Volume 16 Number 21 1988 Nucleic Acids Research

Mutagenesis induced by site specifically placed 4'-hydroxymethyl-4,5',8-trimethylpsoralen adducts

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Received August 30, 1988; Revised and Accepted October 10, 1988

ABSTRACT. Closed circular double stranded M13mp19 DNA containing a site-specifically placed HMT (4'-hydroxymethyl-4-5'-8-trimethyl-psoralen) monoadduct or crosslink was synthesized *in vitro*. The damaged DNA were scored for loss of infectivity by transfection into repair proficient or deficient *E. coli* and into SOS induced *E. coli*. Mutant phages were detected by the loss of α-complementation between the viral and the host Lac Z genes or by the acquisition of resistance to kpn I digestion. Our results indicate that HMT mutagenesis is targeted and that deletion or transversion of the modified thymidine is the predominant sequence change elicited by a monoadduct or a crosslink. Transfection of the monoadducted DNA into a *Uvr A* deficient strain did not change the mutation pattern but did increase the respective mutation frequencies. Transfection of the crosslinked DNA into a SOS induced host resulted in the appearence of other types of mutations attribuable to an increase in both targeted and untargeted mutations.

INTRODUCTION.

Psoralens are a naturally occuring class of aromatic compounds consisting of a furan ring fused to a coumarin (1). These linear tricyclic compounds intercalate into DNA and photoreact with it in a well characterized manner (1,2). In addition to their clinical use in treating skin diseases such as psoriasis and vitiligo, psoralens have been used extensively in studying the secondary and tertiary structures of nucleic acids (1) and in investigating the molecular basis of DNA repair processes (3,4). Upon near UV irradiation (320-380 nm) intercalated psoralens photoreact by a two stage process primarily with thymidines in DNA and uridines in RNA (1). In the first step of the reaction, either a furan side or a pyrone side monoadduct is induced depending upon whether the 4',5' or the 3,4 double bond of the psoralen molecule reacts with the pyrimidine. By

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absorbing a second photon, the furan side monoadduct can be converted to a crosslink if there is an adjacent pyrimidine base available for photoreaction on the other strand of the helix (5). In contrast, the pyrone side monoadduct cannot be driven to a crosslink because it does not absorb in the near UV (6). All of these adducts are photoreversed by far UV in a wavelength dependent fashion (7).

These two types of DNA lesions lead to important genotoxic effects when induced in living organisms (8). Like most physical and chemical carcinogens interacting primarily with DNA, psoralen adducts are mutagenic (9). The elucidation of mutagenic mechanisms is a central task for the understanding of carcinogenesis (10), heritable diseases (11), and evolutionary processes (12). Results of studies on the specificity of a particular mutagen may yield insights into the nature of the premutational lesion as well as the molecular mechanisms of the mutagenic pathway involved. Mutations are induced by psoralen adducts in a wide variety of biological systems but seemingly conflicting conclusions have been reported for the respective contributions of crosslinks and monoadducts in both wild type and repair deficient background (13,14,15,16). Early studies by Drake and Mc Guire (17) employing the rII gene of bacteriophage T4 indicated the preferential induction of transitions relative to other classes of mutagenic events. More quantitative data has been generated in recent years using bacterial systems, such as those involving the M13 lac Z gene (18) and the lac I gene of *E. coli* (19,20) in combination with DNA sequencing technologies. These studies have shown that psoralen mutations appear to be targeted and that both tranversion and frameshift events are induced. However, since these approaches utilize randomly modified DNA, it is difficult to precisely correlate the type of mutation with the type of psoralen adduct.

We have felt that the genetic and biochemical analysis of the mutagenic process would be more straightforward if DNA vectors containing a single well defined site of damage were available to unambigously establish a cause and effect relationship between a lesion and any subsequent mutations. The well understood photochemistry between psoralens and DNA has enabled us to construct a double stranded circular DNA containing a HMT

thymidine monoadduct specifically placed in a non-essential part of the lac Z gene carried by M13mp19 DNA. Upon irradiating with 320 - 380 nm light, the furan side monoadduct can be converted into an interstrand crosslink. Taking advantage of having two well defined DNA molecules bearing either a HMT monoadduct or a HMT crosslink, we have determined the transfection efficiencies of these molecules in various recipient bacteria and characterized the type and frequency of mutagenic events induced by the two types of adducts.

