and II are definitely different, possibly due to slight changes in the stacking of the loop bases.

These studies on a set of related sequences indicate that there can be a substantial effect of the nucleotide sequence on the conformational properties of DNA hairpins. Most of the DNA hairpins studied to date have loop regions that consist solely of thymidine residues (Haasnoot et al., 1980, 1983; Hare & Reid, 1986; Ikuta et al., 1986). By studying a diverse set of sequences, both RNA and DNA, with different combinations of stem sequences, loop sequences, and loop sizes, we can approach a comprehensive understanding of hairpin structure. We are currently undertaking a study of the thermodynamics of this set of related hairpins to look for a correlation of the structural variations with stability.

ACKNOWLEDGMENTS

The 500-MHz NMR spectrometer was funded by grants from the National Science Foundation (DMB-8515942) and from the National Institutes of Health (1-51-RR-2733-01). We thank Dr. Jack Ohlms and Karen Wert at Beckman Instruments, Palo Alto, CA, for the generous gift of d(CTGCTCTTGTTGAGCAG). We also thank Dr. Hans Heus for helpful comments on the manuscript.

Registry No. Hairpin I, 115427-43-5; hairpin II, 119242-88-5; hairpin III, 119242-93-2.

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Mutation Induced in Vitro on a C-8 Guanine Aminofluorene Containing Template by a Modified T7 DNA Polymerase†

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Received August 9, 1988; Revised Manuscript Received November 18, 1988

ABSTRACT: We reacted uracil-containing M13mp2 DNA with *N*-hydroxy-2-aminofluorene to produce a template with *N*-(deoxyguanosin-8-yl)-2-aminofluorene adducts. This template was hybridized to a non-uracil-containing linear fragment from which the *lac z* complementing insert had been removed to produce a gapped substrate. DNA synthesis using this substrate with the modified T7 DNA polymerase Sequenase led to an increase in the number and frequency of *lac*$^-$ mutations observed. *Escherichia coli* DNA polymerase I (Kf) did not yield a comparable increase in mutation frequency or number even though both Sequenase and the *E. coli* polymerase had similar, low, 3′ → 5′ exonuclease activities as compared to T4 DNA polymerase. We did not observe an increase in mutations when synthesis was attempted on a template reacted with *N*-acetoxy-2-(acetylamino)fluorene to give *N*-(deoxyguanosin-8-yl)-2-(acetylamino)fluorene adducts. Both *E. coli* and T7 enzymes terminate synthesis before all (acetylamino)fluorene lesions. Only some of the putative aminofluorene adducts produced strong termination bands, and there was a difference in the pattern generated by Sequenase and *E. coli* pol I (Kf) using the same substrate. Analysis of the mutations obtained from Sequenase synthesis on the aminofluorene-containing templates indicated a preponderance of −1 deletions at G's and of G → T transversions.

Most mutations occur as a result of errors during replication. A large literature deals with the problem of the fidelity of polymerases synthesizing DNA on normal templates (Kirkwood et al., 1986). An early analysis of the factors involved in mutation induced by prokaryotic polymerases which carry their own 3′ → 5′ exonucleases (Fersht, 1979) pointed out that the probability of fixing a mismatch depended on at least three factors: (a) the rate of incorporation of the mismatched base; (b) the rate of excision of the mismatch by the

† This work was supported in part by grants from the National Institute of General Medical Sciences (GM 07816) and the National Cancer Institute (CA32436) and by a contract with the Department of Energy (DE-AC02-76ER02040).

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0006-2960/89/0428-2836$01.50/0

3′ → 5′ exonucleolytic activity; (c) the rate of elongation since elongation of the chain with the production of a properly matched base pair protects the mismatch from exonucleolytic attack. There is therefore a "next nucleotide" effect (Kunkel et al., 1981) in which fidelity in vitro can be affected by the presence of dNTP's which increase the elongation rate.

Mutations are induced by alteration of the template nucleotides in DNA. A class of mutagen alters the base composition of the DNA while still permitting synthesis at approximately normal rates. We have classified the lesions produced by such mutagens (e.g., aminopurine, bromouracil, nitrous acid) as instructive since the altered bases still specify particular base pairings, albeit the "incorrect" pairs (Strauss et al., 1982). Mutations induced in *Escherichia coli* by agents producing instructive lesions do not, as a rule, require gene products (i.e., proteins) other than those involved in normal replication.

A second class of mutagen produces lesions which block DNA synthesis, at least temporarily. The mechanism by which such lesions are converted into mutations must be more complex, since errors must be made and the DNA block at the damaged site must also be bypassed, possibly as part of the same event. That the mechanism is indeed more complex is demonstrated by the isolation of proteins involved in the mutation process, distinct from those involved in DNA replication (Burkhardt et al., 1988). The *recA* and the *umcC-D* gene products have been shown to be essential. These gene products are part of the "SOS repair" system, a set of about 18 genes which is induced in response to treatment of *E. coli* with DNA damaging agents (Walker, 1984; Nohmi et al., 1988). The situation is complex since *N*-hydroxy-2-aminofluorene is a poor inducer of the *umuC-D* system and does not block DNA synthesis with high efficiency (Lutgerink et al., 1985) although mutations are produced by this agent only in SOS-induced cells (Salles et al., 1983).

Many of the components of the SOS system that are related to mutagenesis have been cloned and sequenced and the proteins isolated (Burkhardt et al., 1988). It is not yet known whether they affect the fidelity of the polymerase to make the insertion of mismatched bases more frequent. It has been proposed that the recA protein combines at UV-induced lesions in double-stranded DNA and decreases fidelity by inhibiting 3′ → 5′ exonuclease activity (Lu et al., 1986; Lu & Echols, 1987). In the absence of SOS gene products, DNA polymerases rapidly turn over nucleotides opposite damaged sites in DNA, indicating a rapid addition and removal from the site (Sagher & Strauss, 1983). In the presence of exonuclease inhibitors it is possible to demonstrate the termination of DNA synthesis reactions opposite, rather then 3′ to, the lesion. The addition of a nucleotide opposite a noninstructive site in DNA therefore need not be the rate-limiting step in bypass. Rather, it may be the elongation step which is rate limiting, and it may be this step in which the *umuC-D* gene products are involved. It is possible to obtain mutants in organisms unable to manufacture the *umuC-D* gene product, by UV irradiating and incubating for several hours before photoreactivation. Bridges' et al. (1987) interpretation of this experiment is that insertion of an incorrect base occurs opposite the lesion which remains a block to further elongation in the absence of the *umuC-D* gene products. Removal of the lesion by photoreactivation permits DNA synthesis to occur which "fixes" the mispairing as a mutation.

