Processing of DNA Base Damage by DNA Polymerases

DIHYDROTHYMINE AND β-UREIDOISOBUTYRIC ACID AS MODELS FOR INSTRUCTIVE AND NONINSTRUCTIVE LESIONS*

Hiroshi Ide†, Lynn A. Petrullo§, Zafer Hatahet, and Susan S. Wallace¶

From the Department of Microbiology and Molecular Genetics, University of Vermont, Burlington, Vermont 05405

(Received for publication, July 25, 1990)

The processing of unrepaired DNA lesions is a key to understanding and predicting the biological endpoints of particular DNA damages. In this study, we prepared single-stranded f1 phage (f1-K12) DNA containing dihydrothymine or β-ureidoisobutyric acid as models for instructive or noninstructive base lesions and assessed the potential biological consequences of these lesions in vitro and in vivo. To determine the effect of the two lesions on in vitro DNA synthesis, the extent of DNA synthesis was measured by 3H-labeled nucleotide incorporation, and the newly synthesized DNA was analyzed by DNA sequencing gels. The results showed that dihydrothymine in the template was at most a weak block to synthesis, whereas β-ureidoisobutyric acid was observed. The potential effects of the structures of base lesions on lesion-polymerase interactions are discussed.

In living cells, DNA is exposed continuously to environmental agents such as ionizing radiation (1-3), UV light (4), and a variety of chemical agents (5, 6). Recently, much attention has been focused on DNA damages produced by metabolically activated oxygen species (7-9). In fact, accumulating chemical and physicochemical evidence indicates that a major portion of DNA damage results from free radicals (10, 11). Fortunately, both prokaryotic and eukaryotic cells have evolved repair systems which efficiently repair DNA damage in an error-free way (12-15). However, DNA lesions may be encountered by the replication fork before they have been removed by excision repair. In this case, cellular DNA replication may be arrested in the vicinity of the lesion, or alternatively, the DNA lesion may be bypassed by the replication apparatus. If the lesion lacks proper hydrogen bonding and stacking interactions that are indispensable for selecting the correct nucleotide by DNA polymerases, then an incorrect nucleotide may be incorporated. The arrest of cellular DNA replication leads to loss of viability of cells, whereas translesion DNA synthesis can lead to mutation fixation. Accordingly, understanding the response of the cellular replication machinery to the encountered DNA lesion is essential for predicting the biological consequences of particular DNA damages.

Recent in vitro studies using defined DNA templates containing unique base damages such as apurinic/apyrimidinic (AP) sites (16-19), urea residues (20, 21), thymine glycols (20-24), pyrimidine photo dimers (25, 26), and polyaromatic hydrocarbon adducts (27) have shown that the response of a particular DNA polymerase to the encountered DNA lesion is a complicated process. The termination site of DNA synthesis and the efficiency of translesion bypass, if it occurs, are dependent on the enzymatic properties of the DNA polymerase used, the structure of the lesion, and the sequence context of its location. Although there are exceptions, in general, DNA synthesis tends to be arrested one base prior to (or 3' to) noninstructive lesions such as AP sites or urea glycosides and opposite instructive lesions such as thymine glycols. When the fidelity of DNA synthesis is relaxed by using DNA polymerases lacking proofreading (3' → 5' exonuclease) activity (mostly mammalian DNA polymerases) or

* This work was supported by Grants CA33657 from the National Institutes of Health and DE-AC02-86EV from the United States Department of Energy (to S. S. W.) and in part by grants from the ACS Vermont Division and BSCI90-11 from the University of Vermont (to H. I.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Present address: Dept. of Polymer Science and Engineering, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606, Japan.

§ Present address: Division of Natural Sciences and Mathematics, College of New Rochelle, New Rochelle, NY 10805.

¶ To whom correspondence should be addressed.
by replacing the divalent cation Mn"+ for Mg"+ as a cofactor, a nucleic acid is incorporated opposite noninstructive lesions with A being preferred (16, 18, 19). In some cases, DNA synthesis proceeds beyond the lesion (19, 20). Translesion bypass of an instructive lesion such as thymine glycol is also dependent on the sequence context and the exonuclease activity of T4 polymerase (21, 24). Interestingly, Goodman and co-workers recently have identified an inducible Esche-
ricia coli DNA polymerase (tentatively assigned to Pol II) that is able to incorporate nucleotides efficiently opposite AP sites and then continues the elongation process (28).

In this study, we have developed a method to introduce specifically dihydroxyethymine and 3'-uridoisobutyric acid into single-stranded fl DNA to study DNA base lesion-polymerase interactions. Dihydroxyethymine is a major radiolysis product of thymine under anoxic conditions (29–33). Although 3'-uridoisobutyric acid is not produced directly by radiolysis of DNA, it serves here as a model for pyrimidine ring fragmentation products formed by ionizing radiation or free radicals. Because our goal is to assess the biological consequences of particular DNA base lesions produced by free radicals, fl DNA containing dihydroxyethymine or 3'-uridoisobutyric acid was also used to measure biological end points. We found that dihydroxyethymine was not an efficient block to DNA polymerases in vitro nor was it a lethal lesion in vivo. In contrast, 3'-uridoisobutyric acid was a strong block to DNA polymerases in vitro and was an inactivating lesion in phage-transfecting DNA.