MATERIALS AND METHODS.

Chemicals.

Unlabeled deoxynucleoside triphosphates and M13 sequencing primers were purchased from Pharmacia. (α-^{32}P) dATP was obtained from Amersham. (^{3}H) HMT and unlabeled HMT were gifts from HRI Associates (Berkeley, CA). All other chemicals were reagent grade.

HMT-monoadducted oligonucleotides.

A DNA oligomer (5' GCTCGGTACCCGG 3') complementary to the Kpn I site of M13mp19 was photochemically modified with HMT on the 3' side of the central thymidine as described by Gamper *et al.* (21). The photoproducts were resolved by electrophoresis on a 20% polyacrylamide-7M urea gel, extracted into 1mM EDTA-1OmM NaCl and then ethanol precipitated. HMT furan side and pyrone side monoadducted oligomers were isolated for use as a two to one mixture.

Construction of the HMT modified M13 DNA.

Single stranded circular M13 mp19 DNA was mixed with a five fold molar excess of unmodified or HMT modified oligomer and incubated at 37°C for 20 min. The resulting hybrids served as templates for minus strand synthesis during in the presence of T_4 43,45 and 44/62 gene proteins, rATP and dNTP's for 30 min at 37°C (22). The nicked double stranded circular product was ligated with T_4 DNA ligase, extracted with phenol, chloroform-isoamylalcohol (24/1,v/v), ether and precipitated with ethanol Covalently closed DNA was isolated using the Exonuclease III - BND cellulose procedure of Gamper *et al.* (23). For the preparation of crosslinked M13mp19 DNA, the HMT monoadducted DNA was driven to the crosslinked

form prior to the BND cellulose (Benzoylated naphtoylated DEAE cellulose) extraction step. Irradiation of the DNA was carried out for 5 min at 4°C with 400 mW/cm^2 of 320-400 nm light from an elliptically focused 500W Hg/Xe arc lamp. Non crosslinked DNA was irreversibly denatured by a denaturation-renaturation treatment carried out in phosphate buffer (21). The denatured DNA was then removed in the BND-cellulose extraction step. Control M13mp19 DNA was obtained by using an intact oligonucleotide as primer for the minus strand synthesis. The evaluation of the contaminating unmodified or monoadducted DNA in the crosslinked DNA preparation was done by restricting DNA with Bgl II, end labeling with T4 polynucleotide kinase in the presence of (γ)-ATP and electrophoresis on 1% alkaline agarose gel. After autoradiography, the gels was sliced and counted to evaluate the percentage of contaminating material in the crosslinked DNA preparation.

Culture, transfection and phage plating.

E. coli K12 JM105 (lac, pro,sup E,thi, str A, sbcb 15, hsd 124,F tra D36, pro AB, lac Iq Zm 13, 24) K12 AB1157 (arg E3, his 4, leu 6, pro A2, thr-1, str-31, gal K2, lac Y1, xyl-5, ara- 14, tsx-33, sup E44, thi-1) and K12 AB 1886 (UvrA6, an isogenic derivative of AB 1157, 25) were grown in YT medium (26). For the transfection procedure, the cells were resuspended in 50 mM $CaCl_2$ and incubated 40 min at O°C with the site specifically modified M13mp19 DNA. The cells were then plated on YT agar containing 0.07% 5-bromo, 4-chloro, 3-indolyl β -D-galactopyranoside (X-gal) and 0.7 mM isopropyl β-D- thiogalactopyranoside (IPTG). Inactivating mutations in the lacZ gene of M13mp19 gave rise to light blue or colorless plaques in the selection medium. Mutant phage were plaque purified on YT agar containing X-gal and IPTG. The remaining blue plaques from the initial transfection were pooled and used to infect *E. coli* K12 JM105. After 5 hours at 37°C in liquid culture, M13 mp19 replicative form was extracted and its infectivity determined before and after restriction with Kpn I at 37°C for 2 hours. The Kpn I restricted M13mp19 RF DNA was then used for a second round of transfection of *E. coli* K12 JM105 at 0°C for 40 min. Cells were plated on agar containing X-gal and IPTG and the phage were plaque purified on the same medium. Pure mutant clones were grown 5 hours at 37°C in liquid

