Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9974–9978, November 1991
Biochemistry

The vinyl chloride DNA derivative N^2,3-ethenoguanine produces G → A transitions in *Escherichia coli*

(environmental carcinogenesis/chloracetaldehyde/site-specific mutagenesis/cyclic DNA adducts/mutagenicity tests)

K. C. CHENG[†], B. D. PRESTON[‡], D. S. CAHILL[†], M. K. DOSANJH[§], B. SINGER[§], AND L. A. LOEB[†¶]

[†]Department of Pathology SM-30, University of Washington, Seattle, WA 98195; [‡]Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, NJ 08855; [§]Donner Laboratory, Cell and Molecular Biology Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720

Communicated by Heinz Fraenkel-Conrat, August 16, 1991

ABSTRACT **Vinyl chloride is a known human and rodent carcinogen that forms several cyclic base derivatives in DNA. The mutagenic potential of these derivatives has been examined *in vitro* but not *in vivo*. One of these derivatives, N^2,3-ethenoguanine (εG), is known to base pair with both cytosine and thymine during *in vitro* DNA synthesis, which would result in G → A transitions. To determine the base pairing specificity of this labile guanine derivative in *Escherichia coli*, we have developed a genetic reversion assay for guanine derivatives. The assay utilizes DNA polymerase-mediated analogue insertion into a bacteriophage vector, M13G*1, which detects all single-base substitutions at position 141 of the *lacZα* gene by change in plaque color. After the insertion of a single εG opposite the template cytosine at position 141 by use of εdGTP and DNA polymerase and further extension with all four normal dNTPs, the DNA was transfected into *E. coli*. Transfection of M13G*1 containing εG at the target site yielded 135 mutants among 26,500 plaques, 134 of which represented G → A transitions. The uncorrected mutation frequency was 0.5%, as compared with the control value, ≈0.02%; when corrected for εG content and penetrance, the calculated mutagenic potential of εG (mutations/analogue) was about 13%. We thus conclude that εG specifically induces G → A transitions during DNA replication in *E. coli*. The M13G*1 assay may permit the testing of other labile guanine derivatives not otherwise amenable to mutagenesis studies.**

Vinyl chloride is an important industrial chemical used worldwide in plastics manufacture. Human exposure to this compound causes liver hemangiosarcomas (1, 2). Vinyl chloride is metabolized by the monooxygenase P-450 system into chloroethylene oxide (3), a very unstable alkylating agent (4). The stable metabolite, chloroacetaldehyde, is formed by chemical rearrangement (5) and, as a bifunctional agent, generates 1,N^6-ethenoadenine, 3,N^4-ethenocytosine, N^2,3-ethenoguanine (εG), and 1,N^2-ethenoguanine (refs. 6–8; reviewed in ref. 9) as well as DNA–DNA crosslinks (10). Each of these etheno derivatives except for 1,N^2-ethenoguanine has been found in the liver DNA of rodents exposed to vinyl chloride (reviewed in ref. 11). The extreme lability of the glycosyl bond of N^2,3-ethenodeoxyguanosine (εdG; structure in Fig. 1), particularly as the monomer, has made synthesis of εG-containing DNA difficult and consequently has hindered studies of its mutagenic potential.

The recently synthesized triphosphate of εdG, εdGTP (12), serves as a substrate for polymerases and can be incorporated into DNA site-specifically opposite either C or T template residues (13). εG also readily pairs with both C and T during copying of εG-containing ribocopolymers by reverse transcriptase *in vitro* (14). We thus expect, upon

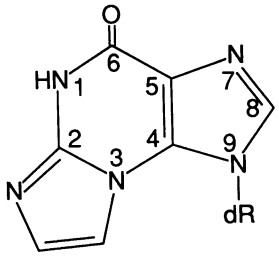

FIG. 1. Structure of εdG. dR, 2′-deoxyribosyl.

replication of εG-containing DNA, a high level of εG → A transitions resulting from εG·T pairing. This pairing resembles that of the wobble pair, G·T (15), and would not be expected to distort the helix significantly.

