, wild type for DNA repair.

UV irradiation. Phage were diluted into T7 diluent to a concentration of approximately 1×10^8 PFU/ml and irradiated in 0.5 ml portions in 7-cm watch glasses with constant stirring at room temperature. Survival of phage irradiated at an incident dose rate of 0.5 J/m^2 per S was determined on UV-sensitive strains AB2499 (uvrB), TN207 (uvrA), and WM359 (uvrB umuC::Tn5). Survival of phage irradiated at an incident dose rate of 0.8 J/m^2 per S was determined on all other strains.

Bacterial cells were grown in L-broth at 37° to a density of 3.5×10^8 cells per ml. Approximately 200 ml portions were irradiated at room temperature in 17-cm dishes on a rotating platform. The depth of liquid was approximately 1 cm. (Under these conditions the absorbed UV dose is expected to be considerably lower than the incident dose.) UV-sensitive strains AB2499 (uvrB), TN207 (uvrA), and WM359 (uvrB umuC::Tn5) were irradiated at an incident dose rate of 0.7 J/m^2 per S, and strains JC2926 (recA13) and WM295 (recA56) were irradiated at an incident dose rate of 0.1 J/m^2 per S. All other strains were irradiated at an incident dose rate of 1.4 J/m^2 per S. After irradiation, cells were grown for 15 min at 37° (unless otherwise indicated), followed by the addition of 1.0 ml portions to appropriate dilutions of T7 phage (0.1 ml). After the addition of 2.5 ml top agar the mixture was poured on T plates which were incubated at 37° . All manipulations and incubation subsequent to irradiation were carried out under dim yellow lights.

Thermal induction of a dnaB mutant. Strain KLC474 (dnaBts) was grown in L-broth at 30° to a density of 3.5×10^8 cells per ml. Cultures (approximately 40 ml) were then shifted to growth at 42° . Immediately before the temperature shift or at various times thereafter, 1 ml portions

were added to appropriate dilutions of T7 phage (0.1 ml). After the addition of 2.5 ml top agar the mixture was poured on T plates which were incubated at 30°.

Results

To examine the possibility of inducible reactivation of T7 containing lesions introduced by alkylating agents and to determine the possible roles of umu/muc gene products in phage survival, we performed the experiment shown in Figure 11. Survival of T7 after treatment with various concentrations of MMS and infection of noninduced E. coli strains was not affected by the presence of the pKM101 plasmid nor by a umuC mutation in the host. When phage survival was determined on host cells preirradiated with a single inducing dose of UV light, these same strains permitted higher survival than what was seen with noninduced cells. The ratio of phage survival on preirradiated host cells to survival on cells of the same strain not induced by prior DNA damage is referred to as the "reactivation factor" and is also plotted in Figure 11. The extent of reactivation was a function of the amount of alkylation damage suffered by the phage but was not influenced to any large degree by the chromosomal umuC or plasmid-encoded muc gene products. This finding of inducible reactivation of MMS-damaged T7 is especially interesting in view of the recent report (Janion, 1982) that the viability of lambda or T7 treated with MMS is not enhanced when host cells are induced for the SOS response.

We next examined the reactivation of UV-irradiated T7 in order to make comparison with our results obtained using MMS and, since UV has been used as a damaging agent in several prior studies, to facilitate

Figure 11. Survival and inducible reactivation of MMS-damaged T7. Suspensions of T7 phage were alkylated with various concentrations of MMS as described in Chapter II. After treatment, the phage were diluted and survival was determined on the indicated strains preirradiated with 0 or an incident dose of 200 J/m^2 (noninduced and induced, respectively) as described in the text. (Upper Frame) Phage survival on noninduced (mock-irradiated) strains. (Lower Frame) The ratio of phage survival on induced cells to survival on noninduced cells of the same strain. Different symbols indicate the bacterial strains used for determining phage titers: (○) AB1157 (wt); (□) AB1157 (pKM101); (Δ) GW2100 (umuC::Tn5).

