Site directed substitution of 5-hydroxymethyluracil for thymine in replicating φX-174*am3* DNA via synthesis of 5-hydroxymethyl-2′-deoxyuridine-5′-triphosphate

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ABSTRACT

5-hydroxymethyluracil (HmUra) is formed in DNA as a product of oxidative attack on the methyl group of Thy. It is removed from DNA by HmUra-DNA glycosylase. To determine whether the replacement of Thy by HmUra is mutagenic, which might explain the repairability of HmUra, a HmUra residue was substituted for Thy in a target (*amber*) codon by *in vitro* extension of an oligonucleotide primer annealed to φX-174*am3* virion DNA. This was accomplished by synthesizing HmdUTP and using DNA polymerase to effect primer extension. *E.coli* spheroplasts were transfected with the HmUra-containing DNA and the yield of revertant phage determined following replication in the bacterial host. Since *E. coli* do not express HmUra-DNA glycosylase activity, mutagenesis could be assessed in the absence of repair. χ^2_c analysis showed that replacing Thy with HmUra did not result in an increase in revertant phage. These data indicate that the oxidation of Thy to HmUra in cellular DNA probably does not result in substantial mutagenesis.

INTRODUCTION

5-Hydroxymethyluracil (HmUra) is formed in DNA as a product of oxidative attack on the methyl group of Thy (1). It was detected in DNA of cells exposed to ionizing radiation (2, 3) or to active oxygen species released by activated leukocytes (4). A corresponding DNA repair enzyme, HmUra-DNA glycosylase, was first identified in mouse plasmacytoma cells (5) and subsequently purified from calf thymus (6).

The biologic effects of HmUra in DNA were studied using 5-hydroxymethyl-2′-deoxyuridine (HmdUrd) which acts as a dThd analogue. Bacteria and mammalian cells grown in medium containing [^{3}H]HmdUrd incorporated HmdUrd into their DNA (7, 8, 9, 10). HmdUrd induced point mutations in *Salmonella* tester strains and SOS functions in *E.coli* (11, 12). V79 hamster cells grown in medium containing HmdUrd displayed a low level of mutagenicity (1/30,000 HmUra residues) at the HGPRT locus (13).

These results raised the possibility that HmUra in DNA was repaired because it was weakly mutagenic. This mutagenicity might result from the electron withdrawing properties of the hydroxymethyl group rendering HmUra ambiguous when compared to Thy with respect to base pairing (14). SPO1 phage DNA, which normally contains HmUra in place of Thy, melts at 10 degrees less than DNA of corresponding Thy content, indicating that base stacking interactions and/or hydrogen bonds involving HmUra are weaker than in DNA containing Thy (15).

However, the mutagenicity studies which used HmdUrd could not distinguish between incorporative mutagenesis resulting from misincorporation of the 5′-triphosphate of HmdUrd (HmdUTP) into DNA opposite bases other than Ade as opposed to replicative mutagenesis. Replicative mutagenesis would result from misinsertion of nucleotides other than adenylate opposite HmUra residues in template DNA. This distinction is critical in assessing whether the oxidation of Thy to HmUra in DNA is sufficiently mutagenic to promote the evolutionary development of a repair enzyme. Incorporative mutagenesis is a phenomenon of growth in culture since HmdUTP is not normally present in living organisms.

We used site directed mutagenesis to evaluate the potential of HmUra to cause replicative mutagenesis in the absence of incorporative mutagenesis. HmdUTP was synthesized and used to introduce HmUra in place of Thy in the *amber* codon of φX-174*am3* DNA. Replication of this phage DNA in an *E.coli* host produces progeny phage which may be easily screened for point mutations since any such mutation at the target locus results in a revertant wild type phenotype. Since bacteria do not express HmUra-DNA glycosylase activity, the mutagenicity of HmUra in replicating DNA could be assessed *in vivo* in the absence of repair.

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MATERIALS AND METHODS

Materials

Organic chemicals and enzymes were from Aldrich or Sigma Chemical Companies. Radiochemicals were from New England Nuclear.

Spheroplasts were made from *E.coli* KT-1 (*Su*$^+$, ϕX insensitive), a gift of L.Loeb of the University of Washington. Screening of progeny phage was done on *E.coli* HF-4704 (*Su*$^-$, *ThyA*) and *E.coli* HF4714 (*Su*$^+$) from the *E.coli* Genetic Stock Center at Yale University. Serum Biotest Institute bovine serum albumin and ϕX-174*am3* virion DNA used in mutagenesis experiments were gifts from L.Loeb and B.Preston (Rutgers University, Piscataway NJ), respectively.