culture on *E. coli* K12 JM105. Phage were precipitated from the culture supernatant by 5% polyethyleneglycol-0.5 M NaCl. The single stranded M13 DNA was purified by extraction with phenol and chloroform prior to being sequenced.

Frequency of mutation.

The frequency of mutations induced during the initial round of transfection of repair proficient or deficient *E. coli* cells was determined in two steps. We have determined the frequencies of either the frameshift mutations and the large deletions using simply the lac Z forward mutation assay. To these values, we have added the frequencies of mutations due to base substitutions which have been obtained simply by sequencing the DNA of the lac Z+ phage issued from the second round of transfection.

DNA sequencing.

M13mp19 DNA was sequenced according to the dideoxynucleotide procedure described by Sanger *et al.* (27). Two 17 base long primers having the sequences 5' GTTTTCCCAGTCACGAC 3' and 5' CGCACTCCAGC-CAGCTT 3' were utilized. The sequencing products were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. Autoradiography was carried out at -20°C with Fuji X ray films.

RESULTS.

Synthesis of M13 DNA with site-specifically placed HMT adducts.

In the presence of all four dNTP's and rATP, the T4 DNA polymerase holoenzyme catalyses synthesis of the M13 minus strand using hybridized oligonucleotide as a primer. This synthesis provides a nearly quantitative yield of form II DNA. Relaxed covalently closed circular DNA in 40 to 80 % yield is obtained after ligation with T4 DNA ligase (Fig. 1A). The presence of an HMT monoadduct on the 3' side of the central thymidine of the priming oligonucleotide (Fig. 1B) does not interfere with the annealing, the synthesis or the ligation procedures. This is expected since a previous study had indicated that stability of the DNA helix is only slightly altered by the presence of a HMT furan side or pyrone side monoadduct (28). The site specifically placed furan side HMT monoadduct is converted to an interstrand crosslink by irradiation with 320-400 nm light. Purification of

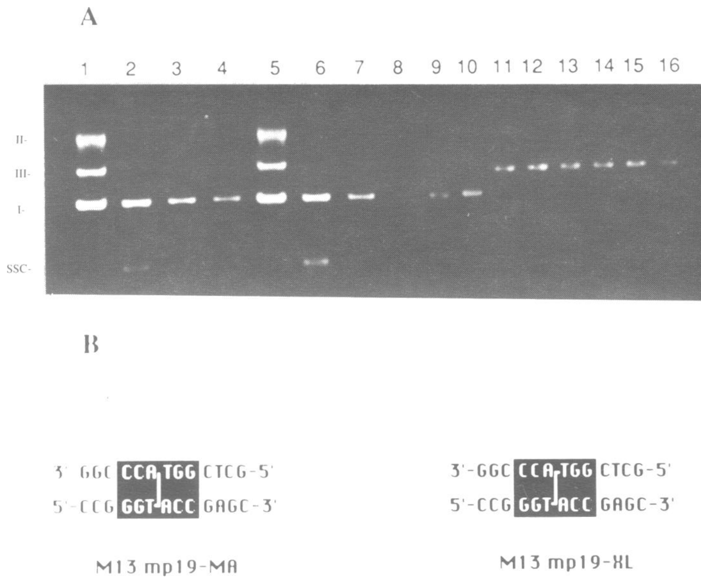

Figure 1. Preparation and characterization of M13mp19 DNA site-specifically modified by HMT