To determine the mutagenic potential of εG-containing templates in *Escherichia coli*, we have developed an assay for guanine analogues, the M13G*1 assay. This assay was designed to test the mutagenic potential of guanine analogues without subjecting them to chemically harsh conditions or long incubations. Site-specific insertion of the analogue is accomplished using a DNA polymerase. After replication of εG-containing templates in *E. coli*, all single-base substitutions at the target site can be detected by a change in plaque color from parental white to dark blue revertants, based upon restoration of β-galactosidase activity. Using this assay, we show here that copying of εG in *E. coli* causes G → A transitions.

MATERIALS AND METHODS

***E. coli* Strains and Medium.** An F′ derivative of strain CSH50 [Δ(*pro–lac*) *thi ara strA*/F′ (*proAB lacI*^{q}*–ZΔ*M15)], used for bacterial lawns, was received from T. A. Kunkel (National Institute of Environmental Health Sciences). *E. coli* strain NR9161 [*mutL*::Tn*10 hsdR*$^-$ *hsdM*$^+$ *araD* Δ(*ara,leu*) Δ*lacIPOZY lacU galK strA*; equivalent to MC1061 *mutL*] was kindly provided by R. Schaaper (National Institute of Environmental Health Sciences). *E. coli* strain CJ236 (*ung-1 dut-1 relA1 spoT1 thi-1*, pCJ105) was obtained from B. Bachman (*E. coli* Genetic Stock Center, Yale University). Per liter, 2×YT medium contains 16 g of Bacto tryptone, 10 g of Bacto yeast extract, and 5 g of NaCl.

Nucleotides, Oligonucleotides, DNA, and Enzymes. dATP, dCTP, dTTP, and dGTP were obtained from Pharmacia. [γ-^{32}P]ATP was obtained from Amersham. εdGTP was prepared as described (12, 13, 16) to >99.9% purity. Oligonucleotides were synthesized and HPLC-purified by Operon Technologies (Alameda, CA) and were further purified from

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: εG, N^2,3-ethenoguanine, usually in a base-pair context; εdG, N^2,3-ethenodeoxyguanosine.

¶To whom reprint requests should be addressed.

denaturing 15% polyacrylamide sequencing gels (17) with elution from Nensorb 20 columns by 50% methanol. Double-stranded replicative-form marker DNA and uracil-containing single-stranded M13G*1 template DNA were made by growing the phage in CJ236 (*dut ung*), as described (17). The uracil-containing DNA was treated with bovine pancreatic RNase A (type IIA; Sigma) at 20 μg/ml for 30 min at 45°C. Oligonucleotide-directed site-specific mutagenesis was performed as described by Kunkel *et al.* (18), except that native T7 DNA polymerase was substituted for T4 DNA polymerase (19). The nucleotide sequence of the M13G*1 construct and of mutants was confirmed by dideoxy DNA sequencing (20). Immunoaffinity-purified calf thymus DNA polymerase α, a DNA polymerase–primase complex devoid of exonuclease or endonuclease activities (21), was the kind gift of F. Perrino (Wake Forest University). Cloned bacteriophage T7 DNA polymerase was obtained from United States Biochemical.

Site-Specific Incorporation of εdG. HPLC and gel-purified synthetic 17-mer 5′-GCTATTACGCCAGCTGG-3′ (complementary to positions 142–158 of the *lacZ* sequence) was 5′-end-labeled with ^{32}P (17), with subsequent addition of unlabeled 5 μM ATP and incubation for 15 min at 37°C, followed by 10 min of heat inactivation (65°C) of polynucleotide kinase. Labeled 17-mer was hybridized to uracil-containing, single-stranded circular M13G*1 at a 2.5:1 primer/template ratio in 300 mM KCl/100 mM Hepes, pH 7.3, by successive incubations at 75°C, 30°C, and 0°C for 5, 25, and 30 min, respectively. Two micrograms of primed template was then incubated for 1.5 hr (experiment 1) or 1 hr (experiment 2) at 30°C in a 200-μl reaction mixture containing 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 23 mM KCl, 2 mM dithiothreitol, 8.5 units of DNA polymerase α (units as defined in ref. 22), and εdGTP (12) at 50 μM (experiment 1) or 10 μM (experiment 2). Following this reaction, all four dNTPs were added to a final concentration of 250 μM each, and the reaction mixtures were incubated for 30 min at 30°C. Five units of native T7 DNA polymerase (units as defined by the manufacturer) was then added, and the reaction mixtures were incubated for 2 hr at 30°C. The reaction products were stored at −20°C prior to transformation of competent *E. coli* NR9161. Reaction products (50–200 ng in 5–20 μl) were used directly in transformations without further processing (see below).