Ingredients for bacteriological media were from Difco. Bacterial growth was in R-medium except for bacteria being grown for spheroplasts. These were grown as described in Kunkel *et al.* (16).

Analytical Methods

ODS HPLC was done on Altex 5 um Ultrasphere columns (0.46×25 cm for analytical, 1×25 cm for preparative). Anion exchange HPLC was performed using an Altex Ultrasil SAX column (0.46×25 cm). Solvent A was 1 M KH_2PO_4 and Solvent B 100 mM NH_4COOH/10 mM H_3PO_4 (pH 4.8). A clear separation of mono-, di- and triphosphates was provided by elution at 1 ml/min of solvent A for 5 minutes followed by a 15 min linear gradient to 40% B. The UV absorbance spectra of chromatographic peaks analyzed during HPLC runs were obtained using a 9 μl flow cell in a Beckman model 165 detector with the model 450 data analyzer.

Negative ion Fast Atom Bombardment (FAB) mass spectra (glycerol matrix) were obtained by J.Ulrich (Laboratoire de Spectromtrie de Masse des Proteines, Centre d'Etudes Nucleaires, Grenoble, France) on a Kratos model MS 50 spectrometer equipped with an FAB gun (8 KeV Xenon atoms).

Nucleoside and nucleotide syntheses

5-Hydroxymethyl-2′-deoxyuridine-5′-phosphate (HmdUMP, **II**) was synthesized from dUMP (**I**) using paraformaldehyde in 1 M HCl as described by Maley (17), except that we were unable to purify the product as described. Instead, aliquots were purified on the preparative ODS column by elution with 3 ml/min 0.3 M TEAB/2% methanol followed by a 100% methanol wash. HmdUMP eluted at 19 minutes, preceded by unreacted dUMP and HmdUrd.

The identity of the product was confirmed by cleavage of the phosphate bond with alkaline phosphatase, which produced a single product which coeluted with [^{3}H]HmdUrd (see below) on HPLC.

5-Hydroxymethyl-2′-deoxyuridine-5′-triphosphate (HmdUTP, **III**) was synthesized from HmdUMP using the method of Hoard & Ott (18). As a positive control for the methodology, dUMP was phosphorylated, resulting in a high yield of pure dUTP. In contrast, phosphorylation of 76 μMol HmdUMP resulted in a low yield (30−40% by HPLC estimation) of HmdUTP and also produced large quantities of side products. Anion exchange HPLC analysis of the products revealed several compounds which were not resolved by conventional liquid chromatography (AG-1, A-G-2, P2-Biogel and DEAE sephacel). Partial purification was achieved by preparative ODS HPLC chromatography using 0.4 M TEAB/7.5% methanol (3 ml/min), followed by a 100% methanol wash. HmdUTP eluted in a broad peak at about 28 min. Overall yield was 20−30% of the input HmdUMP after the first ODS preparation. Purity was 70−90%.

HmdUTP which had been partially purified by ODS chromatography was evaporated to dryness and further purified by SAX HPLC. The SAX purified HmdUTP was desalted by suspension in water, filtration, and rechromatography on the ODS column using 0.4 M TEAB/7.5% methanol as eluent (3 ml/min). After desalting, the SAX purified triphosphate was >95% pure and the overall yield was 1−4%.

[6-^{3}H]5-Hydroxymethyl-2′-deoxyuridine ([6-^{3}H]HmdUrd, **V**) was synthesized to aid in the biochemical characterization of the compound. The synthesis was originally developed for other studies (10) but is described in detail here for the first time. Carrier-free [6-^{3}H]HmdUrd was synthesized by adapting the synthesis of non radioactive HmdUrd described by Shiau *et al.* (19). The synthesis was scaled down to 0.25−1.0 mCi of carrier free [6-^{3}H]dUrd (**IV**, 25 Ci/mmol) which was evaporated to dryness in a 7×50 mm tube and mixed with a slurry of 9 mg of paraformaldehyde in 250 μl 0.5 M KOH. The stoppered tube was maintained without stirring at 50−55°C. Additional 10 μl aliquots of 5 M KOH were added to maintain the pH above 9 and progress monitored by HPLC. After 13 days at 50°C, 56% of the radioactivity coeluted with UV marker HmdUrd, 9% with dUrd, 3% with Ura and/or HmUra, and the remainder was in the injection front.