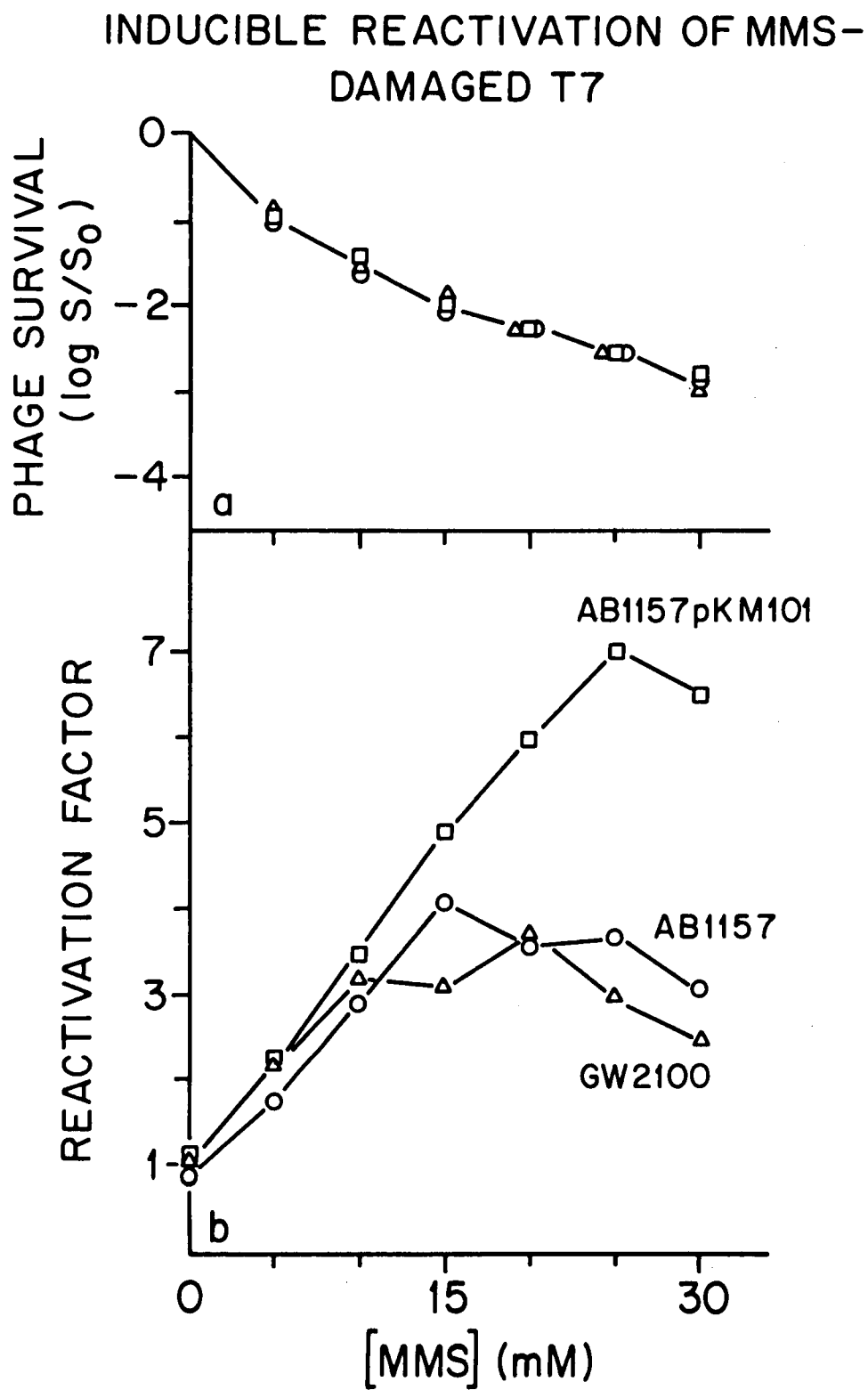


Figure 11.

comparison of T7 with other phages. Figure 12 shows that the survival of T7 when determined on noninduced cells, was similar in umuC⁺ and umuC⁻ strains but was slightly enhanced by the presence of pKM101 (plasmid-mediated reactivation). The levels of reactivation displayed by host cells preirradiated with UV light before phage infection (Figure 12) were similar to those obtained with MMS-treated T7 (Figure 11) even though more extensive killing was achieved for UV-damaged T7. Also, inducible phage reactivation of T7 differed from previous reports of Weigle reactivation of lambda in that the effects of the umuC and muc gene products were minimal and the extent of reactivation was usually below the 10- to 100-fold increases in survival normally observed with lambda. (In some experiments we have observed a reactivation factor as high as 14 with T7, but the results shown in Figures 11 and 12 are more typical of what was found on several repeats of these experiments.)