Estimation of εdG Incorporation. Aliquots used for quantitation of εdG incorporation were dried and resuspended in 98% formamide loading buffer and loaded, with or without incubation in boiling water for 15 min, on a 15% polyacrylamide gel mounted on a Poker-face gel apparatus (Hoefer) to maintain room temperature during electrophoresis. The gel was dried onto Whatman no. 3 filter paper without fixation and analyzed on a Molecular Dynamics (Sunnyvale, CA) Phosphorimager after exposure to the screen for 1 hr. The accompanying ImageQuant software was used to quantitate ^{32}P disintegrations within defined areas. Absolute counts within individual bands were >140,000 for εdG reactions and >50,000 for control dG reactions after correction for background.

Determination of Mutation Frequencies. Competent NR9161 cells were prepared, transfected, and plated as described (22). After overnight incubation at 37°C, dark blue plaques representing revertants were scored and replated for purification and DNA sequencing (see below). After ≥2 days at room temperature, nonmutant white plaques turned faint blue, facilitating counting. Plaque densities of <1000 plaques per plate were used during screening. Mutation frequency was calculated as the ratio of dark blue plaques to faint blue plaques. Multiple independent transformations and platings yielded similar mutation frequencies.

DNA Purification and Sequencing. Individual dark blue plaques were picked into 1 ml of TE (10 mM Tris, pH 7.5/1 mM EDTA) and diluted 10,000-fold in TE, and aliquots were replated to yield well-separated plaques (<200 plaques per plate). For each original mutant plaque, one well-separated plaque from the replating was touched with a toothpick and placed in 2 ml of 2×YT containing a 1:100 dilution of a fresh late-logarithmic growth of F′ CSH50. The culture was grown 6–16 hr and single-stranded DNA was purified (17). The sequencing primer 5′-GCTGCGCAACTGTTGG-3′, complementary to nucleotides 6363–6378 of M13G*1 (numbering based upon Vecbase numbering of M13mp2), was 5′ end-labeled with ^{32}P and used for dideoxy sequencing (20) of the M13G*1 target site, 44 nucleotides away from the primer's 3′ end.

RESULTS

Derivation of M13G*1, a Vector for Studying Guanine Analogue Mutagenicity. We have designed a genetic assay to study the mutagenic potential of guanine analogues. This assay scores reversion mutations at a single site of a missense codon in the β-galactosidase gene of an M13 bacteriophage vector; any base substitution at this site causes white-to-blue plaque color change when plated with 5-bromo-4-chloro-3-indolyl β-D-galactoside, a chromogenic substrate. The mutagenesis vector, M13G*1, was designed to facilitate the incorporation of single guanine analogues by a polymerase-based approach to site-specific mutagenesis (23, 24) but may also be used for synthetic oligonucleotide (25) or RNA ligase-based (26) approaches. Important characteristics in the design of a DNA polymerase-based approach to the study of guanine analogues include (*i*) a template cytosine at the insertion site in the single-stranded form of the DNA and (*ii*) a site that detects all three single-base substitutions by change of parental white plaque phenotype to blue, which would allow easier screening than the opposite color change. A compilation of known mutations in *lacZα* of M13mp2, kindly provided by J. D. Roberts and T. A. Kunkel (National Institute of Environmental Health Sciences), suggested only one apparent missense mutation with these characteristics: cytosine at position 141 (C^{141}). To construct M13G*1 (Fig. 2), three nucleotide substitutions were introduced simultaneously in M13mp2 by oligonucleotide-directed mutagenesis (18). G^{141} (number refers to position relative to the transcription start site as +1) in M13mp2 was changed to cytosine to give a missense mutation that changes plaque color from dark blue to white. C^{140} was changed to thymine (a silent mutation) to eliminate a second successive template C that would encourage double additions of G analogue onto the primer