The reaction mixture, in 2 ml of water, was combined with 5 ml of concentrated ammonia and dried by rotary evaporation at 40°C. After a second ammonia treatment, the residue was dissolved in water and deionized on a 0.7×7.0 cm Biorad A-G50X2[H^+] cation exchange column.

The product was then concentrated and purified by ODS HPLC using water as eluent. The [^{3}H]HmdUrd recovered from this run was 96−98% pure and represented an overall yield of 20−60% of the input [^{3}H]dUrd. 1−2% of the radioactivity was dUrd so that a second HPLC purification was performed to remove all detectable dUrd (<0.1%). The purified material coeluted with authentic UV marker HmdUrd on ODS HPLC. Its identity was confirmed by acetylation (3).

[6-^{3}H]-5-Hydroxymethyl-2′-deoxyuridine-5′-phosphate ([6-^{3}H]HmdUMP, **II***) was synthesized from [^{3}H]HmdUrd by phosphorylation using thymidine kinase prepared by the method of Waldman *et al.* (20). Carrier free [^{3}H]HmdUrd (1 nmol) was added to 12 units of enzyme in a reaction buffer containing 5 mM ATP, 40 mM KCl, 4 mM $MgCl_2$ and 50 mM Tris pH 7.9 (final volume 500 μl). After 2 days at 37°C, about half of the nucleoside was phosphorylated. HmdUMP was purified by HPLC as described above.

[6-^{3}H]5-Hydroxymethyl-2′-deoxyuridine-5′-triphosphate ([6-^{3}H]HmdUTP, **III***). [^{3}H]HmdUMP was diluted with non-radioactive HmdUMP to a specific activity of 1.6 Ci/mmol. The tributylammonium salt (10 nmol) was reacted with 60 μmol of carbonyldiimidazole and tributylammonium pyrophosphate, each in 500 μl dimethylformamide, according to the protocol described above. The yield was 23% HmdUTP and 24% unconverted HmdUMP in the unpurified reaction mixture.

HmdUTP characterization

To demonstrate the synthesis of HmdUTP and its suitability for use in site directed mutagenesis, HmdUTP was analyzed spectroscopically, derivatized by dephosphorylation followed by acetylation, and characterized biochemically by its activity as a substrate for DNA polymerase.