A. 1% Agarose gel electrophoresis performed in the presence of 1 µg/ml ethidium bromide. Analysis of unmodified (lanes 1 to 3), monoadducted (lanes 5 to 7) and crosslinked M13mp19 DNA (lane 8). Lanes 1 & 5: Oligonucleotide extension by T4 replication complex. Lanes 2 & 6: After ligation and digestion by Exonucease III. Lanes 3,5 and 8 : A BND cellulose purification. Lane 4: M13mp19 DNA standards including the single stranded circle and forms I, II and III of the double stranded replicative . intermediate. Lanes 9 & 10: monoadducted and crosslinked DNA digested by Kpn I. Lanes 11 & 12: same DNA but digested by Eco R I. Lanes 13 & 14: same DNA but digested by Sst I. Lanes 15 & 16: same DNA but digested by Sma I.
I, II, III &ssc indicate the positions of supercoiled, relaxed, linear double-stranded M13mp19 DNA and single-straneded circular m13mp19 DNA respectively.

B. Location of the HMT monoadducts and crosslink inserted into the Kpn I site of M13mp19 DNA

the crosslinked product is based on its renaturability after transient exposure to alkaline pH. Contaminating non crosslinked DNA, primarily pyrone side HMT monoadducted form I molecules, is irreversibly denatured by this treatment and removed by extraction with BND cellulose in 1 M NaCl.

The presence of a psoralen adduct at the Kpn I recognition sequence has been verified by restriction analysis (Fig. 1A). Both the HMT crosslinked and the mixed population of furan side (70%) and pyrone side (30%) HMT monoadducted M13mp19 DNA are resistant to Kpn I restriction thus confirming the presence of an HMT adduct at the central 5'TpA3' site. Neither the monoadducts nor the crosslink have an effect on Eco RI, Sst I or Sma I dependent linearization (Fig. 1A).

Transfection efficiencies of the HMT modified M13mp19 DNA.

The biological activity of M13mp19 DNA containing a single HMT monoadduct or crosslink can be evaluated by transfection of competent *E. coli* K12 cells. HMT monoadducted M13mp19 DNA has a transfection efficiency about 60% of that exhibited by the unmodified DNA in either *E. coli* K12 JM105 or *E. coli* K12 AB1157 (Table I). Induction of the SOS network by pre-irradiating the host cells with far UV results in a partial reactivation of the monoadducted DNA. The use of *E. coli* K12 AB 1886, which is *UvrA* deficient, as a host for the transfection, significantly reduces the viability of the modified DNA and is consistent with an increased lethality of the HMT monoadducts in absence of an excision repair pathway. A reactivation is observed when the SOS response is induced in this repair defient host (Table I).

Crosslinked M13mp19 DNA transfects *E.coli* K12JM105 with an efficiency of 1.2×10^3 pfu per µg of DNA (Table I, Fig.2). This value is nearby two orders of magnitude lower than what is obtained with unmodified DNA. Given that the HMT crosslink functions as an absolute block to DNA replication (29) and RNA transcription (30) and that its primary mode of repair requires the presence of homologous undamaged DNA (31), a site-specifically placed psoralen diadduct could conceivably be lethal in every instance. If so, the observed biological activity of the crosslinked phage DNA would probably be attributable to contaminating unmodified or monoadducted DNA. To evaluate this possibility, an aliquot of the crosslinked DNA was restricted with Bgl II, end labeled, and electrophorezed through a denaturing gel. Densitometric analysis and counting indicated that at least 99.5% of the DNA was site-specifically crosslinked. Based on the transfection frequencies reported here for

Table I: M13 mp19, M13 mp19-MA and M13mp19-XL transfection efficiencies (pfu/μg) measured on various *E. coli* K12 cells.