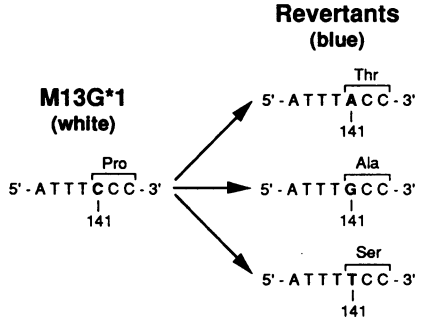

FIG. 2. Detection of targeted substitutions in M13G*1. Only nucleotides 137–143 (reading from left to right) are shown. The nucleotides indicated at positions 137, 140, and 141 were simultaneously changed in M13mp2 by oligonucleotide-directed mutagenesis. Each of the three single-base substitutions for C^{141} of M13G*1 results in a change in plaque phenotype from white to blue. The primer used for site-specific mutagenesis contains a 3′-terminal G residue across from C^{142}; the next nucleotide is therefore inserted across from the target site, C^{141}.

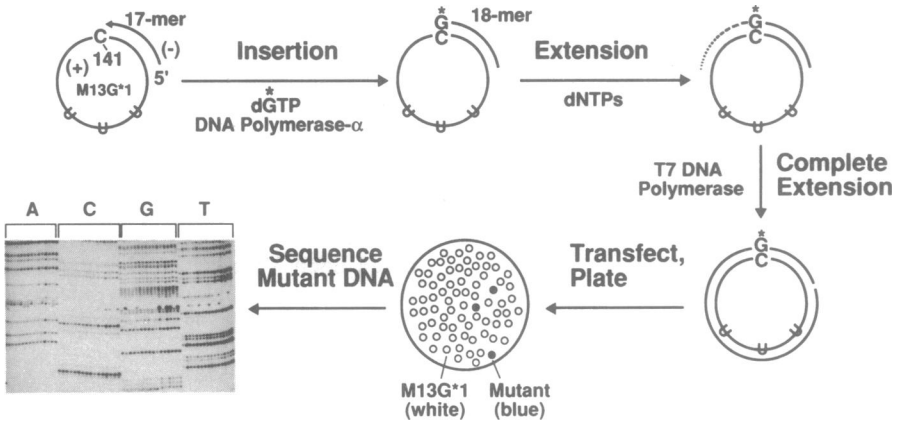

FIG. 3. The M13G*1 assay. After priming of uracil-containing plus-strand M13G*1 with 17-mer 5′-GCTATTACGCCAGCTGG-3′ such that the next template nucleotide is C^{141}, a guanine analogue (G*) is inserted opposite C^{141} by using a DNA polymerase and dG*TP as the only substrate nucleotide. After analogue insertion, all four normal dNTPs and, finally, native T7 DNA polymerase are added to complete extension. The copied circle is then transfected into repair-deficient competent *E. coli* and plated in the presence of isopropyl β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl β-D-galactoside for detection of blue revertant plaques. The revertants are sequenced to determine the types of base substitutions.

during polymerization. T^{137} was changed to adenine (also a silent mutation). In control experiments, the single base substitutions indicated at the right of Fig. 2 were made in M13G*1 by oligonucleotide site-directed mutagenesis (27) to confirm the plaque color phenotype of each potential mutation (data not shown). Thus, all three base substitutions at position 141 are scorable in this assay.

Site-Specific Incorporation of εG into M13G*1. The generation of double-stranded M13G*1 containing εG at the target position was accomplished in three steps (Fig. 3). Uracil-containing circular single-stranded M13G*1 DNA was first hybridized to an oligonucleotide such that the next base to be added onto the 3′ primer terminus was opposite the target cytosine, C^{141}. Insertion of εG opposite C^{141} was accomplished using εdGTP as the sole nucleotide substrate for DNA polymerase α (Figs. 3 and 4*A*). The nucleotide analogue was then sealed into the nascent strand by the subsequent addition of dATP, dCTP, dTTP, and normal dGTP at a high concentration. Native T7 DNA polymerase was finally added to generate fully double-stranded product (Fig. 4*B*).