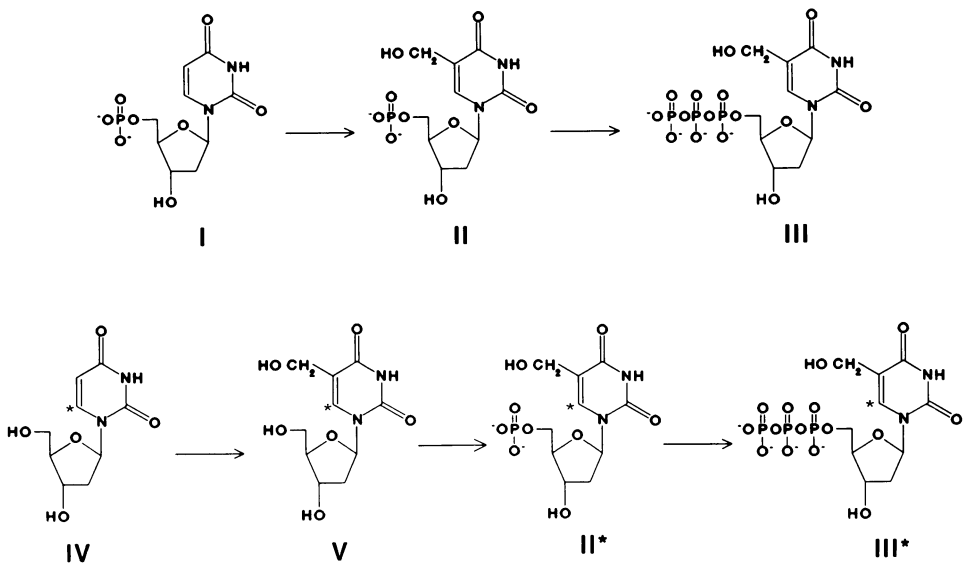

Figure 1. Syntheses of HmdUTP. Upper: dUMP (**I**) was reacted with $(CHO)_n$/HCl to form HmdUMP (**II**). The tributylammonium salt of **II** was phophorylated by addition of carbonyldiimadazole followed by P_2O_7 to form HmdUTP (**III**). Lower: Synthesis of [6-^{3}H]HmdUTP (**III***) began with dUrd (**IV**) labelled at carbon 6 with ^{3}H (*). This was converted to [^{3}H]HmdUrd (**V**) using $(CHO)_n$/KOH. **V** was phosphorylated using ATP and thymidine kinase to yield [^{3}H]HmdUMP (**II***) which was then reacted in identical fashion to **II** to generate **III***.

On ion exchange HPLC, its retention time was close to those of dUTP and dTTP and different from ATP and 5'-nucleotide diphosphate markers. Treatment with alkaline phosphatase regenerated the nucleoside, which coeluted with [^{3}H]HmdUrd marker.

FAB-Mass spectroscopic analysis of the product yielded large peaks at m/e of 497 and 519, consistent with molecular ions (M−H and M+Na, respectively) of HmdUTP. Pairs of peaks corresponding to the ions of the diphosphate (417 and 439) and monophosphate (337 and 359) were also observed.

To demonstrate the accuracy of primer extension, a 15-nucleotide primer was synthesized with sequence complementary to the sequence 3' to position 587 of the virion (+) strand of ϕX-174*am3* (see Figure 2). The primer was 5'-end labelled with γ-^{32}P-ATP (3000 Ci/mMol) and polynucleotide kinase (IBI). The radioactive primers were purified using Sep-Pac ODS Cartridges (Waters).

Primers were annealed to the virion (template) DNA by adding a 2-fold molar excess of primer to virion DNA in 10−25 μl SSC (10 ng/μl) at 75°C followed by slow cooling. Virion DNA (100 ng), with annealed primer, was combined with primer extension buffer (20 mM Tris, 20 mM HEPES, 60 mM KCl, 5 mM $MgCl_2$, 2 mM DTT,pH 7.3), 6 units of Klenow polymerase with 2 nmol of HmdUTP, dUTP, or dTTP and 2 nmol of each the following nucleotides: 1) none; 2) dATP; 3) dATP and dCTP; or 4) dATP, dCTP, and dGTP (final volume 50 μl). Reactions were terminated by addition of EDTA and formamide gel loading buffer, and electrophoresed on 38×50 cm×0.4 mm 20% acrylamide/7 M urea gels at 1600 volts.

For the mutagenesis experiments the ratio of primer to template was 5:1. Primer annealed to 100 ng of virion DNA was combined with 0.4 nmol dATP and 2 nmol HmdUTP (or dTTP or dUTP) in primer extension buffer at 37°C (final volume 40 μl). After 20 min, 10 μl of a mixture of the 4 normal dNTPs (20 nMol each) were added followed, 15 min later, by another 6 units of polymerase (in 2 μl of primer extension buffer). After a final 15 min, the reaction was terminated by addition of 3 μl 0.5 M EDTA (pH 8.0), vortexed, and stored at 4°C.

Agarose gel electrophoresis was used to check the extent of primer extension. Aliquots from each reaction mixture were compared with double stranded (RF) and single stranded (virion) ϕX DNA markers (New England Biolabs) on 5×7 cm 1% agarose gels run at 70 V in TBE.

Nearest neighbor analysis was used to demonstrate that, in the presence of dTTP, the proofreading activity of DNA polymerase I did not replace HmdUMP with dTMP in the extended primer. A 5-fold excess of primer was annealed to a virion template and extended with 2 nmol HmdUTP and 0.4 nmol dATP as above except that the mixture contained 75 Ci/nmol [α-^{32}P]dATP. A replicate tube was treated identically, except after 20 min, a mixture of the normal triphosphates (20 nmol each) was added. After 3 min, the reaction was terminated with EDTA and the triphosphates removed on Sephadex spin columns (Worthington) which had been preequilibrated in succinate buffer (20 mM sodium succinate, 10 mM $CaCl_2$, pH 6.0). The DNAs were then digested 17 h at 37°C in 260 μl succinate buffer containing 10 units micrococcal nuclease and 0.35 units spleen phosphodiesterase. The resulting digest contained 3'-nucleoside monophosphates which were separated, after filtration, by analytical ODS HPLC, with isocratic elution by 200 mM KH_2PO_4. Radioactivity was measured using the in-line scintillation counter.