**EXPERIMENTAL PROCEDURES**

_Chemicals—Thymidine and dihydroxyethymine (a mixture of 5S and 5R stereoisomers) were obtained from Sigma. HPLC-purified deoxyribonucleoside triphosphates were from Pharmacia. [2-14C]Thymidine (50.5 mCi/mmol), [methyl-3H]thymidine (73 Ci/mmol), [methyl-3H]dThd (66 Ci/mmol) were from ICN and [gamma-32P]ATP (3000 Ci/mmol) was from Du Pont-New England Nuclear. [methyl-3H]Dihydrothymine was synthesized by the method of Cohn and Doherty (34). [methyl-3H]Thymidine (10 mCi) in 10 ml of distilled water was hydrogenated at 1 atm for 6 h in the presence of 30 mg of rhodium-on-alumina with stirring. The catalyst was removed by centrifugation, the supernatant was evaporated, and then the evaporated residue was taken up with 0.5 ml of distilled water. The solution of [3H]-labeled dihydroxyethymine was filtered with a 0.25 µm filter and subjected to HPLC purification. The details of the HPLC have been described previously (35). The peak fractions containing stereoisomers of dihydroxyethymine (5S and 5R forms) were combined, evaporated and resuspended in 3 ml of distilled water. Since dihydroxyethymine was separated completely from thymidine during HPLC purification, no experimental artifacts should be introduced due to contamination of [methyl-3H]dihydroxyethymine by [methyl-2H]thymidine._

_Bacteria and Phage—E. coli SMH77 was provided by J. E. LeClerc, University of Rochester (36), and the genotype of SMH77 is Δ(pro-
 lac), thr-1, his-4, thi-1, argE3, galK2, ara-14, xyl-5, nth-1, tsx-33, rps-
 L3I1, supE44, F' prolac2 ΔM15. The strain LAP201 was derived from BW372, which contains a nth:KmR mutation (37), first by conjugation with KL584 (38) to make it Δpro-lac, followed by transformation to it the F+ episome from SMH77 which carries lacZ ΔM15, Δ(pro-lac), nth:KmR, thr-1, his-4, thi-1, argE3, galK2, ara-14, xyl-5, nth-1, tsx-33, rps-
 L3I1, supE44. LAP201, a thymidine-requiring derivative of LAP201, was isolated by selecting for trimethoprim resistance (39, fl-K12 phage, an F' lacZΔ2 (a) hybrid phage, was provided by J. E. LeClerc (36). Enzymes—E. coli DNA polymerase I Klenow fragment (Pol I), Pol I that totally lacks a 3' → 5' exonuclease activity (exonuclease-free Pol I), T4 DNA polymerase, and T4 polynucleotide kinase were purchased from United States Biochemical Corp. (Cleveland, OH).

_DNA—fl DNA containing dihydroxyethymine was prepared as follows. Appropriate host cells (SMH77, LAP201, LAP201) were grown at 37 °C with vigorous shaking in glucose-M9 medium (40) supplemented with necessary amino acids (arginine, histidine, threonine, 20 µg/ml), FeCl3 (10 µM), and thiamine (1 µg/ml). For LAP201, a thy- auxotroph, thymidine (50 µg/ml) was also included in the medium. At a cell density of 2 × 109/ml, fl-K12 phage stock was added to the medium at a multiplicity of infection of 5. After 10 min of infection, dihydroxyethymine (final concentration 0.3–2 µg/ml) was added to the medium, and incubation was continued for 4 h. The phage DNA was isolated as described by Messing (41). To prepare fl DNA containing 3'-uridoisobutyric acid, the excised fragment of fl-K12 DNA containing dihydroxyethymine was dialyzed against 40 mM Na2HPO4, (pH 12.0), 2 mM EDTA at 25 °C for 16 h, then dialyzed against 10 mM Tris-HCl (pH 7.5), 1 mM EDTA extensively at 4 °C. fl-K12 DNA containing thymine glycols or urea residues was prepared as described previously (20)._
m CaCl₂ (0.5 volume of the original culture), and kept on ice for 20 min. The cells were collected by centrifugation and resuspended in 0.1 m CaCl₂ (0.1 volume of the original culture). SOS-induced competent cells were prepared in a manner similar to uninduced cells, except that the cells were suspended in 10 mM MgSO₄, 0.85% NaCl, and irradiated with 254 nm UV light (30-150 J/m², General Electric G15T8 germicidal lamp) before being made competent. The UV fluence was determined using a Black-Ray UV meter model J-225 (UltraViolet Product). The irradiated cells were collected, resuspended in prewarmed YT medium, and incubated at 37 °C for 30 min to allow SOS expression. Following expression, induced competent cells were prepared as described for uninduced competent cells. Transformation of φ1 DNA was performed as follows. 25 ng of φ1-K12 DNA in 10 μl of 10 mM Tris HCl (pH 7.5), 1 mM EDTA was added to 200 μl of competent cells and gently vortexed. Tubes were kept on ice for 40 min, followed by a 42 °C heat pulse for 2 min, and again placed on ice. Typically, 3 ml of YT top agar containing 0.2 ml of late-log phase SMH77, isopropyl β-D-thiogalactoside (final concentration 0.4 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (final concentration 0.04%) were added to 10 or 100 μl of transfected cells, and the mixture poured on YT plates. The plates were incubated at 37 °C overnight and the number of plaques counted. Under these conditions, 1 ng of untreated DNA yielded about 10⁶ plaques. The transformation efficiency was linear with the DNA concentration in this range.

**Phage Growth in the Presence of Dihydrothymine—** φ1-K12 phage was grown as described above using LAP201 as the host cell, except that the concentration of dihydrothymidine was 0-50 μg/ml. After addition of dihydrothymidine, incubation was continued for 4 h and the phage titer was determined in the same manner as for the transfection assay.

**RESULTS**

Detection and Quantitation of Dihydrothymine in φ1 DNA—Phage φ1-K12 DNA, isolated from culture medium containing dihydrothymidine, was examined for the presence of dihydrothymine by using a direct ELISA with an antibody specifically elicited to dihydrothymine (for characterization of the antibody and detailed ELISA procedure, see Ref. 33). Dihydrothymine could be detected in φ1-K12 DNA isolated from wild-type (SMH77), a strain lacking endonuclease III (LAP200), or a thy⁻ auxotroph (LAP201); however, DNA isolated from the thy⁻ auxotroph (LAP201) showed a significantly higher signal than the other two strains grown at the same dihydrothymidine concentration (2 μg/ml) in the culture medium (Fig. 1). These data indicate that exogenous dihydrothymidine can be incorporated into φ1 phage DNA and that the amount of dihydrothymidine incorporated into DNA depends on the concentration of dihydrothymidine in the medium as well as on the phenotype of the host cell used.