Electrophoretic analysis of the products from the insertion step provided evidence for the incorporation of εG at C^{141} (Fig. 4*A*). About 12% of the 17-mer primers were extended by 1 nucleotide in the presence of εdGTP; this yielded 18-mers with presumed $\varepsilon G{\cdot}C^{141}$ base pairs at their 3′ termini. In addition, about 8% of the original primers were extended by 2 nucleotides to yield 19-mers. The relatively high yield of 19-mers is consistent with the ability of εdGMP to be incorporated opposite both C and T residues (13). On addition of unmodified dNTPs (Fig. 3, "Extension" step), the εdGTP-derived 18- and 19-mer products were extended slowly by DNA polymerase α, resulting in about 50% of the 18-mers extended to products ≥23 nucleotides in length after 30 min at 30°C; products derived from initial insertion of unmodified dGTP (Fig. 4*A*, lane 4) were extended with higher efficiency (>95%; data not shown). This is consistent with previous observations that primers containing 3′-terminal εG residues are relatively poor substrates for some polymerases (B.S. and M.K.D., unpublished observations). Subsequent addition of T7 DNA polymerase (Fig. 3, "Complete Extension" step) resulted in extension of nearly all oligomers to products >1000 nucleotides in length (Fig. 4*B* and data not shown).

To determine whether the 18- and 19-mer products contained εG, we took advantage of the heat lability of the εG-deoxyribose glycosyl bond (28). Upon heating (100°C for 15 min), about two-thirds of the 18-mers and nearly all of the 19-mers disappeared, with a concomitant increase in two new bands with altered electrophoretic mobilities (18* and an analogous band between 18- and 19-mer positions; Fig. 4*A*,

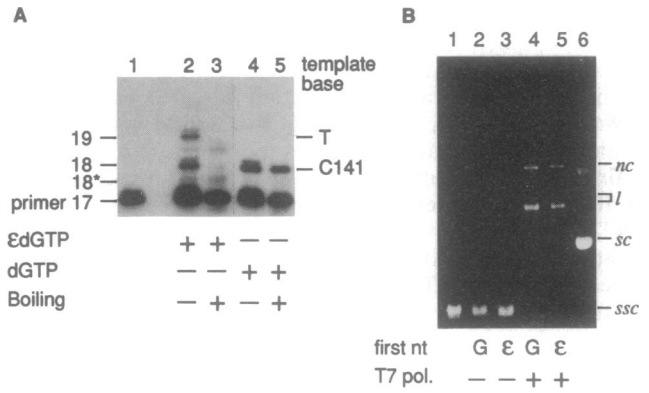

FIG. 4. Analogue incorporation into M13G*1. (*A*) Site-specific incorporation of εdGMP by DNA polymerase α. The 5′-end-labeled 17-mer was annealed to uracil-containing M13G*1 and extended as described in *Materials and Methods*. Products were then analyzed by urea/polyacrylamide gel electrophoresis with or without incubation at 100°C for 15 min prior to loading, as indicated. Lane 1 represents unannealed 17-mer primer. εdGTP (50 μM) or dGTP (0.05 μM) was added to the reaction mixtures as indicated. The 18* band is due to the heat lability of εG (28), resulting in a change in the mobility of the resulting apurinic product (29). The doublet appearance of many bands in both εdGTP and dGTP reactions is probably due to secondary structure, since some faint upper bands disappear when the samples are heat-denatured prior to analysis (compare lanes 4 and 5). (*B*) Final steps in extension of primer. Reactions were carried out as described in Table 1 and *Materials and Methods*. The reactions shown are after the addition of all four normal nucleotides, before and after the addition of T7 DNA polymerase (T7 pol), as indicated ("Extension" and "Complete Extension" steps of Fig. 3). Aliquots of the reaction mixtures, each containing about 200 ng of DNA, were run in a 0.8% agarose minigel containing ethidium bromide at 0.5 μg/ml. Lane 1 represents unprimed uracil-containing single-stranded circular M13G*1. Lanes 2 and 3 show products resulting from initial insertion of either dGMP (lane 2) or εdGMP (lane 3) followed by extension with all four dNTPs by DNA polymerase α (see Fig. 3). The reaction mixtures to which T7 DNA polymerase were added, shown in lanes 4 and 5, represent transfected material. Double-stranded marker M13G*1 DNA was loaded in lane 6 to show the position of nicked circular double-stranded form. We surmise that the doublet bands labeled *l* may represent linear forms derived from extension of single-stranded linear molecules known to be present in the template DNA preparation. *nc*, Nicked circle; *l*, "linear forms"; *sc*, supercoiled circular; *ssc*, single-stranded circular; nt, nucleotide.