	JM 105	AB 1157	UV treated AB 1157 (100 J/m^2)	AB 1886	UV treated AB 1886 (10 J/m^2)
M13 mp19	$0.72\pm0.20\times10^5$	$0.28\pm0.06\times10^5$	$0.27\pm0.08\times10^5$	$0.27\pm0.07\times10^5$	$0.28\pm0.08\times10^5$
M13 mp19-MA(1)	$0.43\pm0.08\times10^5$	$0.16\pm0.02\times10^5$	$0.25\pm0.03\times10^5$	$0.06\pm0.01\times10^5$	$0.08\pm0.01\times10^5$
M13 mp19-XL(2)	$1.2+0.2\times10^3$	$0.5\pm0.1\times10^3$	$1.3\pm0.2\times10^3$	N.D(3).	N.D(3).

(1): Monoadducted M13mp19 DNA, (2): crosslinked M13mp19 DNA, (3): Not Done.

unmodified and monoadducted DNA, it is unlikely that the transfection activity is due to non-crosslinked DNA.

Mutagenicity of the HMT monoadduct and crosslink.

Inactivation of the phage DNA by HMT is accompanied by an increase in the mutation frequency in the surviving viral population as scored using the lac Z forward mutation system (32). M13mp19 is a lac Z hybrid phage containing the M13 genome together with a cloned insert encoding the regulatory region and part of the lac Z gene (the α-peptide) of *E. coli* (33). Functional β-galactosidase is produced upon infection by protein complementation between the amino portion of the enzyme encoded by the lac Z gene of the phage and the carboxy portion provided by the host bacteria. Because the HMT adducts are located in a non essential part of the lac Z gene, only frameshift mutations or large deletions lead to an inactivation of the α-complementing activity. Mutations due to base substitutions at the Kpn I insertion site are detected after pooling the phage which express an active β- galactosidase in *E. coli* K12 JM105 and extracting the M13mp19 replicative form I. The DNA is restricted with Kpn I and the resistant form I DNA is used to transfect *E. coli* K12JM105 a second time. Clones expressing an inactive β-galactosidase together with those issued from the second round of transfection are sujected to DNA sequence analysis.

Table II summarizes the data obtained after sequencing the DNA of these clones. The types and frequencies of nucleotide changes due to the presence of either a monoadduct or a crosslink are enumerated.

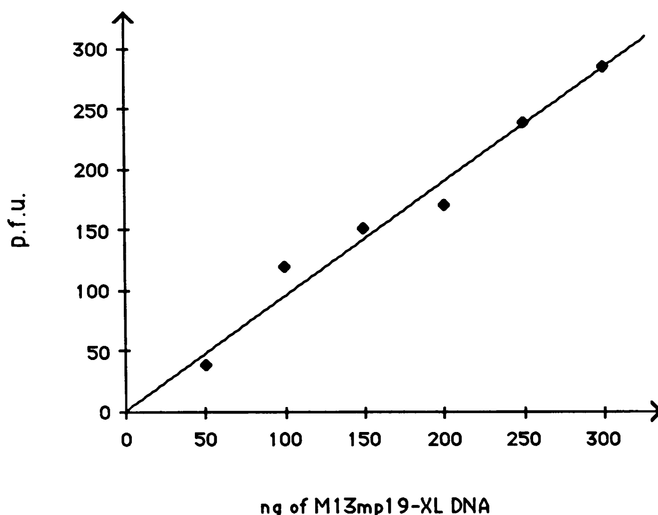

Figure 2. Plaques obtained after transfection of *E. coli* K12 JM105 by M13mp19 DNA containing an HMT crosslink.
The number of pfu detected after the transfection of 6×10^8 $CaCl_2$ treated *E. coli* K12 JM105 is plotted *vs* the amount of crosslinked DNA used.

Spontaneous lac Z defective phages (mutation frequency = 3×10^{-4}) have been characterized after transfection of *E. coli* K12 JM105 with unmodified M13mp19 DNA and are attribuable to single base transitions (G:C to A:T) occuring at position 6364 of the lac Z structural gene or to deletions eliminating the central part of this gene. Single base deletions in repetitive tracts of G:C have also been detected in both the regulatory and the structural part of the lac Z gene (34). This type of frameshift deletion is observed between positions 6272 and 6274 (Table II) and may be due to a spontaneous slippage of DNA strands within this tract at the time of replication (35). With unmodified M13mp19 DNA no mutations are observed at the Kpn I 5'TpA3' locus.