Alkaline hydrolysis of φ1-K12 DNA containing dihydrothymine abolished the ELISA signal almost completely (Fig. 1). This is consistent with the reported result that dihydrothymine undergoes ring opening to give β-ureidoisobutyric acid (Fig. 2) under alkaline conditions (45). In order to clarify the quantitative aspect of the conversion of dihydrothymine to β-ureidoisobutyric acid, ¹⁴C-labeled dihydrothymidine was treated in the same manner as φ1-K12 DNA containing dihydrothymine and the product analyzed by HPLC. We found that alkaline hydrolysis of dihydrothymine yielded a single product, β-ureidoisobutyric acid attached to deoxyribosyl, thus this conversion process was essentially quantitative (data not shown). It is also noteworthy that, in agreement with the previous reports on the preparation of phage M13 or PM2 DNA containing urea glycosides (20, 46), alkaline treatment of φ1-K12 DNA containing dihydrothymine did not result in detectable strand breaks or AP sites under the conditions used (data not shown).

Although the ELISA assay of dihydrothymine was very useful for screening φ1-K12 DNA containing dihydrothymine as well as for confirming the conversion of dihydrothymine in DNA to β-ureidoisobutyric acid, it only provided us with information on the relative number of dihydrothymines in DNA. The exact number of dihydrothymines in φ1-K12 DNA was determined by measuring the incorporation of ³²P-labeled dihydrothymidine and ¹⁴C-labeled thymidine into DNA using the thy⁻ strain (LAP201) as a host cell. Based on the ratio of radioactivity incorporated into DNA and the specific activity of dihydrothymidine and thymidine, the number of dihydrothymines per φ1-K12 DNA (8100 bases) was calculated.

In Vitro DNA Synthesis with an φ1 DNA Template Containing Dihydrothymine or β-Ureidoisobutyric Acid—In order to determine whether dihydrothymine and β-ureidoisobutyric acid constituted a block to in vitro DNA synthesis, φ1-K12 DNA containing these lesions was isolated from LAP201 and used as a template for E. coli DNA polymerase I Klenow fragment (Pol I). With the φ1 DNA template containing

---

**Fig. 1.** Enzyme-linked immunosorbent assay of φ1-K12 DNA isolated from culture media containing varying concentrations of dihydrothymidine (DHT). Host cell: LAP201 (before (C) and after alkaline treatment (D)), LAP200 (E), and SMH77 (F). DNA bound to a polystyrene microtiter plate was assayed using the polyclonal antibody specifically elicited to dihydrothymidine monophosphate conjugated with bovine serum albumin (primary antibody) and goat anti-rabbit IgG alkaline phosphatase conjugate (secondary antibody). For details of the ELISA, see Ref. 33.

**Fig. 2.** Alkaline conversion of dihydrothymine (DHT) to β-ureidoisobutyric acid (UBA).
dihydrothymines, the extent of primer elongation, as measured by [methyl-\(^3\)H]dTMP incorporation, decreased slightly as the number of dihydrothymines in the template increased (Fig. 3, a–d, and Fig. 4, open symbols). In contrast, when dihydrothymine in the template was converted to \(\beta\)-ureidoisobutyric acid, DNA synthesis was greatly inhibited (Fig. 3, a–d, and Fig. 4, closed symbols). Essentially the same results were obtained for \(\phi\)-K12 DNA isolated from SMH77 or LAP200 (Fig. 4, e and f). We have calculated the average number of replication blocks per molecule in the templates containing \(\beta\)-ureidoisobutyric acid using the data from Fig. 4 and an equation \(s = (1 - e^{-r})/r\), where \(s\) is the relative amount of DNA synthesis and \(r\) is the average number of replication blocks per DNA molecule (47). Comparison of the calculated average number of replication blocks with those determined experimentally (see above) gives a figure of approximately 0.8 molecule of \(\beta\)-ureidoisobutyric acid per replication block. This indicates that, within experimental error, \(\beta\)-ureidoisobutyric acid is an absolute block to \textit{in vitro} DNA synthesis catalyzed by Pol I. On the other hand, the amount of DNA synthesis on the template containing one molecule of dihydrothymine is estimated to be around 96% (Fig. 4), suggesting that under these conditions, the majority (around 96%) of dihydrothymines in the template are bypassed by Pol I.

**DNA Sequencing Gel Analysis of Newly Synthesized Products**—\(\phi\)-K12 DNA templates containing dihydrothymines or \(\beta\)-ureidoisobutyric acid residues were replicated by DNA polymerases and the newly synthesized DNA was analyzed at the sequencing level by the method pioneered by Moore and Strauss (25). For comparison, polymerization reactions were also carried out with templates containing thymine glycols or urea residues, which we and others have reported previously (20–24). When the template containing dihydrothymine was replicated by Pol I, essentially no termination bands due to the arrest of DNA synthesis were observed (Fig. 5, lane 5). Further, Pol I lacking a 3'→5' exonuclease activity (data not shown) and T4 DNA polymerase containing a highly active 3'→5' exonuclease activity (48) also bypassed putative dihydrothymine sites (lane 13). These results confirm the prediction based on the \textit{in vitro} nucleotide incorporation data that dihydrothymines in the template are at most weak blocks to DNA synthesis (see above). On the other hand, with thymine glycol that is a structural analog of dihydrothymine, DNA synthesis catalyzed by Pol I was arrested significantly with the termination sites appearing primarily opposite the lesion (lane 2). Interestingly, DNA synthesis catalyzed by Pol I lacking 3'→5' exonuclease passed putative thymine glycol sites and was arrested one base beyond the lesion (lane 8). Removal of the 3'→5' exonuclease activity from Pol I appeared to promote bypass at certain thymine glycol sites so that termination bands at particular positions (lane 8, positions 54, 64, 80, and 81) disappeared almost completely. Bypass at position 22 was independent of the 3'→5' exonuclease activity of Pol I (lanes 2 and 8).