In this paper we report the development of an in vitro system to study mutation specificity. A DNA polymerase is allowed to copy a sequence that has been reacted with a chemical mutagen. We used two DNA polymerases in these experiments. We used *E. coli* DNA pol I, Klenow fragment (Kf), to compare its properties in the system we describe with the termination experiments previously reported [see Strauss (1985)]. An altered T7 DNA polymerase, Sequenase, was used because of its processive properties. T7 DNA polymerase combined with thioredoxin differs from *E. coli* pol I (Kf) DNA polymerase in at least two major properties. First, it is highly processive. In addition, the native T7 enzyme contains a far more active 3′ → 5′ exonuclease activity (Kornberg, 1980; Tabor & Richardson, 1987). Sequenase is manufactured by chemical treatment of T7 DNA polymerase plus thioredoxin to remove much of this exonuclease activity although some still remains (Tabor & Richardson, 1987). The products of the in vitro polymerase reaction are transfected into host organisms under conditions such that any mutants selected after transfection must have arisen during the in vitro synthesis step. We find that mutations are produced after synthesis catalyzed by the more processive DNA polymerase on a template reacted with *N*-hydroxy-2-aminofluorene. The mutagenic specificity observed is similar to that observed by Bichara and Fuchs (1985) and by Ames et al. (1972) in vivo. If the in vitro results can be applied to in vivo conditions, they suggest that mutagen specificity is determined by polymerase and that the *umuC-D* products play some other role, possibly in elongation.

Materials and Methods

Reagents. *N*-Acetoxy-2-aminofluorene, *N*-acetoxy-2-(acetylamino)[9-^{14}C]fluorene, *N*-hydroxy-2-(acetylamino)fluorene, and *N*-hydroxy-2-acetyl[ring-G-^{3}H]fluorene were products of Chemsyn Science Laboratories, Lenexa, KS. Deoxynucleoside triphosphates (HPLC purified) were obtained from Pharmacia; [α-^{35}S]dATP used for sequencing and for the termination experiment was obtained from Amersham.

Enzymes. Restriction enzymes were supplied by New England Biolabs (*Eco*RI) and Boehringer Mannheim Biochemicals (*Pvu*I, *Pvu*II). *E. coli* DNA polymerase I (Klenow fragment) was purchased from Boehringer Mannheim Biochemicals, Sequenase was purchased from U.S. Biochemicals Inc., and T4 DNA polymerase and T4 DNA ligase were purchased from New England Biolabs. AMV reverse transcriptase was purchased from Life Sciences Inc. Cloned *E. coli* single-stranded binding protein was purchased from Pharmacia LKB Biotechnology Products.

Enzyme Assays. The 3′ → 5′ exonuclease activity of the DNA polymerases was determined by a slight modification of the method described by Rogers and Weiss (1980). Nick-translated salmon sperm DNA was prepared by treatment of salmon sperm with pancreatic DNase followed by incubation with [^{3}H]dTTP in a reaction mix containing all four dNTP's. The substrate as used had a specific activity of 1.74 Ci/mol of DNA. DNA polymerase assays were performed by determining the amount of [^{3}H]dTTTP incorporated into acid-insoluble material after 30-min incubation at 37 °C in a 300-μL reaction mixture containing 100 mM Hepes, pH 8.0, 100 mM KCl, 16 mM $MgCl_2$, 6 mM DTT, 50 μg of denatured salmon sperm DNA, and 50 μM each of dATP, dCTP, dGTP, and dTTP to which 0.5 μCi of [^{3}H]-dTTP and 20 μL of diluted enzyme had been added.

Bacterial strains used in these experiments are described in Table I.

Isolation of M13mp2 Uracil-Containing DNA. M13mp2, an original Messing strain without amber mutations, obtained from Dr. E. LeClerc was grown on BW313 (*ung*$^{-}$*dut*$^{-}$). The phage used was taken through two cycles of growth on the

Table I: Strains Used

strain	markers	origin
BW313	Hfr KL16 PO-45 [*lysA*(61) → → serA (62)] chromosomal *ung-1*, *dut-1*, *thi*, *relA1*, *spot1*	B. Weiss
CSH-50	*ara*⁻, *thi*⁻, Δ (*pro* – *lac*)/F′ *traD36*, *proAB*, *Lac18ZΔM15*]	T. Kunkel
S90C	[Δ (*pro* – *lac*), *ara*⁻, thi⁻, *strA*]	T. Kunkel
S90CRecA56	[Δ (*pro* – *lac*), *ara*⁻, *thi*⁻, *recA56*, *strA*]	T. Kunkel

ung⁻*dut*⁻ bacteria incubated in YT medium modified as described in Sagher and Strauss (1983), isolated by CsCl density gradient centrifugation, dialyzed, and extracted with phenol–chloroform.

Construction of Gapped Templates. Replicative form I DNA was prepared from M13mp2 as described in Sagher and Strauss (1983). RF was linearized with *PvuI*. *Pvu*II was added, and after incubation an aliquot of the reaction mix was electrophoresed to demonstrate both small (268 bp) and large (6.8 kb) fragments. The digest was then applied to a preparative 0.8% agarose gel and electrophoresed. The large fragment was electroeluted, and the eluate was filtered, concentrated with 1-butanol, and then precipitated with ethanol. Yields of 30–50% were obtained. Preparations were analyzed by electrophoresis before further use to make sure that only a single sharp band characteristic of the large fragment was present.