The most common mutation induced by both monoadduct and crosslink is deletion of the thymidine residue bearing the HMT adduct. The frequency of this mutation is actually doubled when the monoadducted DNA is transfected into an *Uvr A* deficient host. This can be simply explained by an increase in the residence time of the monoadducts in the bacteria. On

Table II: Mutations induced by site-specifically placed HMT adducts.

			M13mp19-XL +*E.coli*JM105		M13mp19-XL +UV treated *E.coli*JM105		M13mp19-MA +*E.coli* AB1157		M13mp19-MA +*E.coli* AB1886	
Class of mutation	Position	Type	Number	MF*	Number	MF	Number	MF	Number	MF
Base substitution	6275	T:A–>G:C	2	1.6×10^{-4}	10	5.6×10^{-4}	3	0.5×10^{-4}	4	1.8×10^{-4}
	6276	A:T–>T:A	0	-	2	1.1×10^{-4}	0	-	0	-
	6277	C:G–>A:T	0	-	1	0.5×10^{-4}	0	-	0	-
	6275	T:A–>A:T	0	-	0	-	0	-	1	0.45×10^{-4}
One base deletion	6275	-T	20	16.0×10^{-4}	27	15.2×10^{-4}	45	7.5×10^{-4}	39	17.7×10^{-4}
	6276	-A	0	-	3	1.7×10^{-4}	0	-	0	-
	6272–6274	-G	2	1.6×10^{-4}	6	3.4×10^{-4}	0	-	0	-
Large deletion	6020 to 6469		0	-	3	1.7×10^{-4}	0	-	0	-
	6125 to 6520		0	-	1	0.5×10^{-4}	0	-	0	-
Total mutations			24	19.2×10^{-4}	53	29.7×10^{-4}	48	8×10^{-4}	43	20.0×10^{-4}

*: Mutation frequency (mean relative error=25%)

the other hand, UV irradiation of the host bacteria prior to transfection with the crosslinked DNA, increases the overall mutation frequency without changing the frequency of the thymidine deletion (Table II). Base sustitutions and especially the T:A to G:C transversion are also observed but the frequency of this substitution is always several times lower than the frequency of the thymidine deletion. These two types of mutations account for the majority of the targeted changes induced by either a HMT monoadduct or crosslink. For reason previously mentioned this similarity is not expected since some DNA in the crosslinked preparation may contain a monoadduct.

The situation is somewhat changed when the error-prone repair pathway is induced in the host bacteria prior to transfection with site-specifically crosslinked M13mp19 DNA. Induction of the SOS network increases the frequencies of the mutations already observed in a non induced state as well as promoting other types of mutational changes (Table II). For example, the adenine residue at position 6276 which is

coupled to one of the two crosslinked thymidines is deleted with a frequency of $1.7x10^{-4}$. Targeted A:T to T:A transversions at position 6276 and a C:G to A:T transversion at position 6277 are also observed. The frequency of untargeted mutations is also increased. For instance, several example of a single guanine deletion situated in a run of guanine residues between positions 6272 and 6274 have been sequenced. In the presence of the SOS functions, two large deletions which eliminate half of the lac Z gene have also been detected. As expected these deletions abolish β-galactosidase activity

DISCUSSION.

Site specific placement of psoralen monoadduct or crosslink into a viral DNA has been accomplished by (i) the preparation of an oligonucleotide containing an uniquely positioned HMT monoadduct, (ii) its hybridization to viral single stranded DNA and (iii) the use of the T4 replication complex to carry out an extremely efficient primer extension reaction. The processivity of this complex together with its ability to synthesize through secondary structure without performing strand displacement, leads to high yield of the covalently closed product in the presence of DNA ligase. The monoadducts (70 % furan-side and 30% pyrone-side) are formed on the 3' side of the thymidine located within the Kpn I recognition site of M13mp19. As previously shown (36), the 5'TpA3' sequence is at least one order of magnitude more susceptible to psoralen photoadduction than thymidine in other surroundings. The furan side monoadduct when placed into this sequence can easily be converted into a crosslink by near UV irradiation (320 to 380 nm). These molecules have been used to study the effects of psoralen and near UV irradiation on the phage progeny resulting from transfection of $CaCl_2$ treated *E. coli*. Selective placement of adducts should permit the assignment of specific biological effects to specific lesions.