Table 1. Mutations resulting from *in vivo* replication of site-specifically incorporated εdG

Target base	No. of plaques screened	% blue plaques	Mutation		
			Base change	No.	Frequency × 10^4
εG (Exp. 1)	6,860	0.50	G → A	34	50
			G → C	0	<2
			G → T	0	<2
εG (Exp. 2)	19,640	0.51	G → A	100	51
			G → C	1	0.5
			G → T	0	<0.5
G (Exp. 3)	11,500	0.02	G → A	2	2
			G → C	0	<1
			G → T	0	<1

Uracil-containing single-stranded M13G*1 was primed with 5'-end-labeled 17-mer in reaction mixtures containing DNA polymerase α and εdGTP at 50 μM (Exp. 1) or 10 μM (Exp. 2); in both experiments, priming was followed by addition of 250 μM dNTPs and finally native T7 DNA polymerase. In Exp. 3, the normal guanine base was positioned at the target site by hybridization of an 18-mer that contained a 3'-terminal dG. Copying reactions, transfections, platings, and sequencing were as outlined in Fig. 3.

lane 3). In contrast, there was no heat-induced degradation of 18-mers derived from the incorporation of unmodified dGMP (Fig. 4*A*, lanes 4 and 5). The heat lability of the εdGTP-derived products reflects depurination of εdG residues and provides evidence for the presence of this analogue in the product oligomers. The quantitative degradation of the 19-mer εdGTP reaction products suggests that the conditions of depurination were exhaustive. Since dGTP pairs with C much more readily than εdGTP, the residual nondegraded 18-mers in lane 3 may also represent incorporation of trace amounts of dGTP in our εdGTP preparation (<0.1%).

εG Causes G → A Transitions. We have carried out two independent experiments to determine the mutagenic potential and spectrum of mutations induced by template εG during DNA replication in *E. coli*. In both experiments, incorporation of εdGMP resulted in a mutation frequency of about 0.5%, which is 25-fold higher than that arising from DNA containing unmodified G at the target position (Table 1). Of the 135 εG-induced mutants, 134 exhibited G → A transitions at the target site; no other sequence changes were detected within a 150-nucleotide region containing the target site.

DISCUSSION

Most chemical carcinogens produce a multiplicity of DNA lesions *in vivo*; as a result, it is difficult to identify the specific lesion (or lesions) responsible for a given carcinogen's mutagenic, and ultimately carcinogenic, potency. Techniques for site-directed mutagenesis have made it possible to place certain specific analogues at predetermined sites in DNA and then to measure the frequency and types of mutations produced *in vivo* (30–32). However, many of these techniques (32) require either chemical synthesis of oligonucleotides or long incubations during ligation, which can result in degradation of labile analogues before mutagenesis can be determined. A major barrier to the study of εG is the extreme lability of its glycosyl bond ($t_{1/2} \approx 25$ hr at 37°C and pH 7; ref. 10). This facile depurination precludes the synthesis of εG-containing oligonucleotides by conventional chemical methods. In an alternative approach, dNTP analogues are used as substrates for DNA polymerases *in vitro*. Preston *et al.* (23, 24) used DNA polymerase to rapidly insert alkyl-dTTP analogues opposite the *am3* target-site A in φX174*am3* DNA, thereby permitting an analysis of mutation frequency and base changes induced by labile thymine analogues in *E. coli*.

Since guanine analogues are among the most common lesions produced by chemical mutagens and carcinogens (32), a vector for specifically studying mutagenesis by guanine analogues is highly desirable. We have developed a phenotypic reversion assay that allows one to introduce labile dGTP analogues site-specifically by using DNA polymerase. The M13mp2-derived vector M13G*1 allows detection of all three base substitutions induced by a single guanine analogue (Fig. 2). Since the presence of an analogue may significantly inhibit the ability of that strand to be replicated (33), we have also utilized a uracil-containing template strand to increase the yield of progeny derived from the analogue-containing strand (27, 34). While there is currently no evidence for an effect of the *mutHLS* mismatch correction system in the repair of nucleotide analogues in DNA, we performed all experiments in a *mutL* background, which is deficient in mismatch correction (35, 36), in the event that this system can recognize and repair a G analogue·C base pair.