To more thoroughly characterize the T7 reactivation phenomenon we performed the experiment in Figure 13. In this experiment T7 was exposed to a single concentration (20 mM) of MMS and survival was determined on host cells that had received various inducing doses of UV light. These data show that the extent of T7 reactivation was approximately proportional to the UV-inducing dose inflicted upon each strain. Also, the fact that survival of phage that were not exposed to MMS remained constant regardless of the induction protocol means that the observed reactivation is entirely dependent upon phage DNA damage. Reactivation of UV-irradiated T7 was also proportional to the inducing dose applied to the bacteria (data not shown). The T7 reactivation also showed induction kinetics similar to that reported for lambda (Defais et al., 1971). When

Figure 12. Survival and inducible reactivation of UV-irradiated T7. Suspensions of T7 phage were irradiated with various doses of UV light as described in the text. After irradiation, the phage were diluted and survival was determined as described in the legend to Figure 11. Symbols are the same as those in Figure 11.

INDUCIBLE REACTIVATION OF UV-IRRADIATED T7

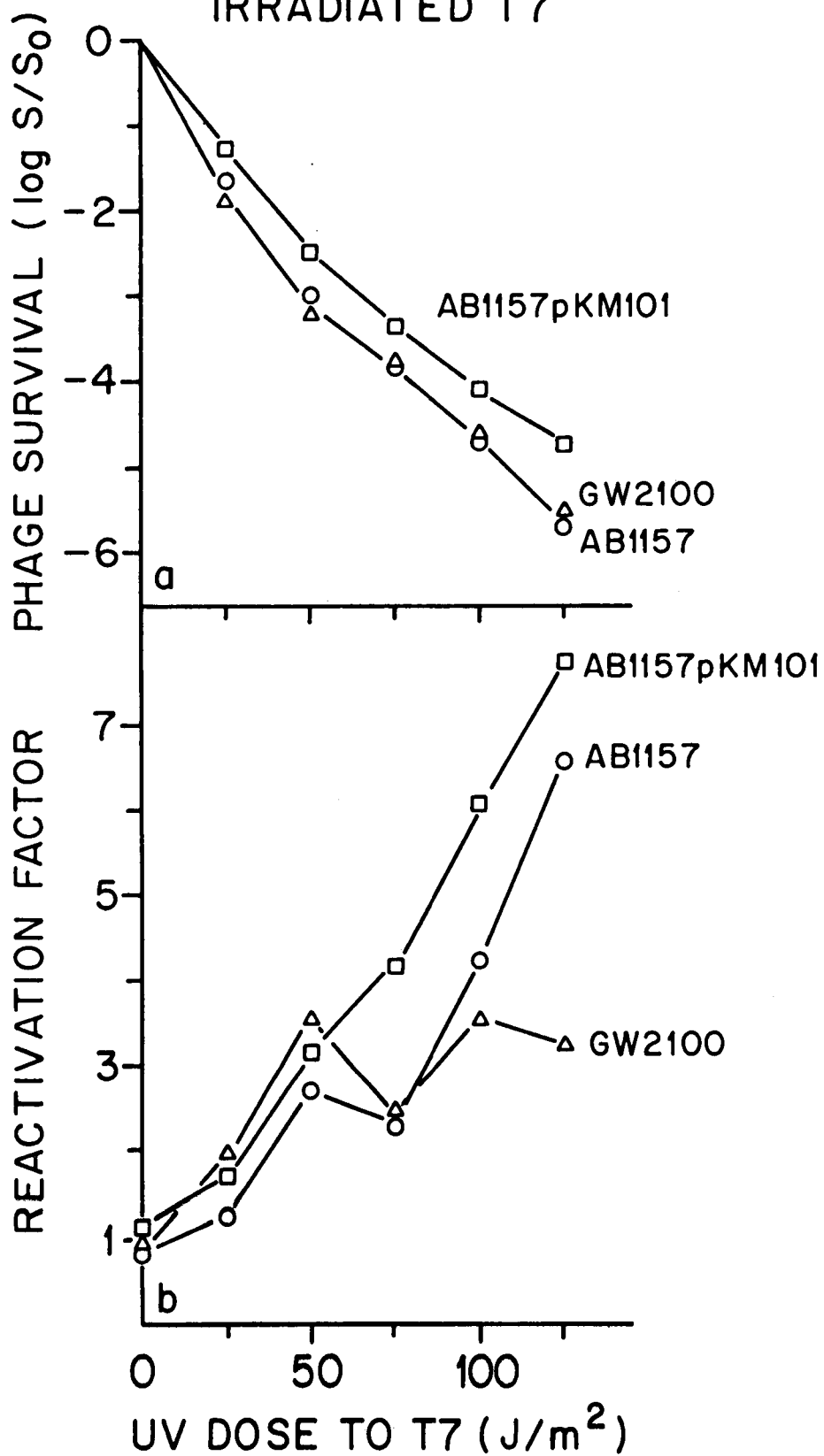


Figure 12.

our "zero-time" point was chosen as the time of absorption of alkylated phage to host cells immediately after UV irradiation, the yield of surviving phage was maximal when the host cells were allowed approximately 30 min post-irradiation incubation in growth medium prior to infection. Reactivation decayed rapidly to less than 50% of the maximum level by 60 min of post-irradiation growth (data not shown).