M13mp2 (+) strand was reacted with mutagen (see below) before hybridization. The 6.8-kb fragment (175 μg) was chilled 5 min in an ice bath. M13mp2 (+) strand (175 μg) and 1 M KCl were added to give a final concentration of 100 mM and a final volume of 2333 μL. The mixture was heated at 65 °C for 30 min and cooled slowly to room temperature (30 min). Samples were analyzed by gel electrophoresis and then stored at 4 °C. This procedure creates a gapped hybrid substrate capable of serving as a template for in vitro DNA synthesis. In an experiment using *E. coli* DNA polymerase I (Kf) and a mixture of dNTP's including [^{3}H]dTTP, 5263 dpm was incorporated with the (+) strand DNA alone as a template, 8850 dpm with the nonhybridized mix of (+) strand DNA and large fragment, and 48 793 dpm with the gapped hybrid mix, all corrected for a blank of 666 dpm.

Reaction with N-Hydroxy-2-aminofluorene and N-Acetoxy-2-(acetylamino)fluorene. DNA was reacted with *N*-hydroxy-2-aminofluorene by the method of Lutgerink et al. (1985) and of Fuchs and Seeberg (1984). DNA in sodium citrate (10 μg/mL, pH 5.5, 10 mM) was reacted with [^{3}H]-*N*-hydroxy-2-aminofluorene added to give a final concentration of 0.71 mM in 20% ethanol. Reaction was carried out at 20 °C in the dark under N_2. The mixture was extracted with phenol–chloroform/isoamyl alcohol (1:1) twice and with chloroform–isoamyl alcohol (24:1) twice and then ethanol precipitated. The number of adducts per molecule was determined from the UV absorbancy and the radioactivity bound.

DNA was reacted with *N*-acetoxy-2-(acetylamino)fluorene (AAAF)[1] by the procedure described by Rabkin and Strauss (1984) except that the reagent was dissolved in tetrahydrofuran. Nonradioactive AAAF was used, and the number of adducts added was calculated from the biological inactivation of the ss M13mp2 (uracil) DNA transfected into the *ung*⁻*dut*⁻ strain BW313, assuming that each adduct produces a lethal hit (Lutgerink et al., 1984).

[1] Abbreviations: AAAF, *N*-acetoxy-2-(acetylamino)fluorene; AAF, 2-(acetylamino)fluorene; AF, 2-aminofluorene.

Synthesis on Gapped Substrates. All syntheses were carried out at a concentration of 1 μg of DNA per 50 μL of reaction mixture consisting of 20 mM Hepes, pH 7.8, 5 mM DTT, and 10 mM $MgCl_2$. The four dNTP's (dATP, dCTP, dGTP, dTTP) were added to a final concentration of 300 μM. ATP was added to all reaction mixtures to a final concentration of 500 μM. In some experiments we added dGMP to a final concentration of 10.3 mM to inhibit exonuclease activity. *E. coli* pol I (Kf) was used at 1–2.5 units/μg of DNA, Sequenase at 0.8 unit/μg of DNA, and T4 DNA ligase, when used, at 40 units/μg of DNA, and *E. coli* ssb (Pharmacia) was used at a ratio of 4.2 μg/μg of DNA. Enzyme incubations were at 10 or 37 °C as indicated for 30 min. EDTA (0.25 M) was then added to a final concentration of 15 mM, and the samples were held on ice until used for transfection.

Termination reactions were carried out with the same DNA substrates but with the substitution of [^{35}S]dATP (final concentration 1.2 μM) for dATP during the first 15 min of incubation and with 0.4 unit of either *E. coli* pol I (Kf) or Sequenase and with 0.5 μg of DNA in a total volume of 17 μL. Incubation was for 15 min at 10 °C at which time 1 μL of dATP was added to give a final concentration of 267 μM and incubation continued for an additional 15 min. An aliquot was removed for determination of TCA-precipitable radioactivity. NaCl (1 μL of 1.2 M) and *Bgl*I (1 μL of 2 units/μL) were added, and the mixture was digested 60 min at 37 °C. Stop mix (13 μL; 0.3% xylene cyanol, 0.3% bromphenol blue, 10 mM EDTA, pH 7, 95% formamide) was added, and 15-μL aliquots of the samples were electrophoresed. A sequence ladder was prepared by synthesizing on unreacted gapped uracil hybrid template with a reaction mixture (5.5 μL) including template, DTT (9 mM), [^{35}S]dATP, and 25–30 units of AMV reverse transcriptase.

Transfection and Isolation of Mutants. Transfection was carried out by slight modification of published procedures (Maniatis et al., 1982). We used the *recA*⁻ recipient S90C, described by Kunkel (1985a). Exponential cultures were monitored to give an absorbancy at 550 nm corresponding to (2–4) × 10^7 cells/mL; the cells were suspended in a $CaCl_2$ solution and held in the cold. DNA was added, and the cells were incubated 40 min in an ice bath before a 2-min heat shock at 42 °C. Soft agar was added, and the cells were plated on prewarmed plates. The agar was allowed to harden, and the plates were inverted and incubated overnight at 37 °C. They were removed from the incubator and allowed to develop color for 24 h at room temperature. Plaques were counted, and all mutants were numbered and listed on a record sheet that indicates the experiment, date, mutant color, and plate number to permit possible siblings to be eliminated. All putative mutants were picked into fresh medium and incubated to prepare a fresh stock. The stock was replated alongside of bona fide wild-type phage to confirm their mutant nature. Miniprep DNA preparations of mutants were used for sequencing by the dideoxy technique using AMV reverse transcriptase as the polymerase and a 15-mer (5′-CTGTTGGGAAGGGCG-3′) which primes at the template, 3′ to the gap. Dideoxynucleotide mixes were used with [^{35}S]dATP and with dITP substituted for dGTP.