In contrast to dihydrothymine, strong termination bands of DNA synthesis were observed when templates containing \(\beta\)-ureidoisobutyric acid were replicated by Pol I, Pol I lacking a 3'→5' exonuclease activity, and T4 DNA polymerase. The termination sites of DNA synthesis were dependent on the 3'→5' exonuclease activity associated with the DNA polymerases used. With T4 DNA polymerase containing an active 3'→5' exonuclease (48), DNA synthesis was arrested primarily one base prior to the putative \(\beta\)-ureidoisobutyric acid (lane 11). With Pol I, that contains an intermediate level of 3'→5' exonuclease activity, termination occurred either one base before or both one base before and opposite the putative sites (lanes 3). DNA synthesis catalyzed by Pol I lacking a 3'→5' exonuclease activity was terminated almost exclusively opposite the putative \(\beta\)-ureidoisobutyric acid sites (lanes 9). The patterns of termination of DNA synthesis with a template containing urea residues (lanes 4, 10, and 12) were similar to those obtained with \(\beta\)-ureidoisobutyric acid (lanes 3, 9, and 11) for the three DNA polymerases used in this study.

**Determination of the Nucleotide Incorporated Opposite \(\beta\)-Ureidoisobutyric Acid or Urea Residues—** In order to determine which nucleotide was incorporated opposite \(\beta\)-ureidoisobutyric acid or urea residues, we used a two-stage reaction (43). In the first stage reactions, the primer-template containing \(\beta\)-ureidoisobutyric acid or urea residues was elongated by T4 DNA polymerase, which predominantly terminates DNA synthesis one base prior to the lesions (Fig. 5, lanes 11 and 12). Then, the template-primer isolated from the first stage reaction mixture was incubated with Pol I lacking 3'→5'
exonuclease in the presence of a single dNTP. Fig. 6 shows the results of the second stage reaction. The stepping of the termination bands occurred most efficiently with purine nucleotides for both templates containing β-ureidoisobutyric acid or urea residues (lanes 2, 3, 8, and 9), indicating that purine nucleotides were incorporated preferentially opposite these lesions. When the putative lesion was followed by T or C in the 5' side of the template, stepping occurred by an additional base in the presence of dATP (lanes 2 and 8, position 10) or dGTP (lanes 3 and 9, position 22), respectively, since they are correct combinations of the template base and dNTP. Pyrimidine nucleotides were incorporated less efficiently than purine nucleotides, and thymine was slightly preferred over cytosine (lanes 4, 5, 10, and 11). It is likely that the incorporation of pyrimidine nucleotides was sequence-dependent since incorporation at positions 14 and 20, where the putative lesions are in the sequence 3' G lesion A 5', was more efficient than at the other positions.

**FIG. 6.** DNA sequencing gel analysis of second stage reaction products. The first stage reaction was performed with F1-K12 DNA templates containing β-ureidoisobutyric acid (UBA) or urea (Urea) and T4 DNA polymerase. In the second stage reaction, the template-primer obtained from the first stage reaction was incubated with Pol I lacking 3'→5' exonuclease (0.5 unit) and a single dNTP (50 μM): dATP (lanes 2 and 8), dGTP (lanes 3 and 9), dCTP (lanes 4 and 10), dTTP (lanes 5 and 11), no dNTP (lanes 6 and 12). Lanes 1 and 7 show the first stage reaction products. The sequence of the template is also shown next to lane 1.

**FIG. 5.** DNA sequencing gel analysis of products synthesized by DNA polymerases using F1-K12 DNA templates containing base lesions. DNA synthesis was carried out with Pol I (lanes 2-6), Pol I lacking 3'→5' exonuclease (lanes 8-10), or T4 DNA polymerase (lanes 11-14) as described under "Experimental Procedures." The DNA templates contained thymine glycol (TG, lanes 2 and 8), β-ureidoisobutyric acid (UBA, lanes 3, 9, and 11), urea (Urea, lanes 4, 10, and 12), dihydrothymine (DHT, lanes 5 and 13), or no damage (Cont, lanes 6 and 14). Lanes 1 and 7 show standard dideoxy-A ladders that indicate the position of thymine in the template. The sequence of the template and the nucleotide position from the primer terminus are also shown next to lane 1.

**Inactivation of F1 DNA Containing Dihydrothymine or β-Ureidoisobutyric Acid—In vitro DNA synthesis** data with DNA templates containing dihydrothymine or β-ureidoisobutyric acid suggested that dihydrothymine in the template should not constitute a strong lethal lesion in *vivo*, whereas β-ureidoisobutyric acid should. To test this hypothesis, the survival of F1-K12 transfecting DNA containing these lesions was measured. Transfections of competent SMH77 cells were carried out with F1-K12 DNA containing the average 1.2, 2.6, or 5 lesions per molecule. As shown in Fig. 7, inactivation of F1-K12 DNA containing dihydrothymine was very inefficient (open triangles) with the survival of the transfecting DNA containing 5 dihydrothymines per molecule being over 80%. However, DNA containing β-ureidoisobutyric acid was inactivated very efficiently (open circles), with one lethal hit corresponding to about 0.9 β-ureidoisobutyric acid residue per F1-K12 DNA molecule. These data suggest that, within error, β-ureidoisobutyric acid constitutes an absolute block to DNA synthesis *in vivo*.

When host SMH77 cells were UV-irradiated (60 J/m²) prior to the competence regimen to induce the SOS response, a slight increase in survival of F1-K12 DNA containing β-ureidoisobutyric acid was observed (Fig. 7, closed symbols). The extent of reactivation of F1-K12 DNA containing β-ureidoisobutyric acid (2.6 residues/DNA) was also dependent on the UV dose to the host cells (Fig. 8). However, the extent
performed using fl-K12 DNA containing dihydrothymine (DHT) or β-ureidoisobutyric acid (UBA). Transfection was carried out using fl-K12 DNA containing dihydrothymine (Δ, uninduced host; ▲, SOS-induced host) or β-ureidoisobutyric acid (○, uninduced host; ✧, SOS-induced host) and SMH77 as the host cells.