There are several mechanisms by which the SOS response can be induced in bacteria. For example, comparable degrees of lambda phage reactivation have been reported after preirradiation of host cells with UV light or after incubation of unirradiated tif hosts at 42°C before phage infection (Castellazzi et al., 1972). Temperature-sensitive dnaB mutants of E. coli are similar to tif strains in that they express SOS functions at 42°C (Witkin, 1976). In dnaB mutants, the immediate cessation of replicative DNA synthesis at the restrictive temperature is known to trigger induction of SOS effects (Witkin, 1976). Since bacteriophage T7 replicates its DNA independently of the DnaB protein, it seemed likely that if the results seen in Figures 12 and 13 are indeed true SOS phenomena the survival of damaged T7 should be enhanced by thermal induction of a dnaB strain. Table 7 shows that levels of reactivation similar to those already presented (Figures 11, 12, and 13) were obtained with this induction protocol. As expected, the reactivation capacity decayed with time of incubation at 42°.

Our current understanding of the regulation of SOS functions (Little and Mount, 1982) suggests that activation of RecA protein as a protease results in the cleavage of LexA repressor, thereby increasing the level of expression of several genes (din) whose products participate in the

Figure 13. Dependence of reactivation on bacterial inducing dose and phage damage. T7 phage were treated with 0 or 20 mM MMS as described in Chapter II. After treatment, the phage were diluted and survival was determined on the indicated strains preirradiated with various inducing doses of UV light as described in the text. The survival of alkylated phage determined on noninduced cells was similar for all three strains (approximately 0.4%). The data are plotted as the ratio of phage survival determined on cells preirradiated with the indicated UV dose to the survival on unirradiated cells of the same strain. Symbols: Phage exposed to 0 mM MMS and plated on strains (○) AB1157, (□) AB1157 (pKM101), and (△) GW2100 (umuC::Tn5); phage exposed to 20 mM MMS and plated on strains (●) AB1157, (■) AB1157 (pKM101), and (▲) GW2100 (umuC::Tn5).