The HMT modified DNA has been characterized with regard to infectivity and mutagenesis. From the results presented above, the presence of one HMT monoadduct per M13mp19 DNA is less inactivating than one HMT crosslink per DNA molecule. Indeed, both the furan side and pyrone side monoadduct can be excised from DNA by the action of *UvrABC*

excinuclease, which cuts the 8th phosphodiester bond 5' and the 5th phosphodiester bond 3' to both HMT pyrone side and furan side thymidine monoadducts (4). We have shown that a defect at the level of *Uvr A* decreases the infectivity of the monoadducted DNA thus confirming that the excision repair pathway efficiently removes these two type of adducts from DNA. Reactivation of the monoadducted DNA can be observed after a pretreatment of competent bacteria with UV light. Induction of the SOS network may increase the efficiency of excision repair or allow more frequent bypass of the lesion by *E. coli* DNA polymerase III (37).

By contrast, the HMT crosslink is higly toxic reducing infectivity of the modified DNA to just 1.6% of the control. Some of the residual infectivity may be due to a slight contamination of the crosslinked DNA with monoadducted or unmodified M13mp19. Indeed, the primary pathway for HMT crosslink repair consists of three sucessive steps: (i) an incision of one strand on both sides of the diadduct by the *UvrABC* excinuclease, (ii) a rec A directed recombination event involving the participation of an undamaged DNA homologous to the sequence containing the crosslink, and (iii) a second pair of *UvrABC* excisions on the remaining damaged strand followed by replacement synthesis and ligation. It is difficult to envision homologous recombination between two crosslinked DNA. The results presented here suggest the existence of alternative error prone repair pathways which do not involve recombination. For instance, Zhen *et al.* (38) have proposed a pathway based upon the activity of a novel glycosylase while Yatagi *et al* (39) have suggested a pathway in which the recombination step is substituted by a gap filling reaction catalyzed by DNA polymerase I in the presence of DNA helicase II. As is the case with monoadducted DNA, the induction of SOS funtions in the host bacteria improves the infectivity of the crosslinked DNA. The SOS network controls the expression of genes whose products are known to play roles in the repair of many different types of DNA lesions such as thymidine dimers and mixed population of psoralen crosslinks and monoadducts (40). The reactivation observed here may simply be due to a higher level of expression of the enzymes involved in excision and recombinational repair.

The main goal of this study has been to characterize the types and relative frequencies of nucleotide changes induced by the HMT monoadducts

and crosslink. M13 lac Z phage has been used because it permits the detection of forward mutations in a non essential gene without selection pressure. Furthermore, the adducts are situated in a 5'TpA3' sequence which is not prone to spontaneous mutations (34) thereby allowing the unambiguous correlation of nucleotide changes with the type of HMT adduct. Of the mutational events sequenced, deletion of the modified thymidine is the most frequent change elicited by either a monoadduct or a crosslink. This is in agreement with Yatagi *et al.* (39) who observed a high frequency of thymidine deletion in the lac I gene of *E. coli* after random modification with 8-methoxypsoralen. Our current understanding of frameshift mutagenesis suggests that single base deletions are the result of misalignments occuring during replication of direct repeats or runs (35). The deletion observed here at the 5'TpA3' site does not occur in a run or as part of a direct repeat, and so cannot be explained on the basis of a misalignment. This strongly suggests that the mutation is a direct consequence of replication past the lesion or its misrepair. For the monoadduct in particular, it is quite possible that the adducted thymidine can be displaced from its template position and not copied by DNA polymerase I. The other type of frameshift deletion detected in this study is the loss of a guanine which occurs in a run of G's located near the 5' side of the 5'TpA3' locus. Since this type of mutation has already been detected among spontaneous M13mp19 mutants, it is highly likely to be due to a misalignment of the growing DNA irrespective of the presence of a HMT adduct.