Mutagenesis experiments

E.coli spheroplasts were transfected with primer extended DNA as described by Kunkel *et al.*(16). SOS-induced spheroplasts were prepared by irradiation with 50 J/M^2 under a 254 nm germicidal lamp as described in Schaaper and Loeb (21) except that a second dose of 25 J/M^2 was administered immediately prior to transfection. After treatment with $CHCl_3$ to liberate progeny phage, they were diluted and screened by mixing with indicator bacteria in soft agar.

The yield of *amber* and revertant phage were calculated from the number of plaques on soft agar plates containing Su^+ and Su^- bacterial lawns, respectively, corrected for dilution. Samples were generally spread over three plates, although when transfection frequencies were low up to 9 plates containing *E.coli* HF4704 were prepared so that plaques could be counted for each data point.

The number of plaques counted are expected to be binomially distributed and thus χ_c^2 testing was performed as described in Snedicor and Cochran (22). Because the data was uncorrected for the dilutions made prior to plating, only samples in which the same volumes of the same dilutions were plated could be compared directly. Each experiment consisted of replicate samples of 2 independent transfections. Replicate plates were evaluated for reproducibility using 2×2 contingency tables to compare the ratio of Su^- and Su^+ plaques prior to summing the totals for use in 2×2 or 2×3 contingency tables to compare the effects of various bases in the target codon.

RESULTS

Syntheses

HmdUTP was synthesized so that an HmUra residue could be incorporated at a specific site in φX-174*am3* DNA using primer extension with the Klenow fragment of DNA polymerase I. To do this, it was first necessary to synthesize HmdUMP. This was purified, characterized, and used to synthesize the corresponding 5′-triphosphate. The triphosphate was characterized chemically by obtaining its UV and mass spectra and by the chromatographic properties of the purified triphosphate and its dephosphorylated and dephosphorylated/acetylated derivatives. A second strategy was used to generate [^{3}H]HmdUTP. This compound was synthesized to prove that HmdUTP could indeed be used as a substrate for DNA polymerase to effect primer extension.

The products of the two synthetic routes, ^{3}H labelled and non-radioactive HmdUMP, coeluted on reversed phase HPLC. The yield was considerably lower than for the synthesis of dUTP, probably due to side products formed at the 5-hydroxymethyl group of the pyrimidine base. The two synthetic routes are summarized in Figure 1.

Site directed mutagenesis

The goal of these experiments was the evaluation of the mutagenicity of HmUra when present as a base in template DNA, i.e. replicative mutagenesis. The use of site directed mutagenesis eliminated the effects of incorporative mutagenesis which may have contributed to the mutagenicity observed when *Salmonella* and hamster cells were grown in the presence of HmdUrd.

The system (16, 23) is sensitive to mispairing which causes reversion of φX-174*am3* to wild-type phenotype. The base under study is placed at position 587 of the φX sequence (Figure 2, upper). Position 587 is the middle base of the *amber* codon in strain φX-174*am3*, which lies within the coding region of genes E and D. Gene E is the lysis protein, which lyses the host cell wall to release progeny phage at the conclusion of infection. The lysis protein is not required for DNA replication or phage assembly. Thus, mutants with defective gene E products will replicate normally within a host, with no selection in favor of revertants.

The reversion frequency is the ratio of plaques counted from plating the progeny phage on a wild type *E.coli* (Su^-) host lawn to those counted on a lawn of bacteria with an *amber* suppressor (Su^+). Thus, the reversion frequency is an estimate of the mutagenic frequency, i.e. the rate at which errors are made when DNA containing HmUra is replicated *in vivo*. The presence of a base on the (−) template strand which results in insertion of a base other than Ade in the nascent (+) strand will lead to an increase in the reversion frequency relative to that of controls. Revertants can be recovered from the non-suppressor plates and their DNA sequenced to determine the nature of the replication errors made.

Since we had hypothesized that HmUra might be weakly mutagenic, an accurate estimate of the lower limit of the assay was essential. Therefore, deliberate mismatches were constructed at the target codon site to determine the maximal mutagenic frequency. This was accomplished by annealing primers with each of the normal bases at position 587 (Figure 2, lower) over the *amber* codon of φX-174*am3* virion DNA and transfecting as before.