Results

Substrate for in Vitro Mutagenesis Studies. Our general scheme involves reaction and DNA synthesis of a gapped M13 molecule in vitro, transfection of the product molecule into *E. coli*, and scoring of mutations in the progeny phage. It is important to be sure that the mutations observed occur in vitro rather than during replication of transfected DNA in the

Table II: Transfection Efficiency of Different Substrates in S90CRecA56

substrate	av pfu/ng of DNA
(1) RF I (wild type)	866.0
(2) single-strand (+) DNA (w.t.)	93.0
(3) single-strand (+) DNA (uracil)	0.03
(4) linear fragment (w.t.) (*Pvu*I–II)	0.22[a]
(5) uracil hybrid (3 and 4)	0.31
(6) (5) after DNA synthesis with *E. coli* pol I (Kf)	10.9
(7) wild-type hybrid (2 and 4)	169.0
(8) (7) after DNA synthesis with *E. coli* pol I (Kf)	145.0

[a] Data from a different experiment in which RF I had a transfection efficiency of 825 pfu/ng.

recipient *E. coli*. Since the AF-induced mutations studied in vivo require activation of the SOS repair system for their production (Bichara & Fuchs, 1985), the use of a *recA*$^-$ recipient (Kunkel, 1985a) precludes most host-derived mutations. In order to make the method more general and applicable to mutagens not requiring SOS participation, we use the method of Kunkel (1985b) for destruction of the (+) strand. M13mp2 (+) strand DNA containing uracil is prepared by growth of the M13 vector in an *ung*$^-$*dut*$^-$ host. Molecules prepared in this way contain about 5% of their thymines substituted with uracil (Sagher & Strauss, 1983). We hybridize the uracil-containing M13mp2 (+) strand with a large fragment prepared by digestion with *Pvu*I and *Pvu*II of RF from M13mp2 grown on *ung*$^+$*dut*$^+$. This hybridization produces a molecule with a gap of 391 nucleotides which includes the promoter and initial portion of the *lac z* β-galactosidase complementing peptide. Since the uracil-containing (+) strand will be destroyed on introduction into an *ung*$^+$*dut*$^+$ host, recovery of viable phage in our system depends on in vitro synthesis to close the gap and produce a complete (−) strand.

We used DNA reacted with *N*-acetoxy-2-(acetylamino)-fluorene or with *N*-hydroxy-2-aminofluorene in these experiments. These compounds react with DNA to give a simple spectrum of adducts, and there is an extensive literature documenting the mutagenic spectra produced in vivo by these adducts [e.g., Bichara and Fuchs (1985)]. Although addition of aminofluorene to the C-8 of guanine is the major reaction, it is possible to alter the substrate by opening of the ring (Tang & Lieberman, 1983). We are grateful to Dr. Moon-shong Tang for the analysis of the *N*-hydroxy-2-aminofluorene-reacted substrate used in the experiments reported below. He found that after hydrolysis of the DNA as described by Tang and Lieberman (1983) 95% of the radioactivity chromatographed at the position of authentic *N*-(deoxyguanosin-8-yl)-2-aminofluorene (G-C8-AF). Less than 2% of the radioactivity chromatographed at the position of the ring-opened adduct 1-[6-(2,5-diamino-4-oxopyrimidinyl)-N^6-deoxyribosyl]-3-(2-fluorenyl)urea. The material used for the studies on mutation and termination therefore contained almost all of the adduct as intact *N*-(guanin-8-yl)-2-aminofluorene.

In vitro synthesis following hybridization increases the transfection efficiency of uracil-containing DNA by 35-fold (Table II, lines 5 and 6). However, even after synthesis we found that the transfection efficiency of the uracil hybrid was only 7.5% that of the wild-type hybrid. We suppose that at least two factors account for this difference. In the wild-type hybrid, both (+) and (−) strands can serve as templates in vivo. When the uracil hybrid infects an *ung*$^+$*dut*$^+$ host, only the (−) strand can replicate. In addition, any inefficiency of in vitro synthesis so that a proportion of the gapped molecules are not completely replicated will contribute to the apparent strand bias.

Table III: Results of Synthesis on an AAF-uracil-Containing Template[a]

sample	pfu	pfu/ng of DNA	mutants total	mutants blue	% mutants total	% mutants blue
no synthesis	120	0.03	0	0	0	0
T7 Sequenase, 10 °C	1587	1.1	13	10	0.8	0.6
AAF (3.8)						
no synthesis	61	0.0038	4	0	6.6	0
T7 Sequenase	422	0.05	7	6	1.7	1.4
AAF (5.9)						
no synthesis	36	0.002	3	0	8.3	0
T7 Sequenase	64	0.008	2	0	3.1	0
non-uracil DNA						
no synthesis	4998	83	6	6	0.12	0.12
T7 Sequenase	5606	93	10	7	0.18	0.12

[a] Reactions were carried out as described under Materials and Methods. The calculated number of lesions in the (+) strand is indicated in parentheses.

Synthesis with Sequenase on undamaged templates gave significantly more mutants when synthesis was at 37 °C as compared to 10 °C (3.4% compared to 0.99%). This temperature effect was not as pronounced with *E. coli* pol I (Kf) (0.62% vs 0.34%). Addition of dGMP (10.3 mM) to the *E. coli* pol I reaction mixture increased the error frequency at 37 °C (1.2% vs 0.43%). We also measured the yield of mutants at 10 and 37 °C after synthesis with Sequenase on an aminofluorene-containing (AF) template (see below). The yield of mutations using an AF-containing template was about 40% higher at 37 °C than at 10 °C. However, the ratio of "induced" (from an AF-containing template) to "spontaneous" (synthesis using an unaltered template) was only 1.5 for total mutations and 0.83 for light blue mutations (the class made up mainly of single-base substitutions) as compared to a ratio of 5.6 for total mutations and 3.7 for light blue mutations at 10 °C in this experiment. Identification of an "induced" class would be much more difficult at 37 °C, and we therefore ran the remainder of our synthetic reactions at 10 °C.