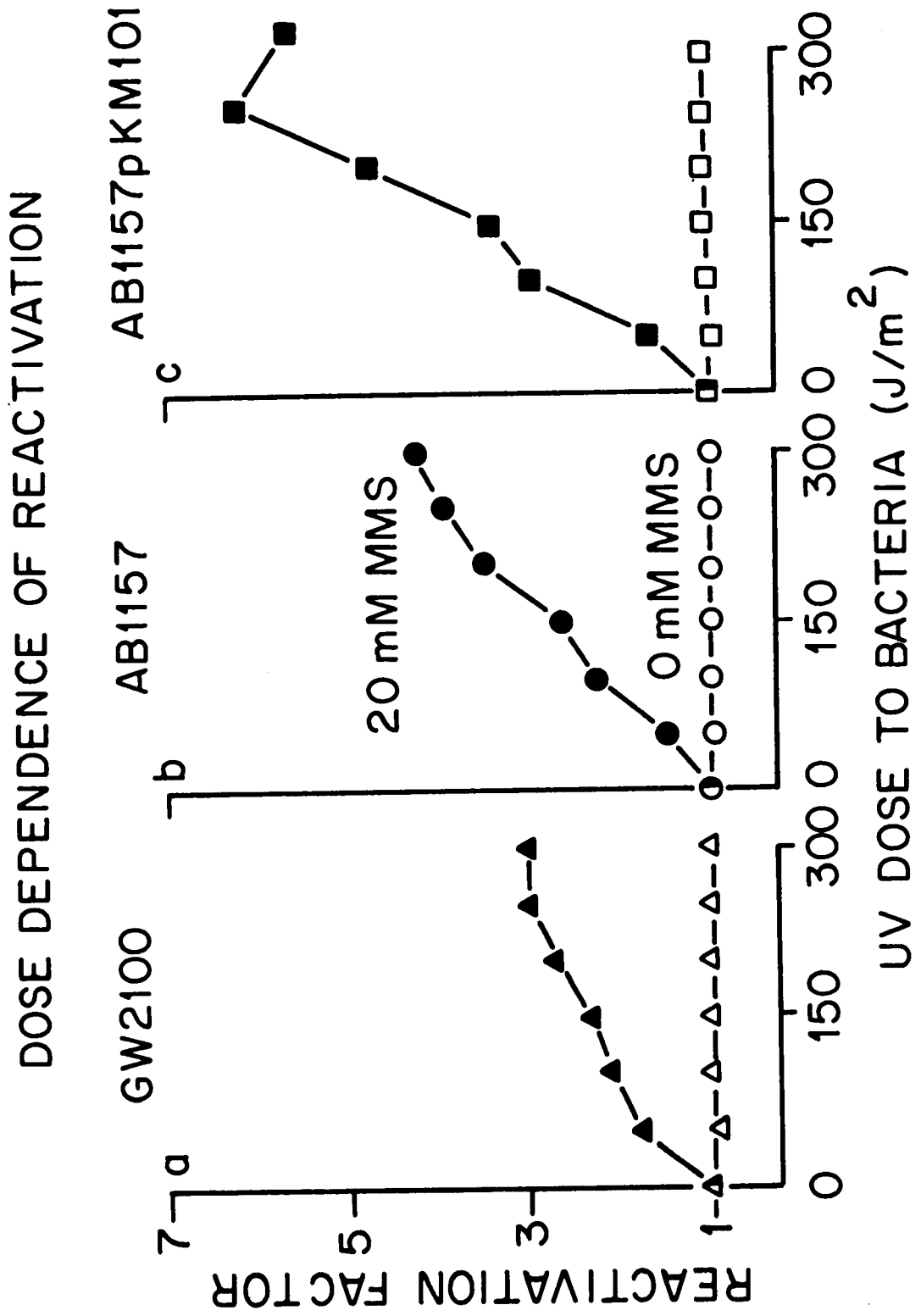


Figure 13.

Table 7. Phage Reactivation in a dnaB Mutant^a

Phage treatment	Incubation time at 42° (min)			
	0	30	60	75
10 mM MMS	1.0 (2.3%)	1.6	3.1	2.2
100 J/m ²	1.0 (6.7 x 10 ⁻⁴ %)	4.6	4.6	2.1

^aEnhanced survival of damaged T7 after thermal induction of a dnaB mutant. Suspensions of T7 phage were alkylated with 10 mM MMS or irradiated with 100 J/m² as described in the text. Phage survival was determined immediately before (0 min) or at various times after shifting an exponentially growing culture of strain KLC474 (dnaB) from 30° to 42°. Phage titers determined at various times after the temperature shift are expressed relative to the titer obtained at 0 min. The numbers in parentheses are the survival of damaged phage relative to undamaged controls determined at 0 min. The titers of undamaged control phage remained essentially constant with incubation of host cells at high temperature (phage reactivation equaled 1.0 ± 0.2).