![Graph showing survival of host cells](image)

**Fig. 7. Inactivation of transfecting fl-K12 DNA containing dihydrothymine (DHT) or β-ureidoisobutyric acid (UBA).** Transfection was performed using fl-K12 DNA containing dihydrothymine (Δ, uninduced host; ▲, SOS-induced host) or β-ureidoisobutyric acid (○, uninduced host; ✧, SOS-induced host) and SMH77 as the host cells.

of the reactivation was at most 2-fold at the highest dose used (150 J/m²). In contrast, as has been observed previously with single-stranded and double-stranded DNA (36, 49, 50), fl-K12 DNA containing thymine glycols (about 2 residues/fl-K12 DNA) was reactivated very efficiently under the same conditions (Fig. 8).

**Fig. 8. Reactivation of fl-K12 transfecting DNA containing ureidoisobutyric acid or thymine glycol.** Transfection was performed using fl-K12 DNA containing β-ureidoisobutyric acid (○) or thymine glycol (●). Host cells (SMH77) were irradiated with the indicated UV dose to induce the SOS response before performing the competence regimen. Transfection was carried out using fl-K12 DNA containing 2.6 β-ureidoisobutyric acid residues or 2 thymine glycols per molecule as described under “Experimental Procedures.”

of the reactivation was at most 2-fold at the highest dose used (150 J/m²). In contrast, as has been observed previously with single-stranded and double-stranded DNA (36, 49, 50), fl-K12 DNA containing thymine glycols (about 2 residues/fl-K12 DNA) was reactivated very efficiently under the same conditions (Fig. 8).

**Phage Growth in the Presence of Dihydrothymidine—**Although dihydrothymines appear to be much weaker replicative blocks both in vitro (Fig. 5) and in vivo (Fig. 7) than β-ureidoisobutyric acid, accumulation of a significant number of dihydrothymines in DNA might result in some biological consequences. In order to obtain fl-K12 DNA containing a relatively large number of dihydrothymines, we attempted to isolate fl phage from infected LAP201 host cells grown in culture medium containing a high concentration of dihydrothymidine. With the exception of the experiment with 3H-labeled dihydrothymidine, fl-K12 DNA was isolated from culture medium containing less than 2 μg/ml dihydrothymidine. Because the phage titer was originally low in minimal medium, and it decreased dramatically at a dihydrothymidine concentration greater than 2 μg/ml (data not shown), the amount of recovered viral DNA was not sufficient to allow us to perform subsequent quantitative studies. However, we were able to calculate that approximately 4 μg/ml dihydrothymidine resulted in 37% phage survival. Because the number of dihydrothymines incorporated in this experiment is about 18, it appears that it takes about 18 dihydrothymines to constitute an inactivating event in fl-K12 phage.

**DISCUSSION**

**Preparation of DNA Containing Dihydrothymine—**Free radicals generate a wide spectrum of DNA damages including strand breaks and damages to base and sugar moieties (1, 2, 10, 11). One of the difficulties in assessing the biological consequences of particular base lesions produced by free radicals is that very few methods are available to introduce unique damages into DNA. In the present study, we have prepared biologically active single-stranded DNA containing dihydrothymine, a major radiolysis product of thymine produced under anoxic conditions (29–33), by in vivo incorporation of exogenously supplied dihydrothymidine. To date, two in vitro approaches have been used to prepare DNA containing dihydrothymine. Schulhof et al. (51) developed a new set of protecting groups for alkali-labile nucleotides that can be removed from the synthesized oligonucleotide without degrading the labile moiety during the ammonia treatment. Alternatively, our approach has been to synthesize dihydrothymidine triphosphate chemically and to incorporate it into DNA by DNA polymerase (35, 52). These two approaches have an advantage over the present in vitro method insofar as dihydrothymine can be incorporated into DNA by Pol I, which has served as a useful substrate to screen and characterize DNA repair enzymes from E. coli that recognize dihydrothymine (53–56).

**Biological Consequences of Dihydrothymine—**Processing of unrepaired DNA lesions is a key to understanding and predicting the biological consequences of particular DNA damages. In this study, we used dihydrothymine and β-ureidoisobutyric acid as models for instructive and noninstructive thymine lesions produced by free radicals. In previous studies (35, 52), we suggested that the presence of dihydrothymine did not create a significant disorder in DNA; and, based on the ability of dihydrothymidine-5'-monophosphate to be incorporated into DNA by Pol I, we predicted that dihydrothymine should not be a premutagenic lesion. In the present study, we have demonstrated that dihydrothymines in a DNA template constitute very weak blocks to in vitro DNA synthesis catalyzed by DNA polymerases including Pol I, Pol I lacking a 3' → 5' exonuclease activity, and T4 DNA polymerase (Figs. 3–5). During the reaction period measured, translesion bypass by Pol I at dihydrothymine sites in vitro was very high, about 96%. The survival data of transfecting fl-K12 DNA containing dihydrothymine has supported further the conclusion that dihydrothymine is not a strong replicative block (Fig. 7). Although we could not estimate the exact inactivation efficiency of dihydrothymine in fl DNA,
phage growth data in the presence of dihydrothymidine sug-
gest that it takes about 18 dihydrothymines to constitute an inactivating event in \( f_{1}-K12 \) DNA. When these results are compared to those obtained with thymine glycol, a structural analog of dihydrothymine, the differences in the biological consequences of these lesions are rather striking. Dihydroyth-
mine and thymine glycol share common structural features. Both lesions lack planarity and aromatic character of the pyrimidine ring due to saturation of the C5–C6 bond, and both assume half-chair conformation (57). The coding regions of both lesions are also essentially intact. The primary differ-
ence is the substituent groups at C5 and C6; two hydrogen atoms for dihydrothymine and two hydroxy molecules for thymine glycol. These relatively minor structural differences resulted in significant differences in biological consequences. With thymine glycol, in vitro DNA synthesis is arrested almost completely at the damage site (20–23; for some excep-
tions see Ref. 24); whereas with dihydrothymine, DNA polym-
erases can synthesize DNA efficiently beyond the lesion (Fig. 5). In keeping with in vitro results, transfecting \( f_{1} \) DNA was not inactivated efficiently by the presence of dihydrothymine in its DNA (Fig. 7), while \( f_{1} \) or M13 DNA was efficiently inactivated by the presence of thymine glycol with the inac-
tivation efficiency being 1.5–2.8 lesions per lethal hit (36, 58).