Bypass of Aminofluorene Adducts. Using AAF-reacted DNA as a template for synthesis with T7 Sequenase, we found that the total number of plaques declined with increasing number of adducts. The total number of "light blue" mutants also declined (Table III). [Single base substitution mutants in the *lac z* complementing region are often observed as light blue plaques. A large fraction of the colorless plaques turn out to be deletions and frame shifts (Kunkel, 1984).]

The higher frequency of colorless mutants in the treated samples is probably a result of cyclization of the large fragment of the DNA which contains all vital M13 genes but none of the *lac* complementing insert. Sequencing of the "colorless" mutants obtained among the progeny of AAF-treated DNA after synthesis with either *E. coli* DNA pol I (Kf) (data not shown) or Sequenase indicated loss of the complete insert in all cases tested, as would be expected if this explanation were correct. This cyclization within the host, which is a rare event, produces colorless viable phage in proportions that increase as the total number of viable phage is descreased. The data show an increased proportion of light blue mutants in the products of synthesis on a template containing 3.8 AAF adducts per molecule (Table III), but the total number of mutants observed is so low that additional data are required before it is clear whether induced mutation has occurred.

We note that the products of Sequenase-catalyzed synthesis on uracil-containing DNA have a higher spontaneous mutation rate than those on non-uracil-containing DNA, a phenomenon also observed by Kunkel (1985b). We suppose this increase to be the result of the tolerance of cytosine deamination in an

Table IV: In Vitro Mutagenesis on an AF-uracil-Containing Template[a]

sample	enzyme	pfu	pfu/ng of DNA	mutants total	mutants blue	% mutants total	% mutants blue
no synthesis	0	1625	0.16	26	6	1.6	0.37
control	Seq	9057	2.1	87	55	0.96	0.60
AF (2.4)	0	495	0.07	3	1	0.61	0.20
AF (2.4)	Seq	15937	1.9	414	209	2.6	1.3
AF (6)	0	280	0.04	5	3	1.8	1.1
AF (6)	Seq	3312	0.40	178	74	5.4	2.2
no synthesis	0	832	0.18	14	3	1.7	0.36
control	Kf	22827	5.28	64	56	0.28	0.25
AF (2.4)	0	585	0.083	3	0	0.51	0
AF (2.4)	Kf	19642	1.58	89	69	0.45	0.35
AF (6)	0	367	0.052	2	1	0.54	0.27
AF (6)	Kf	1998	0.16	26	8	1.3	0.4

[a] Sequenase and *E. coli* reactions were run at 10 °C according to the protocol given under Materials and Methods. Control templates do not contain AF adducts. Numbers in parentheses refer to average adducts per molecule calculated from the radioactivity bound to DNA. The Sequenase and *E. coli* polymerase experiments were done separately. Seq, modified T7 DNA polymerase, Sequenase; Kf, *E. coli* DNA polymerase I, Klenow fragment.

ung⁻dut⁻ host and survival of the resulting C → U transitions during growth of the virus.

There is an increase in the total number of mutants obtained from a given amount of DNA after Sequenase synthesis on a substrate reacted with *N*-hydroxy-2-aminofluorene reaction as well as an increase in the mutation frequency as compared with that of synthesis on a non-AF-containing control template. It is also clear from the data (Table IV) that there is a difference in the behavior of *E. coli* pol I (Kf) and of Sequenase in these experiments. The decrease in transfection efficiency of treated as compared to untreated templates is less after synthesis with Sequenase, and there is an absolute increase in mutants per unit of DNA with Sequenase but not with the *E. coli* polymerase. This suggests that there has been bypass of the AF lesions with Sequenase if not with *E. coli* pol I (Kf). Such bypass is hardly surprising in view of the published data indicating that multiple AF lesions are necessary to inactivate ϕX174 DNA (Lutgerink et al., 1985) as well as biochemical evidence that these lesions are bypassed (Michaels et al., 1986; O'Connor & Stohrer, 1985).

Since the Sequenase preparations we used still contain some 3′ → 5′ exonuclease activity, we compared the exonuclease activities of the T7 DNA polymerase, T7 Sequenase, and *E. coli* pol I (Kf). As expected, the T7 enzyme has by far the highest activity. Exonuclease activity could be demonstrated with both Sequenase and *E. coli* pol I (Kf) preparations, but this activity was only about 1% of the exonucleolytic T7 DNA polymerase activity. The exonucleolytic activities of the DNA polymerases, Sequenase, and *E. coli* pol I (Kf) per unit of enzyme were indistinguishable in these experiments, the ratio of slopes from the least-squares line [T7/Sequenase or T7/*E. coli* pol I (Kf)] being 206 or 150, respectively. The ratio of exonucleolytic activity to polymerizing activity per unit of polymerizing activity as measured on the same template [denatured calf thymus DNA which is suboptimal for *E. coli* pol I (Kf)] was calculated from the slopes of the least-squares line of exonuclease activity plotted against units of polymerase. The values were 9.9, 0.099, and 0.116 for T7 DNA polymerase, Sequenase, and *E. coli* pol I (Kf), respectively. Differences in the capability of Sequenase and *E. coli* DNA pol I (Kf) to bypass the same substrate must therefore be due to some property other than proofreading exonuclease activity.

Analysis of Mutations. We sequenced a sample of the mutants isolated since a major difference in the kinds or in the location of mutations obtained off an AF template as compared with controls would be expected if in vitro mutation had occurred. We analyzed approximately equal numbers of spontaneous mutants obtained as a result of DNA synthesis by Sequenase on a gapped uracil-containing template and induced mutants obtained after comparable synthesis on the same uracil template but also containing AF lesions. Each of the mutants isolated was plaque purified, and DNA was extracted and sequenced by the dideoxy procedure using AMV reverse transcriptase as the polymerase. In order to avoid counting siblings, only one mutant per particular sequence and color obtained from any one Petri dish was counted. A few of the isolates contained more than one base change. However, all mutations were tabulated as though they occurred independently (Table V). Changes are not uniformly distributed across the sequence but seem to occur primary at a few sites ("hot spots", Figure 1). In particular, there seems to be a hot spot for deletion at a 5′-GGGAAAACCC-3′ sequence starting at Kunkel position 88 (position 6266 in the overall MP2 template). The overwhelming majority of mutations obtained after synthesis on the AF-containing template originate at G's, as would be predicted if mutation were targeted.