SOS response. Consistent with this regulatory scheme is the inability of recA strains of E. coli to support Weigle reactivation of lambda (Defais et al., 1971). In contrast, it has been reported that reactivation of bacteriophage T7 proceeds normally in recA hosts (McKee and Hart, 1975). Our results (data not shown) with reactivation of damaged T7 by recA hosts have been far less consistent than what we found with other strains. In some experiments the recA strains showed virtually no ability to reactivate damaged T7 phage. However, most of our experiments corroborated the earlier studies with T7 and showed that when strains JC2926 (recA13) and WM295 (recA56) were preirradiated with low incident doses of up to 7.5 J/m^2 of UV, these strains had increased capability to support the growth of UV irradiated T7. Our results with a lexA mutant are consistent with this latter result. As seen in Table 8 reactivation of T7 obtained with strain AB2494 (lexA) was less pronounced than what was obtained with the wild type (lexA⁺) strain AB1157 (Figures 11, 12, and 13) but was still significantly higher than what can be dismissed as experimental error. Although these results tend to argue that T7 reactivation does not require the rec-lex functions that are almost the definition of a true SOS phenomenon, the small size of the T7 genome relative to that of E. coli might allow some reactivation in spite of low but non-zero levels of the RecA protein. Our results (Table 8) with strains TN207 (uvrA) and AB2499 (uvrB) are more difficult to interpret. Both of these mutants show minimal reactivation of T7 damaged with either UV or MMS. Also, the double mutant WM359 (uvrB umuC::Tn5) gave values similar to the single mutant AB2499 (uvrB) (data not shown). It is possible that in these strains supplies of proteins normally available to

Table 8. Phage Reactivation in *E. coli* Mutants^a

Bacterial Strain	Phage treatment		
	[MMS] (mM)	UV Dose (J/m ²)	
		30	100
AB2494 (<u>lexA</u>)	2.3 (0.5%)		2.6 (0.002%)
TN207 (<u>uvrA</u>)	1.8 (0.2%)	1.6 (0.007%)	
AB2499 (<u>uvrB</u>)	1.6 (0.9%)	1.2 (0.01%)	

^aPhage reactivation in *E. coli* mutants. Suspensions of T7 phage were alkylated with 20 mM MMS or irradiated with 30 or 100 J/m² as described in the text. Reactivation factors are expressed as the ratio of phage survival on induced cells to survival on noninduced cells of the same strain. Strain AB2494 (lexA) was induced with a single dose of 50 J/m² and strains TN207 (uvrA) and AB2499 (uvrB) were both induced with a dose of 20 J/m². The numbers in parentheses are the survival of damaged phage relative to undamaged controls as determined on noninduced cells.

reactivate phage DNA are depleted as the host strives to use its SOS functions to cope with damage left in its own genome as the result of excision deficiency. But, the use of a low inducing dose in the experiment shown in Table 8 argues against this idea. It has been shown that both uvrA and uvrB are din genes and that higher levels of the UvrA and UvrB incision proteins are produced after SOS induction. Thus in the case of UV damage to the phage, it is possible that some of the T7 reactivation may be attributed to increased pyrimidine dimer excision activity upon which the phage depends for repair of UV damage. However, there is no evidence for direct action of uvrA or uvrB on the repair of MMS induced damage and our (unpublished) data indicate that host cell reactivation of MMS treated T7 is similar irrespective of whether the phage is grown on noninduced wild type, uvrA or uvrB strains. Our data therefore confirm and extend the earlier report (McKee and Hart, 1975) that reactivation of UV and HNO₂ damaged T7 requires a functional uvrB gene.

Discussion

In E. coli, at least two distinct systems are induced by DNA damaging treatments that lead to altered processing of DNA lesions. Cells induced for the adaptive response (Jeggo et al., 1977a and 1977b; Samson and Cairns, 1977) by growth in media containing low concentrations of an alkylating agent are more resistant to the killing and mutagenic effects of a subsequent higher challenge dose. The observation (Jeggo et al., 1977a and 1977b) that polA mutants adapt to the mutagenic but not killing effects of alkylating agents suggested the existence of more than one biochemical mechanism underlying this response. We now know that higher survival results (at least in part) from the induction of a glycosylase

that catalyzes the release of purines methylated at ring nitrogen atoms (Karran et al., 1982) and that lower mutation frequencies result from induction of a special protein that acts as a methyl acceptor for methyl groups bound at the O-6 position of guanine (Schendel and Robins, 1978; Karran et al., 1979; Olsson and Lindahl, 1980). In contrast to the error-free nature of the adaptive response, induction of the SOS cascade makes the cellular machinery error-prone. Despite extensive information concerning the regulation of the SOS cascade, few molecular details are currently known regarding the mechanisms of two SOS functions originally described (Weigle, 1953) thirty years ago, inducible phage reactivation and mutagenesis.