Nick translation of salmon sperm DNA was used to demonstrate that DNA polymerase would incorporate HmUra into DNA when HmdUTP is available as a substrate. Rapid incorporation of radioactivity occurred in the presence of [^{3}H]HmdUTP. The DNA was then enzymatically digested to 2′-deoxyribonucleosides (3). HPLC analysis of the digest revealed that the ^{3}H activity co-eluted with authentic HmdUrd. Acetylation confirmed the identity of this material as HmdUrd. Any side products of the synthesis present were not incorporated into the nick translated DNA since HmdUrd was the only radiolabeled nucleoside recovered from the enzymatically digested DNA.

HmUra was next incorporated at a unique site in a DNA sequence. A synthetic oligomer was annealed to a complementary sequence (Figure 2, upper) in the genome of natural φX-174*am3* virion DNA. The 15-mer was then selectively lengthened by adding only triphosphates of HmdUrd (or dThd) followed by the bases complementary to the succeeding bases of the template virion DNA.

Under these experimental conditions, one and only one nucleotide was added to the 15-mer annealed to virion DNA in

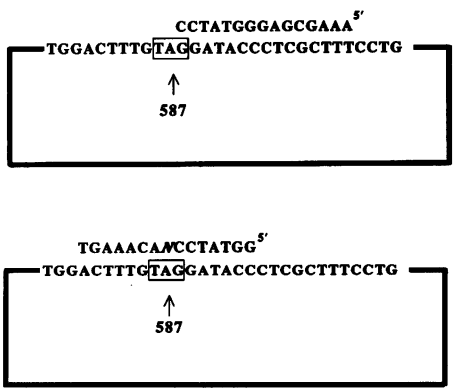

Figure 2. Annealed Primers. Top: Synthetic oligonucleotide annealed to the single DNA strand of φX174*am3* virion DNA, leaving position 587, the middle base in the amber codon (in box), exposed as the next template base for addition of the complementary base (HmUra, Ura or Thy) to the primer using the appropriate 5′-triphosphate and DNA polymerase. Bottom: Synthetic oligonucleotide primer annealed over positions 580−594 of the virion DNA. To evaluate assay sensitivity a primer containing one of the normal bases, three of which represent mismatches with the A at position 587 on the virion DNA, was annealed and the resulting duplex further primer extended and transfected into spheroplasts.

the presence of 50 μM HmdUTP or dTTP as shown in Figure 3. When 10 μM dATP was also added to this mixture, the predicted 17-mer was formed. Addition of HmdUTP or dTTP together with both dATP and dCTP produced the expected 20-mer.

Similar experiments (data not shown) demonstrated the accuracy of insertion of dUTP at position 587, and the inability of DNA polymerase to extend the 15-mer in a mixture containing dATP, dCTP, or dGTP but lacking dTTP or a dTTP analogue.

Nearest neighbor analysis was used to demonstrate that, once added to the primer, HmdUMP was not removed by the proofreading activity of DNA polymerase I. Although no authentic 3'-HmdUMP was available as a marker, a new peak of radioactive material was observed on HPLC analysis of the digested nucleotides, eluting at 10.0 min, between 3'-dUMP (8.9 min) and 5'-HmdUMP (10.8 min). This peak was also observed when the primer was further extended with the normal dNTPs, including dTTP. This peak was not present in control samples

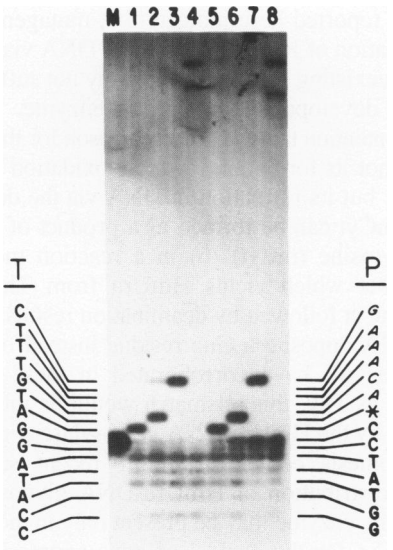

Figure 3. Controlled primer extension. The virion template (T) and primer (P) sequences are indicated with * representing the target site within the *amber* codon which contained either Thy or HmUra. Extended bases are in *italics*. Lanes: M (marker) unextended 15-mer; Triphosphates added: 1, HmdUTP alone; 2, HmdUTP and dATP; 3, HmdUTP, dATP and dCTP; 4, HmdUTP, dATP, dCTP and dGTP; 5, dTTP alone; 6, dTTP and dATP; 7, dTTP, dATP and dCTP; 8, dTTP, dATP, dCTP and dGTP.