Although \( E. coli \) has several DNA repair enzymes (endonucle-
ases III and VIII) that recognize dihydrothymidine and thymine glycol (54, 55, 59), the enzymes are specific for duplex DNA. Thus participation of repair processing in the survival assay of single-stranded DNA is unlikely.

In light of these observations, it can be concluded that dihydrothymine present in DNA is relatively innocuous in terms of constituting a replicative block. This is in contrast to thymine glycol, ring fragmentation products such as urea and \( \beta \)-ureidoisobutyric acid, or AP sites, all of which have been shown to be strong replicative blocks. It appears however, that accumulation of a large number of dihydrothymines in DNA may still be a disadvantage for cells since in vivo they would generate a significantly detectable level of termination of polymerization. Further, the N-glycosyl bond of dihydrothymidine is more susceptible to hydrolysis than thymidine (34), therefore AP sites would be generated more frequently from dihydrothymine than from thymine. It may be for these reasons that cells possess enzymatic activities that recognize and remove dihydrothymine.

Although the mutagenic potential of dihydrothymine was not addressed in this study, it is predicted that dihydrothymine will not be a strong premutagenic lesion in vivo since it appears to retain proper base pairing ability with adenine (52). Thymine glycol, another instructive lesion, was shown not to be a strong premutagenic lesion in vivo when randomly introduced into DNA (36). However, when the lesion was site specifically introduced into M13 DNA in a sequence context where it was readily bypassed, the mutation frequency was about 0.3% (60).

**\( \beta \)-Ureidoisobutyric Acid as a Model for Noninstructive Base Lesions**—In contrast to dihydrothymine, \( \beta \)-ureidoisobutyric acid is a strong block to in vitro DNA synthesis catalyzed by DNA polymerases including Pol I, Pol I lacking a 3' → 5' exonuclease activity, and T4 DNA polymerase (Figs. 3–5). The results of nucleotide incorporation by Pol I on a template containing \( \beta \)-ureidoisobutyric acid (Fig. 4) suggest that this lesion is an absolute block to DNA synthesis in vitro. This observation was confirmed in vivo since it took about 0.9 \( \beta \)-ureidoisobutyric acid residues per molecule to inactivate \( f_{1} \)-K12 transfecting DNA (Fig. 7). To compare the effects of substituent groups present in noninstructive lesions, we also carried out polymerase reactions with a template containing urea residues which are relatively smaller than \( \beta \)-ureidoisobutyric acid residues. It has been shown that urea residues in a DNA template constitute strong blocks to DNA synthesis in vitro (20) and in vivo (50, 55). The termination pattern of DNA synthesis was virtually the same for both \( \beta \)-ureidoisobutyric acid and urea residues, and the positions of the ter-
mination sites relative to the lesion was dependent on the 3' → 5' exonuclease activity associated with the polymerases used. DNA synthesis catalyzed by T4 DNA polymerase ter-
minated primarily one base prior to the putative \( \beta \)-ureidoisobutyric acid or urea residues. This suggests that the nucleotide inserted opposite \( \beta \)-ureidoisobutyric acid or urea would be nonpairing and thus readily excised by extensive 3' → 5' exonuclease activity associated with T4 DNA polymerase (48). DNA synthesis catalyzed by Pol I, which has a less active 3' → 5' exonuclease activity, was terminated either one base prior to or opposite the \( \beta \)-ureidoisobutyric acid and urea residues. We previously observed that DNA synthesis cata-
lyzed by Pol I was terminated almost exclusively one base prior to urea residues (20). To account for this discrepancy, we tested several Pol I preparations from different companies. Furthermore, as done in the previous work, we labeled the newly synthesized DNA with \( [\alpha- \text{P}] \text{dATP} \) instead of using a 5'-end-labeled primer. However, the results from these exper-
iments using \( [\alpha- \text{P}] \text{dATP} \) and a variety of Pol I preparations were essentially the same as those presented above. Although we have no definitive explanation for this discrepancy, we suspect that the balance between the polymerase and 3' → 5' exonuclease activities in the Pol I preparations significantly influences the position of the termination site. This hypoth-
esis appears to be reasonable in light of the correlation be-
tween the termination site of DNA synthesis observed for urea-containing templates and the 3' → 5' exonuclease activity of polymerases used in this study. Interestingly, DNA synthesis catalyzed by Pol I lacking a 3' → 5' exonuclease activity was terminated opposite \( \beta \)-ureidoisobutyric acid and urea residues, suggesting that complete removal of 3' → 5' exonuclease facilitates the incorporation of a nucleotide op-
posite noninstructive lesions; however, it is not enough to allow the polymerase to continue DNA synthesis beyond the lesion.

We also examined potential translesion bypass of \( \beta \)-ureidoisobutyric acid in vivo by transfecting DNA containing this lesion into SOS-induced host cells. In this case, the survival of transfecting \( f_{1}-K12 \) DNA containing \( \beta \)-ureidoisobutyric acid was slightly increased (Fig. 7) and was dependent on the UV dose used to induce the host cells (Fig. 8). This is in contrast to the results observed with single-stranded \( f_{1} \) DNA (36, this work, Fig. 8) and \( f_{0}X174 \) DNA (49) or with \( f_{0}X174 \) RF DNA (50) containing thymine glycol, where very efficient reactivation was observed. With other noninstructive lesions such as AP sites (50, 61) and urea glycosides (50, 55) that totally lack base stacking ability and have no potential to stabilize the primer terminus, no reactivation is observed. To estimate the fraction of \( \beta \)-ureidoisobutyric acid bypassed during replication in SOS-induced host cells, we calculated the number of lethal hits reversed using the survival data for \( \beta \)-ureidoisobutyric acid (Fig. 7). Although there is considerable fluctuation, the average bypass efficiency is about 20%, which is lower than found for thymine glycol (60–80%; 36, 49) but comparable to that for photodimers (see, for example, 62, 63). When \( \beta \)-ureidoisobutyric acid or urea sites are bypassed in vivo, mutation could result especially since the in vitro data...
above show that dGMP as well as dAMP (originally correct base pair) can be incorporated opposite the lesion (Fig. 6).