One model that partially explains the increased survival of damaged phage argues that induction of the SOS cascade promotes more efficient excision repair. This model is supported by observations that reactivation of lambda is partially reduced in uvrA and uvrB mutants (Rothman et al., 1979) and that these uvr genes are included (Kenyon and Walker, 1981; Sancar et al., 1982) in the recently discovered set of din genes (Kenyon and Walker, 1981). However, further studies of inducible reactivation of lambda in other mutants of E. coli indicate a uvrA⁺-independent component dependent on umuC⁺ and demonstrate the availability of alternative pathways leading to higher phage survival. It appears from our data that inducible reactivation of damaged T7 is umuC⁺-independent. The efficiency of repair of lethal lesions as calculated by the method of Devoret et al. (1975) is 12% and 14%, respectively, for UV-irradiated T7 grown on strains GW2100 (umuC::Tn5) and AB1157 (wt) (Figure 12, page 63). For comparison, the extent of reactivation of UV-irradiated lambda in

umuC mutants is less than half the value observed in umuC⁺ strains (24% and 55%, respectively) (Walker and Dobson, 1979). It may be that reactivation of lambda involves two or more components whereas in T7 the umuC⁺-dependent component does not operate. Considering that lambda relies very heavily on host functions for DNA replication while T7 employs virtually all phage-encoded proteins, our results are in accord with the notion that the umuC gene product functions during DNA replication or interacts with one of the E. coli DNA replication proteins. The lower efficiency of repair calculated for T7 also argues against the possibility that T7 codes for a protein functionally analogous to UmuC. It is possible, however, that T7 might code for a function that interferes with the host UmuC protein.

Since the functions of some din genes are as yet unknown and several additional din genes may be identified in the future (Kenyon and Walker, 1980), it is not unreasonable to speculate that other damage-inducible schemes may also contribute to phage survival. Our observation of enhanced survival of T7 damaged by MMS or UV light upon infection of preirradiated lexA cells (Table 8) could be interpreted to mean that we are observing a phenomena other than the typical SOS-inducible "Weigle reactivation," and that this process is also thermally induced in a dnaB mutant (Table 7). These questions underscore the need for understanding the mechanisms behind these processes and augmenting the primarily genetic approach of the present study with a more biochemical approach in which inducible repair mechanisms can be studied in vitro. We are now attempting to extend our experiments to include an in vitro study of the mechanism(s) of inducible reactivation of T7 by using a well-characterized

in vitro system (Kuemmerle and Masker, 1977; Masker et al., 1978; Dodson and Masker, 1981; Dodson et al., 1982) that performs at least some DNA repair of lethal lesions, is capable of extensive DNA synthesis that closely mimics the in vivo DNA replication process, and that has already proved useful as a sensitive probe for in vitro mutagenesis under conditions where the normally accurate DNA replication process is perturbed (Dodson et al., 1982).

CHAPTER V

SUMMARY AND IMPLICATIONS

In vitro studies of the molecular events of DNA replication provide detailed information unobtainable with in vivo systems. However, in vitro systems that employ synthetic DNA templates and purified enzymes represent a major departure from the in vivo situation. Recent studies employing natural DNA templates more closely resemble the in vivo situation. Our initial concern in developing the T7 mutagenesis system was whether the replication of genomic DNA damaged by alkylating agents accurately reflect the in vivo situation when the stringent criteria of phage survival and "biological activity" of DNA are used as endpoints. The data presented in Chapter II support this proposal. In part, we demonstrated that the biological activity of DNA alkylated with MMS or EMS and subsequently packaged into phage heads closely resembled the survival of intact phage similarly exposed to these agents.