Table 1. Reversion frequencies of Thy analogues at position 587

Trial	HmUra	Thy	Ura
1	2.1	4.7	1.9
1SOS	4.8	5.7	ND
2	1.9	1.9	1.4
2SOS	2.4	2.0	ND
3	2.8	3.1	2.4
4	0.10	0.11	
5	1.2	2.7	2.1
6	0.58	1.4	

$10^6 \times$the frequency of reversion to growth on Su^- host lawn with the indicated base at position 587 for six independent trials. Each data point is the average of replicated transfections. Horizontal lines join values which are not significantly different by χ_c^2 analysis. SOS denotes experiments performed with UV irradiated spheroplasts. ND is Not Done. The average titers on Su^- and Su^+ bacterial lawns were 8×10^2 and 5×10^8 plaques/100 ng transfecting DNA, respectively.

with which dUTP was used to insert a Ura at position 587. In those samples a radioactive peak co-eluting with authentic 3'-dUMP was observed. Authentic standards of all normal 3'-dNMPs (N=A,C,T,G) as well as 3'-dUMP were clearly resolved by this chromatographic system.

The results from transfection of *E.coli* KT-1 spheroplasts are presented in Tables 1−3. These experiments detected no significant increase in base pairing errors at the *am3* locus. The reversion frequencies of ϕX DNAs which were primer extended with HmUra, Thy, and Ura at position 587 were quite similar to one another in all experiments (Table 1). Reversion frequencies are reported here as the average of the ratios of revertant to *amber* phage. All values are the average of two transfections of a DNA sample on the same batch of spheroplasts. Each of the six trials were performed on separated days with freshly prepared spheroplasts and primer-extended DNAs. The average titers of progeny phage on Su^- and Su^+ bacterial lawns were 8×10^2 and 5×10^8 PFU/100 ng DNA, which was virtually identical to those reported by the originators of the system (23). χ_c^2 analyses were performed on the sums of counts of plaques on Su^- and Su^+ lawns as described in Methods.

Contingency table analyses of the frequencies of Su^- and Su^+ plaques indicated no significant differences among replicates ($P > .05$). Thus, replicates were combined for all further analyses. The results from transfections of all DNAs extended from position 587 were compared using 2×2 or 2×3 contingency tables, depending on whether a third dUTP-containing reaction was run. With one exception, there were no significant differences ($P > .1$) in the reversion frequencies when an HmUra, a Thy, or a Ura was present at position 587. In the one exception, the difference was highly significant ($P < .005$) but the reversion frequency was *lower* for the progeny from HmUra-containing DNA. While there

Table 2. Positive controls: Reversion frequencies of deliberate mismatches at position 587

Trial	HmUra,Ura,Thy (avg)	Thy+	Cyt+	Gua+	Ade+
1	2.9	4.4	28000	61000	52000
1SOS	5.3	7.2	67000	150000	140000
2	1.7	1.2	12000	28000	27000
2SOS	2.2	1.3	61000	ND	ND
5	2.0	6.1	8900	17000	17000
6	0.99	970.	27000	64000	41000

$10^6 \times$the frequency of reversion to growth on Su^- host lawn. + indicates controls in which the indicated base is at the center of a primer annealed to the template DNA as opposed to insertion during primer extension. Trials were conducted in parallel to those reported in Table 1. Horizontal lines join values which are not significantly different, vertical lines separate values which could not be compared by χ^2 analysis.

Table 3. Assay sensitivity

Trial	Transition	Transversions
1	1:6400	1:13000
1SOS	1:9300	1:20000
2	1:10000	1:23000
2SOS	1:47000	
5	1:1500	1:2800

Assay sensitivity is the ratio of the reversion frequencies measured when primers containing Thy opposite position 587 were compared to those obtained with a mismatched base annealed over the target site (see text).

is no experimental basis for excluding this experiment from the eight others, the data as a whole imply that there was no difference among the three bases.

The mutagenicity of Ura was measured to determine whether DNA repair detectably increased mutagenicity. This was done because host *E.coli* cells contained Ura-DNA glycosylase which removes Ura from DNA. A small amount of dUTP may have been present in the HmdUTP from the synthesis. The results indicate that repair did not affect the fidelity of replication.