The second most common mutation observed is a T:A to G:C transversion at position 6275. The relative frequency of this mutational event compared to the thymidine deletion remains almost constant regardless of whether the lesion is a crosslink or a monoadduct. Base transversions at A:T sites have already been reported for DNA randomly modified by HMT (18), 8-MOP (19) and angelicin (20). Several authors have reported that psoralens can induce base transitions and transversions and that these substitutions can be sequence dependent (41,19). A base mispairing model has been proposed to account for the T:A to G:C transversion (18). In brief, during replication the monoadducted thymidine can rotate 180° about its glycosidic bond forming the monoadducted *syn*-thymidine. Cytosine is preferentially

incorporated opposite this base since only the T:C couple can both accomodate the methyl group on the adducted thymidine and maintain a hydrogen bond. The conversion of the T:C couple to a G:C base pair is done during the second round of replication. This model is also applicable to T-T crosslinks after repair processes have excised one of the modified thymidines (18).

The pattern of mutations recorded after transfection with the monadducted DNA is essentially unchanged when the excision repair pathway is inactivated in the host cells. As expected this repair is error free and the nucleotide changes that do occur probably result from bypass synthesis by DNA polymerase I or III. Induction of the SOS functions in host bacteria prior to transfection with HMT crosslinked M13mp19 increases the mutation frequency and permits the characterization of additional types of mutagenic events. Targeted mutations such as deletion of adenine and A:T to T:A transition are observed at position 6276. In addition, an infrequent C:G to A:T transversion at position 6277 is obtained along with two large deletions. The SOS repair network inhibits the proof reading activity of DNA polymerase III enabling error prone bypass synthesis to take place (37,42). Although base substitutions are enhanced by the SOS response, base deletion events are only weakly increased. Large deletions, which are undetectable using non induced bacteria, are rather frequent using induced bacteria. The explanation for this phenomenon is unknown but may be due to an increase in the frequency of spontaneous untargeted mutations which are too infrequent to be detected using non induced host cells.

As demonstrated in this and in previous studies (43,44), the use of site-specifically modified viral or plasmid DNAs to characterize a mutation spectrum is a powerful technique. Mutagenesis originating from a site-specifically placed 2-acetyl-aminofluorene adduct to guanosine has been described (45,46). The mutations are targeted and consist of transversions or single nucleotide deletions when the modified plasmid is introduced into bacterial or mamalian cells . A pUC19 plasmid containing a site-specifically placed 4,5',8-trimethylpsoralen crosslink has been used to transform *E. coli* (38). Only two mutants were sequenced and each consisted of a A:T to T:A transversion. The infrequency of these

substitution in the study reported here may reflect a variation of the mutation spectrum with the psoralen derivative employed as well as with the DNA sequence surrounding the adduct.

In conclusion, this paper analyses the lethality and the mutagenesis due to site specifically placed HMT adducts. The availability of these substrates has permitted a clear demonstration that crosslinks are far more lethal than monoadducts and that both the monoadducts and the crosslink promote the same kind of nucleotide changes, i.e. a deletion or a transversion of the modified thymidine at the 5'TpA3' sequence. Viability of the site-specifically croslinked phage DNA suggests the existence of one or more error-prone non recombinational repair pathways for the crosslink. Lastly, additional types of mutations are detected when the crosslinked DNA is exposed to the SOS repair functions.

ACKNOWLEDGEMENTS.

J.P. is a research associate supported by the National Fund for Scientific Research (NFSR, Brussels, Belgium). This work was made possible by a NFSR research grant, by the US Department of Energy.under contract number DE-AC030-65SF00093, and by the US National Institutes of Health through grant number GM 11180.

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