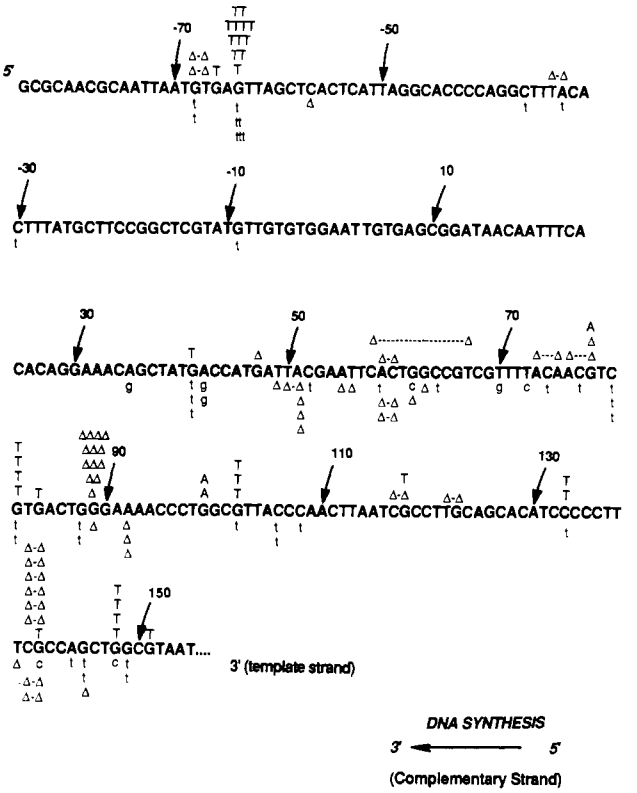

FIGURE 1: Summary of sequence data. The location and nature of the mutants sequenced are indicated above or below the sequence. Lower-case letters or symbols below the sequence indicate spontaneous mutants, i.e., mutants obtained by synthesis on a nontreated, uracil-containing, gapped template. Upper-case letters and symbols above the line indicate results with mutants obtained after synthesis on an AF-containing, uracil, gapped template. (Δ) Deletion; (Δ---Δ) deletion spanning a sequence.

Termination Sites. The data indicate a difference in the ability of Sequenase to bypass AF as compared to AAF lesions in DNA since approximately equal numbers of AAF and AF lesions lead to very different "survivals". After synthesis with Sequenase, 5.9 AAF lesions gave 0.73% of the unreacted plaque-forming efficiency (Table III) as compared with 6 AF lesions giving 19% of the unreacted plaque-forming efficiency (Table IV). Our experimental design permits us to determine the ability of enzymes to bypass particular lesions by a more

Table V: Summary of Sequencing Results[a]

	control	AF containing
transversions		
G → T	21	29
A → T	4	0
G → C	3	0
T → G	1	0
transitions		
G → A	0	2
A → G	3	0
C → T	13	2
T → C	1	0
deletions (3′ → 5′)		
G	4	16
C	1	0
A	7	0
T	3	0
TG	0	2
GC	2	7
GT	0	1
AC	1	0
AT	1	1
CA	1	1
GCA	0	1
ACA	0	1
multiple	0	1
total no. of mutants	59	60
no. of mutations obsd	66	64

[a] The table summarizes the changes observed in mutants obtained by transfection of uracil-containing M13mp2 gapped templates following synthesis with Sequenase (control) or after synthesis with Sequenase on a gapped template containing an average of six AF adducts per molecule.

direct technique. In order to make this comparison, we carried out termination experiments [see Strauss (1985)] using the same preparations of reacted DNA substrates used in the mutation experiments (Figure 2). In these experiments we used either *E. coli* pol I (Kf) or Sequenase as the polymerase.

The results on an AAF-containing template support previous observations from this laboratory (Rabkin & Strauss, 1984). Termination of *E. coli* pol I (Kf) catalyzed synthesis on this template occurs mainly 3′ to G's in the template. At some positions additional bands are observed. Termination bands occur at approximately equal intensity at G's across the sequence. The pattern with Sequenase is similar to that observed with *E. coli* pol I (Kf), including the additional bands, for example, around positions 90 and 100. Our previous interpretation of such additional bands was that termination had occurred opposite, as well as 3′ to, a reacted G (Moore et al., 1982).

The pattern with AF-containing templates is more complex. Although all terminations still occur in the vicinity of G's in the template, the density of the bands is very irregular as though only some sequences are strong terminators. In addition, it appears that many of these strong stops (e.g., at position 79) are opposite rather than before the lesion. The same substrate was used for the Sequenase reaction and for the *E. coli* pol I (Kf) reactions, and similar amounts of radioactivity were loaded (legend of Figure 2). Nonetheless, it appears as though numerous termination bands are missing or much reduced in intensity. A strong band at position 102 is much weaker in the Sequenase lanes. On the other hand, very few Sequenase bands are stronger than their *E. coli* polymerase counterparts.