Because HmdUrd administered to *E. coli* induced the SOS system (12), the assay was repeated using spheroplasts in which the SOS system had been induced by UV irradiation. No significant differences in reversion frequencies were found for templates containing HmUra relative to Thy-containing controls (Table 1).

In order to assess the sensitivity of the mutagenesis assay, positive control RF DNAs with mismatched bases at position 587 were transfected into normal and SOS-induced spheroplasts. This was done to measure the progeny phage which arise from replication of the unmutagenized (+) strand of the transfected DNA.

The data presented in Table 2 show that the assay is about twice as sensitive to transversions as to a transition mutation to wild type genotype. 2×2 contingency table analyses revealed no significant differences ($P > .5$) between progeny of phage DNAs with Ade or Gua in primers annealed over position 587. When the data from the purine bases were then combined and compared to samples with Cyt at that position, the differences were highly significant ($P < .005$) in each of 5 trials.

By dividing the reversion frequencies of the positive control samples containing Thy at position 587 by those obtained from positive control samples containing the mismatched bases at position 587, an estimate of assay sensitivity was obtained. Thus the background mispairing frequency is about 1 in 15,000 (Table 3). The value was not calculated for trial 6 due to the unusually large difference between reversion frequencies of the Thy positive control and the sample in which Thy had been inserted by primer extension.

Estimating the true sensitivity of the assay is further complicated by the fact that the measured reversion frequencies of the positive control samples containing Thy placed at position 587 at the center of an annealed primer were significantly different than reversion frequency observed when either Thy, Ura or HmUra were inserted at that position by DNA polymerase. However, although the differences in reversion frequency and transfection efficiency were significant, they were neither consistently larger or smaller.

DISCUSSION

These experiments assessed the contribution of replicative mutagenesis to the mutagenicity induced by the administration of HmdUrd to bacteria and mammalian cells in culture. This ϕX-174 mutagenesis system was chosen because of its specificity for base changes opposite a Thy analogue. Furthermore, the system is ideally suited for the incorporation of 5′-triphosphates of dThd analogues via the action of DNA polymerase. This second factor was of importance in the study of HmUra mutagenicity since we encountered severe difficulties during attempts to synthesize a protected phosphoramidite for use in solid phase DNA synthesis. Mammalian cell assays, such as shuttle vector based assays, could not be used due to the absence of mutants of mammalian cells deficient in repair of HmUra in DNA.

The sensitivity of the assay proved to be 10^{-4}. This is similar to that obtained by its developers (L.Loeb personal comm.) as well as to the sensitivity of similar assays based on ϕX (24, 25) or M13 (26, 27). We were unable to detect significant differences in mutation rates between DNA templates containing HmUra and those containing Thy. Thus, our data indicate that HmUra in template DNA functions as Thy with respect to replicative base pairing. However, it is possible that replicative errors do occur more frequently opposite HmUra than opposite the normal base Thy, but less frequently than 10^{-4}.

In light of these findings, the mutagenicity of HmdUrd is best attributed to incorporative mutagenesis. Mutations found when *Salmonella* were grown in the presence of HmdUrd were predominantly G:C to A:T transitions (12), suggesting that misincorporation of HmdUTP occurred opposite Gua during replication.

The results reported here indicate that mutagenesis resulting from the formation of HmUra residues in DNA via the oxidation of Thy in a preexisting AT pair is probably not sufficient to have promoted the development of a repair enzyme. These results support our contention that the primary reason for the repairability of HmUra is not its formation from the oxidation of the methyl group of Thy, but its formation in DNA via the deamination of 5-HmCyt. HmCyt can be formed as a product of the oxidation of 5-methylcytosine (mCyt) (6) in a reaction mechanistically identical to that which yields HmUra from Thy. However, oxidation of mCyt followed by deamination results in an HmUra residue in DNA opposite a Gua residue instead of opposite an Ade. Since, as we have corroborated in these experiments, HmUra codes as Thy, this mismatch would be mutagenic were it not repaired by the HmUra-DNA glycosylase. Further support for this hypothesis comes from our recent survey of the phylogenetic distribution of HmUra-DNA glycosylase, which indicates that the enzyme may be present only in those organisms which use mCyt for the control of gene expression (29).

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