Our results indicate that the εG is mutagenic in *E. coli*. In reactions in which 8% of the primers were extended by a single εdGTP opposite the template C^{141}, we observed a mutation frequency of 0.5%, about 25-fold above background. Copying of εdG in *E. coli* resulted in G → A transitions in 134 of 135 εdG-associated mutants. Reconstruction experiments indicated that the penetrance of mutant bases at position 141 in mutagenic primers was ≈50% (data not shown). From the 0.5% mutant frequency, the penetrance of 50%, and the estimated 8% of extended primer containing target-site analogue (see *Results*), we calculate that the mutagenic potency of εG (mutations per analogue) during *in vivo* replication is about 13%. This estimate assumes that the εG^{140} products (19-mers in Fig. 4*A*, lane 2) do not contribute to mutagenesis, since no base changes were detected at position 140 among the 135 mutants sequenced, and there is no evidence that εG can induce untargeted mutations at adjacent nonadducted base positions. εG^{140}-induced mutations in the absence of mutations at position 141 are not likely to be detected, since (*i*) mutagenic coding by the misinserted analogue for T would restore the original sequence and (*ii*) a T → C transition caused by correct coding of εG^{140} for C during copying in *E. coli* would yield a codon (TTC) that codes for the same amino acid (phenylalanine) as the original codon (TTT). The true mutagenic potential of εG may actually be higher than our estimate, because some of the εG residues may be lost prior to replication in the cell as a result of depurination or exonucleolytic removal by T7 DNA polymerase. Nonetheless, our estimate of mutagenic potential (13%) closely approximates the 20% value obtained for εG coding for T during reverse transcription *in vitro* (14).

The monotypic base-substitution pattern of εG most likely results from base pairing of the newly incorporated εG^{141} with T during DNA replication in the host *E. coli*. This mechanism is supported by *in vitro* data showing the formation of the same mispair with εG both as template (14) and as incoming nucleotide precursor (13). The observed mutations are probably not due to polymerase errors during manipulations *in vitro*, since both polymerase α and T7 DNA polymerase are highly faithful (error rates ≈ 1/600,000 and 1/800,000, respectively; ref. 37; R. Doshi and B.D.P., unpublished results). The G → A transitions observed here were also not likely due to depurination of the labile εdG residues, since apurinic sites preferentially induce SOS-dependent G → T transversions (38).

We have inserted the bulky carcinogen analogue εG at a defined position in a guanine-specific *in vivo* mutagenesis assay, using the labile precursor εdGTP and DNA polymerase. Replication across εG-containing template strand specifically generates G → A transitions in *E. coli* at an estimated

frequency of 13%, consistent with *in vitro* data. Although other vinyl chloride-associated analogues remain to be tested in a sensitive *in vivo* mutagenesis assay such as the M13G*1 assay, the high mutagenic potential of εG suggests that this analogue may contribute to the carcinogenic potential of vinyl chloride. The monospecificity of base substitutions induced by εG might be exploited to assess the contribution of this analogue to vinyl chloride-induced cancers.

We thank our colleagues for their support; T. J. McBride for the initial vector construction and sequencing of M13G*1; A. Lee and T. Nguyen for excellent technical assistance; S. Spyropoulos and P. Goodwin of the Fred Hutchinson Cancer Research Center Image Analysis Laboratory for help with Phosphorimager analysis; F. Perrino for DNA polymerase α; T. Kunkel, R. Schaaper, and B. Bachman for *E. coli* strains; and M. Bohidar and K. Carroll for their excellent graphics work. This work was supported by Fellowship DRG-049 from the Damon Runyon–Walter Winchell Cancer Research Fund to K.C.C., National Institutes of Health (NIH) Grant CA48174 to B.D.P., NIH Grant CA47723 to B.S., and NIH Outstanding Investigator Grant R35-CA39903 to L.A.L.