Since mutations are produced on AF templates when Sequenase but not *E. coli* pol I (Kf) is used as a template and it seems likely that Sequenase bypasses lesions that are blocks to *E. coli* (see above), one might expect to see mutations at those sites which terminate *E. coli* synthesized but not Sequenase-catalyzed synthesis. Alternatively, one might expect to see more mutations at the sites of most pronounced termination. Neither of these explanations seems to be correct (Figures 1 and 2). The strongest position for termination with either Sequenase or *E. coli* polymerase appears around position 30 (Figure 2), but this region is devoid of mutations, even though deletions here would certainly affect the phenotype as seen by the isolation of such mutants upstream. Transversions are seen at position 82, and it is true that strong termination bands are seen with both polymerases 3′ to this position. However, most deletions are observed at position 88, but termination is not prominent with either enzyme at this position. Double-base deletions originating at a G occur at position 141 and transversions at position 148. In both cases termination is more prominent with the *E. coli* polymerase. These data, therefore, do not indicate any particular relationship between termination and mutation although it does seem that there is no necessary relationship between intensity

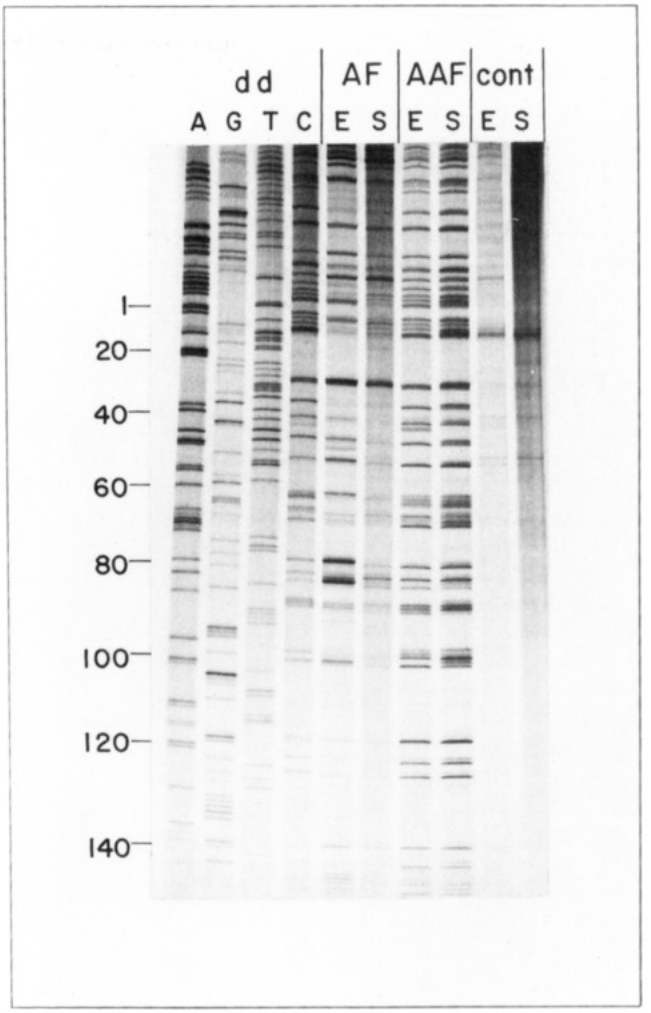

FIGURE 2: Polyacrylamide gel analysis of M13mp2 (uracil-containing) DNA templates either untreated (cont) or treated with *N*-hydroxy-2-aminofluorene (AF) or *N*-acetoxy-2-(acetylamino)fluorene (AAF). AF DNA used for the experiments described in Table IV was used for termination studies as described under Materials and Methods. After synthesis on all templates, the DNA was digested with *Bgl*I which cuts 29 bases 3′ to the *Pvu*I site of the primer. The DNA was denatured in formamide, loaded on an 8% acrylamide–urea buffer gradient gel, and run at 38 constant watts for 5 h. The amounts of TCA-precipitable radioactivity added to each of the lanes were as follows (channel, dpm × 10^{-5}): AF (E), 5.4; AF (S), 5.6; AAF (E), 3.9; AAF (S), 3.4; cont (E), 4.6; cont (S), 5.4. The numbers on the left side of the gel indicate the nucleotide positions as given by Kunkel (1985a). (E) *E. coli* DNA polymerase I (Kf); (S) Sequenase.

Table VI: Distribution of Mutations

	% spontaneous	% induced	% corrected induced
multiple deletions	8	23	26
single-base deletions	23	25	26
transversions	44	45	46
transitions	26	6	2

of termination and likelihood of mutation at any particular site.

Discussion

The sample of "induced mutations" necessarily contains spontaneous mutants, especially since the spontaneous rate in this in vitro mutation system is so high. Among the spontaneous mutations, the C → T transition is particularly frequent. This is expected for M13mp2 grown on an *ung⁻dut⁻* mutant in which uracils resulting from deaminated cytosines would not be removed as discussed above. The C → T transitions account for 13 out of 66 or 20% of the spontaneous class. If all C → T transitions represent spontaneous mutation, the number of such transitions can be used to correct the class of induced mutants for the calculated spontaneous mutations in each class (Table VI).

Overall, and without correction, 58/64 = 91% of the mutations obtained from the AF-containing template originate at G's as compared to 30/66 = 46% in the controls. There are more deletions among the products of Sequenase synthesis on the AF-containing template, and in contrast to the spontaneous class, most involve G's. A major hot spot for deletion occurs in the AF-containing template at position 88–90 (Figure 2), and this position forms part of a potential hairpin structure: GGGAAAACCC. This same position was reported by Kunkel (1984) as a hot spot for spontaneous depurination mutagenesis.

The proportion of transversions is the same in the products of synthesis on control and treated templates, but the distribution is qualitatively different: G → T transversions are the only ones so far observed in the induced sample. A hot spot for G → T transversion at −64 is found for both spontaneous and induced samples. The excess of G → T transversions observed among the products of synthesis on an AF-containing template is an indication that insertion of A occurs opposite damaged sites. However, it is impossible to decide whether this insertion occurs by the incorporation of A opposite noninstructive AF lesions or as a result of incorporation opposite abasic sites formed by decomposition of the altered nucleotide (Loeb, 1985). Since spontaneous transversions are observed at all but one of the hot spots, it could be that these particular sites are unusually susceptible to depurination.