Bypass at Thymine Glycol Sites—DNA synthesis catalyzed by Pol I lacking 3' → 5' exonuclease could bypass the thymine glycol site, yet, the majority of the bypassed sites still showed termination bands one base beyond the lesion. This suggests that conformational perturbation by thymine glycol is manifested by a significant effect on the neighboring base pair 5' side to the thymine glycol in the template. Thus, the destabilized base pair on the 5' side of thymine glycol may not be a good primer terminus for further extension by the polymerase. Based on a computer modeling study, Clark et al. (64) have reached a similar conclusion. According to its sensitivity to the 3' → 5' exonuclease of Pol I, it is likely that the base pair at the 5' side of thymine glycol is more destabilized than the base pair at the thymine glycol site. Although the majority of thymine glycols remained replicative blocks with Pol I lacking 3' → 5' exonuclease, removal of 3' → 5' exonuclease from Pol I promoted bypass at certain thymine glycol sites (Fig. 5, lane 3, positions 54, 64, 80, and 81). The bypass at position 22 was independent of the presence or absence of 3' → 5' exonuclease activity in Pol I (see also Ref. 21). The bypassed sequences observed for Pol I lacking 3' → 5' exonuclease are 3'-GTG-5' (position 54) and 3'-CTT'T-5' (position 80), and 3'-TT'C-5' (position 81), where T' represents the putative thymine glycol. We scanned the template to see if these represented consensus sequences for bypass by Pol I lacking 3' → 5' exonuclease and found similar sequences at other positions that were not bypassed by the polymerase.

Translesion DNA synthesis involves (i) incorporation of a nucleotide opposite a lesion and (ii) subsequent elongation of a primer beyond the lesion. With noninstructive lesions, inhibition or removal of proofreading (3' → 5' exonuclease) activity of DNA polymerases promotes the step (i) as has been demonstrated for AP sites (16-19) or lesions carrying a pyrimidine ring fragment (urea and β-ureidopropionatic acid, this study). However, the removal of 3' → 5' exonuclease activity was not enough to allow the DNA polymerase to elongate a primer beyond the lesion in step (ii) in vitro. The situation for β-ureidopropionatic acid in SOS-induced cells appears to be different from AP sites and urea residues since the bypass efficiency at the β-ureidopropionatic acid site was not negligible compared with the other two noninstructive lesions. To understand this observation, further study on the interaction of E. coli Pol III holoenzyme with SOS-induced gene products such as Rec A, Umu C,D is necessary (65-67). With thymine glycol, which is an instructive lesion thus the correct nucleotide is inserted opposite the lesion in step (i), the removal of 3' → 5' exonuclease activity from Pol I enhanced in vitro bypass at certain sites. However, with the majority of thymine glycol sites, the primer terminus was elongated by only one nucleotide. This is in contrast to the data obtained in vivo in SOS-induced cells where the efficiency of bypass at thymine glycol sites was 60-80% (36, 49). Thus, with instructive lesions like thymine glycol, whose conformational perturbation extends to the 5' side of the template, lesion bypass appears to require processing factor(s) in addition to the removal or inhibition of 3' → 5' exonuclease activity of the polymerase in step (ii).

Acknowledgments—We would like to thank Dr. Karen R. Hubbard for assistance in the immunochromatographic assay of dihydrothymine, and Margaret MacCabe for preparing φ1 K12 DNA containing thymine glycol.