1. Creech, J. L. & Johnson, M. N. (1974) *J. Occup. Med.* **16,** 150–151.
2. Doll, R. (1988) *Scand. J. Work Environ. Health* **14,** 61–78.
3. Zajdela, F., Croisy, A., Barbin, A., Malaveille, C., Tomatis, L. & Bartsch, H. (1980) *Cancer Res.* **40,** 352–356.
4. Zief, M. & Schramm, C. H. (1964) *Chem. Ind.*, 660–661.
5. Laib, R. J., Gwinner, L. M. & Bolt, H. M. (1981) *Chem. Biol. Interact.* **37,** 219–231.
6. Eberle, G., Barbin, A., Laib, R. J., Ciroussel, F., Thomale, J., Bartsch, H. & Rajewsky, M. F. (1989) *Carcinogenesis* **10,** 209–212.
7. Fedtke, N., Walker, V. E. & Swenberg, J. A. (1989) *Arch. Toxicol. Suppl.* **13,** 214–218.
8. Laib, R. J. (1986) in *The Role of Cyclic Nucleic Acid Adducts in Carcinogenesis and Mutagenesis*, eds. Singer, B. & Bartsch, G. (Oxford Univ. Press, London), Int. Agency Res. Cancer Sci. Publ. No. 7, pp. 101–108.
9. Leonard, N. J. (1984) *CRC Crit. Rev. Biochem.* **15,** 125–199.
10. Spengler, S. J. & Singer, B. (1988) *Cancer Res.* **48,** 4804–4806.
11. Singer, B., Spengler, S. J. & Kuśmierek, J. T. (1988) in *Chemical Carcinogens: Activation Mechanisms, Structural and Electronic Factors and Reactivity*, eds. Politzer, F. A. & Martin, F. (Elsevier, Amsterdam), pp. 188–207.
12. Kuśmierek, J. T., Folkman, W. & Singer, B. (1989) *Chem. Res. Toxicol.* **2,** 230–233.
13. Singer, B., Kuśmierek, J. T., Folkman, W., Chavez, F. & Dosanjh, M. K. (1991) *Carcinogenesis* **12,** 745–747.
14. Singer, B., Spengler, S. J., Chavez, F. & Kuśmierek, J. T. (1987) *Carcinogenesis* **8,** 745–747.
15. Brown, T., Hunter, W. N., Kneale, G. & Kennard, O. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2402–2406.
16. Folkman, W., Kuśmierek, J. T. & Singer, B. (1990) *Chem. Res. Toxicol.* **3,** 536–539.
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), 2nd Ed.
18. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382.
19. Bebenek, K. & Kunkel, T. A. (1989) *Nucleic Acids Res.* **17,** 5408.
20. Sanger, F., Coulson, A. R., Friedmann, T., Air, G. M., Barrell, B. G., Brown, N. L., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M. & Smith, M. (1978) *J. Mol. Biol.* **125,** 225–246.
21. Perrino, F. W. & Loeb, L. A. (1989) *J. Biol. Chem.* **264,** 2898–2905.
22. McBride, T. J., Preston, B. D. & Loeb, L. A. (1991) *Biochemistry* **30,** 207–213.
23. Preston, B. D., Singer, B. & Loeb, L. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8501–8505.
24. Preston, B. D., Singer, B. & Loeb, L. A. (1987) *J. Biol. Chem.* **262,** 13821–13827.
25. Basu, A. K. & Essigmann, J. M. (1988) *Chem. Res. Toxicol.* **1,** 1–18.
26. Wood, M. L., Dizdaroglu, M., Gajewski, E. & Essigmann, J. M. (1990) *Biochemistry* **29,** 7024–7032.
27. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 488–492.
28. Fedtke, N., Boucheron, J. A., Walker, V. E. & Swenberg, J. A. (1990) *Carcinogenesis* **11,** 1287–1292.
29. Groebke, K. & Leumann, C. (1990) *Helv. Chim. Acta* **73,** 608–617.
30. Preston, B. D. & Loeb, L. A. (1988) *Mutat. Res.* **200,** 21–35.
31. Singer, B. & Essigmann, J. M. (1991) *Carcinogenesis* **12,** 949–955.
32. Singer, B. & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens* (Plenum, New York).
33. Koffel-Schwartz, N., Maenhaut-Michel, G. & Fuchs, R. P. P. (1987) *J. Mol. Biol.* **193,** 651–659.
34. Reid, T. M., Lee, M.-S. & King, C. M. (1990) *Biochemistry* **29,** 6153–6161.
35. Modrich, P. (1987) *Annu. Rev. Biochem.* **56,** 435–466.
36. Huisman, O. & Fox, M. S. (1986) *Genetics* **112,** 409–420.
37. Reyland, E. M. & Loeb, L. A. (1987) *J. Biol. Chem.* **262,** 10824–10830.
38. Schaaper, R. M., Kunkel, T. A. & Loeb, L. A. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 487–491.