We conclude that Sequenase produces both deletions and base substitution mutations (mostly transversions) in about equal frequency on an AF-containing template. In *Salmonella typhimurium*, aminofluorene adducts are efficient in producing frame shifts (Ames et al., 1972). AF adducts are reported as efficient in producing G → T transversions in SOS-induced *E. coli* (Bichara & Fuchs, 1985). Bichara and Fuchs (1985) conclude that as many as 85% of the mutations induced by AF are base substitutions with the G → T transversion prominent. They did observe both single- and double-base deletion frame shifts and conclude that AF lesions do induce such changes, but at a lower frequency than do AAF lesions. The spectrum of mutations observed in vitro therefore resembles that observed in vivo.

The difference in termination bands on AF- and AAF-containing templates could be due to either differential reaction at the different sites or differential bypass of lesions. Given the number of G-C8-AF adducts (Table IV) and the size of the single-stranded gap, we expect there to be 0.13 AAF or 0.32 AF adduct in each gap. At this low level of reaction it is quite possible that certain sites are overrepresented. It is also possible that certain sites are bypassed more readily by both *E. coli* pol I (Kf) and Sequenase, and we hope in future studies to determine which of these explanations is correct.

Although the T7 DNA polymerase preparation has been chemically treated to lower its 3′ → 5′ exonuclease activity, there is still about 1% of the residual exonuclease activity present (see above), and this activity is very close to that of *E. coli* pol I (Kf). Some factor other than exonucleolytic proofreading must account for the difference in the behavior of these two enzyme preparations. Livneh (1986) has suggested the importance of *E. coli* pol III processivity for the bypass of pyrimidine dimers. Sequenase is highly processive because of the association of the polymerase with thioredoxin (Tabor & Richardson, 1987). We suggest that it is the capacity to remain associated with the template at each polymerization step which facilitates elongation past a damaged base. This tentative conclusion is related to the original question as to the possible role of the *umuC-D* gene products in mutation. Both *E. coli* pol I (Kf) and Sequenase can, at particular positions, add a nucleotide opposite a lesion (see Figure 2). Therefore, the limiting step in bypass of such positions must be elongation. We assume that the processive enzyme is able to carry out the elongation more readily. This formulation of the problem also suggests that the specificity of incorporation opposite the lesion is due to polymerase. Elongation but not mutational specificity would then be due to the SOS gene products as suggested by Bridges et al. (1987) and by Livneh (1986).

Acknowledgments

We thank Dr. Daphna Sagher for her many suggestions and for her reading of successive drafts of the manuscript.

Registry No. AAAF, 6098-44-8; G-C8-AF, 73051-69-1; DNA polymerase, 9012-90-2; *N*-hydroxy-2-aminofluorene, 53-94-1; *N*-(deoxyguanosin-8-yl)-2-(acetylamino)fluorene, 37819-60-6.

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Binding of Captan to DNA Polymerase I from *Escherichia coli* and the Concomitant Effect on 5′→3′ Exonuclease Activity†

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Received October 14, 1988

ABSTRACT: Captan (*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide) was shown to bind to DNA polymerase I from *Escherichia coli*. The ratio of [^{14}C]captan bound to DNA pol I was 1:1 as measured by filter binding studies and sucrose gradient analysis. Preincubation of enzyme with polynucleotide prevented the binding of captan, but preincubation of enzyme with dGTP did not. Conversely, when the enzyme was preincubated with captan, neither polynucleotide nor dGTP binding was blocked. The modification of the enzyme by captan was described by an irreversible second-order rate process with a rate of 68 ± 0.7 M^{-1} s^{-1}. The interaction of captan with DNA pol I altered each of the three catalytic functions. The 3′→5′ exonuclease and polymerase activities were inhibited, and the 5′→3′ exonuclease activity was enhanced. In order to study the 5′→3′ exonuclease activity more closely, [^{3}H]hpBR322 (DNA–[^{3}H]RNA hybrid) was prepared from pBR322 plasmid DNA and used as a specific substrate for 5′→3′ exonuclease activity. When either DNA pol I or polynucleotide was preincubated with 100 μM captan, 5′→3′ exonuclease activity exhibited a doubling of reaction rate as compared to the untreated sample. When 100 μM captan was added to the reaction in progress, 5′→3′ exonuclease activity was enhanced to 150% of the control value. Collectively, these data support the hypothesis that captan acts on DNA pol I by irreversibly binding in the template–primer binding site associated with polymerase and 3′→5′ exonuclease activities. It is also shown that the chemical reaction between DNA pol I and a single captan molecule proceeds through a Michaelis complex. The final, irreversible step results in inhibited polymerase and 3′→5′ exonuclease activities as well as enhanced 5′→3′ exonuclease activity.

Kinetic studies of procaryotic, eucaryotic, and viral polymerases have been employed to examine the inhibitory effects of captan (*N*-[(trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide), an inhibitor which acts on a variety of enzyme systems including nucleic acid synthesis (Lewis & Brown, 1978; Gale et al., 1971; Martin & Lewis, 1979). Although the mechanism of captan inhibition of polymerases has been investigated, efforts have mainly focused on the polymerase activity of the enzymes (Dillwith & Lewis, 1982a,b; Freeman-Wittig & Lewis, 1986). Of the DNA polymerases, DNA polymerase I from *Escherichia coli* has been examined extensively (Dillwith & Lewis, 1982a; Freeman-Wittig & Lewis, 1986). In one report, it was noted that captan caused irreversible loss of DNA pol I polymerase activity which was accounted for by captan–protein interactions. Kinetic studies showed that DNA binding by enzyme and/or initiation of polymerization was inhibited by captan but that elongation of DNA was not. The template–primer binding site was shown to be the locus of captan interaction, and DNA served as a protection against captan's action (Dillwith & Lewis, 1982a).

A subsequent investigation of the effects of captan on DNA pol I revealed that the 3′→5′ exonuclease activity which shares the polynucleotide binding site with the polymerase activity was inhibited in the presence of captan and substantiated that

† This research was supported by a grant from the National Institute of Environmental Health Sciences (ES03058) and is a contribution of the Nevada Agricultural Experiment Station. M.-J.F.-W. is a Stauffer predoctoral research fellow.
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0006-2960/89/0428-2843$01.50/0