REFERENCES
1. Teoule, R., and Cadet, J. (1978) in Effects of Ionizing Radiation on DNA (Hutterman, J., Kohnlein, W., and Teoule, R., eds) pp. 171-203, Springer-Verlag, Berlin
2. Hutchinson, F. (1985) Prog. Nucleic Acid Res. Mol. Biol. 32, 115-154
3. Teoule, R. (1987) Int. J. Radiat. Biol. 51, 573-589
4. Wang, S. Y. (1976) Photochemistry and Photobiology of Nucleic Acids, Academic Press, New York
5. Roberts, J. J. (1984) Photochem. Photobiol. 39, 1111-1146
6. Simic, M. G. (1988) Mechanisms of DNA Damage and Repair, Plenum Publishing Corporation, New York
7. Fridovich, I. (1978) Science 201, 875-880
8. Ames, B. N. (1983) Science 221, 1256-1284
9. Hayes, R. C. (1989) Annu. Rev. Mol. Cell. Biol. 13, 765-766
10. Singh, B., and Kusmiercz, J. T. (1982) Annu. Rev. Biochem. 52, 655-695
11. Fridberg, E. C. (1965) DNA Repair, W. H. Freeman and Company, San Francisco
12. Fridberg, E. C. (1968) Microbiol. Rev. 32, 70-102
13. Wallas, S. S. (1987) Br. J. Cancer 55, Suppl. VIII, 118-128
14. Wallas, S. S. (1988) Environ. Mol. Mutagen. 12, 431-477
15. Sager, D., and Strauss, B. (1983) Biochemistry 22, 4518-4526
16. Sager, D., and Strauss, B. (1985) Nucleic Acids Res. 13, 4285-4298
17. Randall, S. K., Ertija, R., Kaplan, B. E., Piatrouka, J., and Goodman, M. F. (1979) J. Biol. Chem. 254, 6844-6870
18. Takekita, M., Chang, C.-N., Johnson, F., Will, S., and Grollman, A. P. (1979) J. Biol. Chem. 262, 10171-10179
19. Ide, H., Kow, Y. W., and Wallace, S. S. (1985) Nucleic Acids Res. 13, 8035-8052
20. Hayes, R. C., and LeClerc, J. E. (1986) Nucleic Acids Res. 14, 1045-1061
21. Rouet, P., and Eisinger, J. M. (1988) Cancer Res. 45, 6113-6118
22. Clark, J. M., and Beardsley, G. P. (1986) Nucleic Acids Res. 14, 73-749
23. Clark, J. M., and Beardsley, G. P. (1987) Biochemistry 26, 5398-5403
24. Moore, P. D., and Strauss, B. S. (1979) Nature 278, 664-666
25. Moore, P. D., Rose, K. K., Rabkin, S. D., and Strauss, B. S. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 110-114
26. Larson, K. L., and Strauss, B. S. (1987) Biochemistry 26, 2471-2479
27. Bonner, C. A., Randall, S. K., Rayssiguier, C., Redman, M., Ertija, R., Kaplan, B. E., McEntee, K., and Goodman, M. F. (1988) J. Biol. Chem. 263, 18946-18952
28. Vercellati, R., Bani, C., and Fouque, B. (1978) J. Am. Chem. Soc. 100, 6749-6752
29. Wada, T., Ide, H., Nishimoto, S., and Kagiya, T. (1986) Int. J. Radiat. Biol. 42, 125-122
30. Nishimoto, S., Ide, H., Nakamichi, K., and Kagiya, T. (1983) J. Am. Chem. Soc. 105, 6740-6741
31. Purlong, E. A., Jorgensen, T. J., and Henner, W. D. (1986) Biochemistry 25, 4344-4349
32. Hubbard, K., Ide, H., Erlanger, B. F., and Wallace, S. S. (1989) Biochemistry 28, 4382-4387
33. Cohn, W. E., and Doherty, G. D. (1956) J. Am. Chem. Soc. 78, 2859-2866
34. Ide, H., Melamede, R. J., and Wallace, S. S. (1987) Biochemistry 26, 964-969
35. Hayes, R. C., Petullo, M. A., Huang, H., Wallace, B. S., and LeClerc, J. E. (1988) J. Mol. Biol. 201, 239-246
36. Cunningham, R. F., and Weiss, B. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 474-478
37. Miller, J. H., and Low, K. B. (1984) Cell 37, 675-682
38. Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
39. Mancini, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
40. Messing, J. (1983) Methods Enzymol. 101, 20-79
41. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467
42. Moore, P. D., Rabkin, S. D., Osborn, A. L., King, C. M., and Strauss, B. S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 7166-7170
Processing of DNA Base Lesions by DNA Polymerases

44. Hayes, R. C., and LeClerc, J. E. (1983) *Gene (Amst.)* **21**, 1-8
45. Kondo, Y., and Witkop, B. (1983) *J. Am. Chem. Soc.* **105**, 764-770
46. Kow, Y. W., and Wallace, S. S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 8354-8358
47. Masamune, Y. (1976) *Mol. Gen. Genet.* **149**, 335-345
48. Huang, W. M., and Lehman, I. R. (1972) *J. Biol. Chem.* **247**, 3139-3146
49. Achery, P. M., and Wright, C. F. (1983) *Radiat. Res.* **93**, 609-612
50. Laspa, M. F., and Wallace, S. S. (1989) *J. Mol. Biol.* **207**, 53-60
51. Schulhof, J. C., Molko, D., and Teoule, R. (1988) *Nucl. Acids Res.* **16**, 11339-11354
52. The, H., and Wallace, S. S. (1988) *Nucl. Acids Res.* **16**, 11339-11354
53. Ide, H., Melamede, R. J., Kow, Y. W., and Wallace, S. S. (1987) in *Anticancerogenesis and Radiation Protection* (Cerutti, P. A., Nygaard, O. F., and Simic, M. G., eds) pp. 145-150, Plenum Publishing Corporation, New York
54. Melamede, R. J., Kow, Y. W., and Wallace, S. S. (1987) in *Anticancerogenesis and Radiation Protection* (Cerutti, P. A., Nygaard, O. F., and Simic, M. G., eds) pp. 139-144, Plenum Publishing Corporation, New York
55. Wallace, S. S., Ide, H., Kow, Y. W., Laspa, M. F., Melamede, R. J., Petrullo, L. A., and LeClerc, J. E. (1988) in *Mechanisms and Consequences of DNA Damage Processing* (Friedberg, E. C., and Hanawalt, P. C., eds) pp. 151-157, Alan Liss, New York
56. Kow, Y. W. (1989) *Biochemistry* **28**, 3280-3287
57. Karle, I. L. (1976) in *Photochemistry and Photobiology of Nucleic Acids* (Wang, S. Y., ed) Vol. I, pp. 483-519, Academic Press, New York
58. Harinaran, P. V., Achey, P. M., and Cerutti, P. A. (1977) *Radiat. Res.* **69**, 375-378
59. Demple, B., and Linn, S. (1980) *Nature* **287**, 203-208
60. Basu, A. K., Loechler, E. L., Leadon, S. A., and Essigmann, J. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 7677-7681
61. Schaper, R. M., and Loeb, L. A. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 1773-1777
62. Froehlich, B. (1981) *Mol. Gen. Genet.* **184**, 416-420
63. LeClerc, J. E., and Istock, N. L. (1984) *Mol. Gen. Genet.* **197**, 414-419
64. Clark, J. M., Pattabiraman, N., Jarvis, W., and Beardsley, G. P. (1987) *Biochemistry* **26**, 5404-5409
65. Fersht, A. R., and Knill-Jones, J. W. (1983) *J. Mol. Biol.* **165**, 669-682
66. Bridges, B. A., and Woodgate, R. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4193-4197
67. Lu, C., Scheuermann, R. H., and Echols, H. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 619-623