Besides in vitro packaging of DNA into preformed capsids, transfection assays can also be used to measure the biological activity of DNA. Transfection of E. coli spheroplasts with/OX174 DNA and measurement of infective centers has been used extensively to study the fidelity of in vitro DNA synthesis (Weymouth and Loeb, 1978; Fersht, 1979; Liu et al., 1979). The efficiency of transfection, however, is much lower than what is routinely obtained with in vitro packaging. The/OX174 systems have been applied to copying of both single-stranded (Weymouth and Loeb, 1978) and double-stranded replicative form (Fersht, 1979; Liu et al., 1979) DNA.

Errors in DNA replication are often detected by determining the reversion frequency of amber mutations to wild type (Weymouth and Loeb, 1978; Fersht, 1979; Fersht and Knill-Jones, 1981). With development of the T7 mutagenesis system, we can examine replication of a duplex DNA molecule that initiates replication at an internal origin and undergoes multiple rounds of in vitro replication. In addition to assays of reversion, we have now developed assays to measure the generation of forward mutations in both essential and nonessential genes of T7.

In Chapter II, we demonstrate significant levels of in vitro DNA synthesis when alkylated DNA is provided as template. One approach we considered for demonstrating in vitro mutagenesis of T7 was to provide density-labeled DNA as template. In this case, isopycnic centrifugation could be used to separate template DNA (fully-heavy) from newly synthesized product DNA (fully-light) and each examined for biological activity and in vitro fixation of mutation. This approach is less than ideal, however, because alkylating agents introduce a wide spectrum of lesions in DNA and it is impossible to demonstrate conclusively that a particular lesion is mutagenic. Thus, we chose instead to include O^6 -methyldeoxyguanosine triphosphate (m^6dGTP) as a precursor for in vitro DNA synthesis. In addition to examining the effect of a single specific lesion, we were able to capitalize on the major strengths of the T7 system in the initial demonstration of its utility as a means for looking at mutagenesis. These data are presented in Chapter III. During the development of the system, we encountered several technical difficulties which are alluded to in the Methods section. Most notably, problems with high levels of recombination during DNA synthesis were circumvented by genetic means and the

efficiency of packaging DNA into phage heads was enhanced significantly by the addition of crude extract containing the T7 gene 6 exonuclease. Using two distinct assays, reversion of an amber mutation and generation of temperature-sensitive mutants, we demonstrated the mutagenic potential of guanine methylated at the 0-6 position. This system can now be used to determine the mutagenic potential of other modified precursors.

Measurements of mutation frequencies obtained from samples that did not contain m^6dGTP and comparison with the *in vivo* situation established the high fidelity of *in vitro* T7 DNA synthesis. Along with the development of several mutational assays, we can now examine additional parameters that may influence the yield of mutants. For example, the role of the 3'→5' exonuclease as editing function of the T7 DNA polymerase can be readily examined by including deoxynucleoside thiotriphosphates in the DNA synthesis reaction or by reducing the overall rate of DNA synthesis by limiting the concentrations of dNTP precursors.

A novel application of the *in vitro* T7 system is to examine molecular mechanisms of host functions induced by DNA damage. Before initiating this line of research, it was necessary to determine the *in vivo* situation. In Chapter IV, we demonstrate inducible reactivation of T7 phage damaged by MMS or UV light. Our results with MMS are particularly interesting because there does not appear to be any enhanced survival of MMS-damaged λ and T4 phages when the host is induced for the SOS response (Janion, 1982). We also show that inducible reactivation of T7 is independent of a functional chromosomal umuC locus, though the enhanced survival appears by several criteria to result from the induction of host SOS functions.

We do not yet know whether the inducible reactivation observed in vivo is accompanied by an increase in the number of phage mutants. This area of research could prove to be of considerable interest and importance. Also, it may be possible to demonstrate inducible reactivation of T7 in vitro with an induction protocol similar to that employed in vivo. We are encouraged in this pursuit by the knowledge that the in vitro system is capable of extensive constitutive DNA repair of lesions introduced by UV light (Kuemmerle and Masker, 1977; Masker, 1977) and alkylating agents (Chapters II and III). Development of the T7 replication-packaging system for studying molecular mechanisms of host functions induced by DNA damage would add a unique and valuable dimension to in vitro mutagenesis work.

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