Mutation Spectra of M13 Vectors Containing Site-Specific Cis-Syn, Trans-Syn-I, (6−4), and Dewar Pyrimidone Photoproducts of Thymidylyl-(3′→5′)-Thymidine in *Escherichia coli* under SOS Conditions†

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ABSTRACT: The mutation spectra of cis-syn, trans-syn-I, (6−4), and Dewar pyrimidone photoproducts of the TT site of AATTAA and TATTAT in the (−) strand of a heteroduplex M13 vector were obtained in an excision and photoreversal repair deficient *Escherichia coli* host under SOS conditions. Oligonucleotides containing site-specific photoproducts were annealed to a complementary uracil-containing (+) strand that contained one or more unique pairs of nucleotide mismatches and used to prime (−) strand synthesis with a DNA polymerase and dNTPs. Following DNA synthesis, the reaction mixtures were incubated with T4 DNA ligase and ATP and then used to transfect SOS-induced competent CSRO6F′ cells (*uvr*A6 and *phr*-1). The transfectants were plated, gridded, and probed by oligonucleotides specific for progeny of the (−) and (+) strands. Individual progeny of the photoproduct-containing (−) strands were plaque purified and sequenced by the dideoxy method. The cis-syn and trans-syn-I dimers were found not to be very mutagenic (<9%), the Dewar product more so (<33%), and the (6−4) product the most mutagenic (<73%). The mutation spectra were similar to those previously reported for the same photoproducts of the TT site of AGTTGG in the (+) strand of an M13 vector [Lawrence, C. W., et al. (1990) *Mol. Gen. Genet. 222*, 166−168; LeClerc, J. E., et al. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9685−9689] except that −1 deletion mutations were not observed for the trans-syn-I photoproducts, and a lower frequency of 3′-T→C mutations was observed for the (6−4) photoproduct. Evidence that a small percentage of (+) strand repair of a double mismatch to the 3′-side of the photoproduct site was obtained from transfection experiments in which a second double mismatch was introduced opposite or flanking the photoproduct. Analysis of the minor tandem mutations induced by the (6−4) and Dewar products suggests that the SOS polymerase complex is able to elongate what amounts to double mismatches opposite these photoproducts and is consistent with the action of a highly processive polymerase that lacks proofreading ability.

Ultraviolet light (UV) is one of the most mutagenic agents known and is the probable cause of most skin cancers. The majority of UV-induced mutations occur at dipyrimidine sites which are the major sites of DNA photoproduct formation [for reviews, see: Patrick and Rahn (1976), Cadet and Vigny (1990), and Taylor (1994, 1995)]. The cis-syn cyclobutane dimer and the (6−4) product are the major products of UVB and UVC[1] irradiation of duplex DNA (Figure 1), and both have been correlated with UV-induced mutations at these sites [for a review, see Hutchinson (1987)]. Trans-syn dimers are minor photoproducts of DNA and are formed in greater quantity in single-stranded DNA (Liu & Yang, 1978; Kao et al., 1993). The (6−4) product, while being chemically stable, is converted to its Dewar valence isomer by UVA and UVB light (Taylor & Cohrs, 1987), while cytosine-containing photoproducts can spontaneously tautomerize and deaminate [for a discussion, see: Jiang and Taylor (1993)]. Which of these photoproducts is primarily responsible for a mutation at a given site, and the precise mechanisms by which the mutations arise remain unknown. Elucidating the precise structure−activity relationships in UV-mutagenesis is complicated by competition between further chemical transformations, repair, and replicative bypass of the different photoproducts. It is probably the modulation of the rates of these processes by the flanking DNA sequence that results in a lack of correlation between photoproduct hot spots and mutation hot spots (Brash et al., 1987).

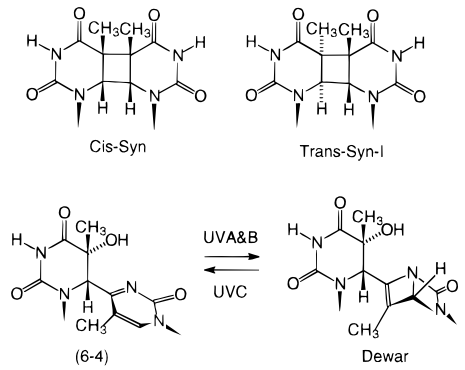

FIGURE 1: Photoproducts of a TT site.

Great progress has recently been made in sorting out DNA photoproduct structure−activity relationships by determining the mutation spectra bacteriophage vectors containing site-specific photoproducts in both bacterial and eukaryotic hosts.

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[1] Abbreviations: ATP, adenosine triphosphate; dNTP, 2′-deoxynucleotide triphosphate; exo⁻, 3′→5′ exonuclease deficient; NMR, nuclear magnetic resonance; RF, replicative form; TpT, thymidylyl-(3′→5′)-thymidine; UVA, 320−400 nm; UVB, 280−320 nm; UVC, 240−280 nm.

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By using single-stranded vectors, Lawrence and co-workers have determined the mutation spectra of the cis-syn, a trans-syn, (6−4), and Dewar photoproducts of TT, the cis-syn and a trans-syn dimer of UU (the complete deamination product of the cis-syn dimer of CC), and the (6−4) and Dewar products of TC in the sequence AGxyGG in both *Escherichia coli* (LeClerc et al., 1991; Gibbs & Lawrence, 1993) and in yeast (Gibbs et al., 1993; Lawrence et al., 1993; Horsfall & Lawrence, 1994). Recently, we reported the mutation spectra for the cis-syn dimers of TT and TU (the deamination product of the cis-syn dimer of TC) in the sequence TAxyAT (Jiang & Taylor, 1993) that were obtained by a modification of Kunkel's method for site-directed mutagenesis (Kunkel, 1985). The major conclusion from these limited studies has been that the cis-syn and, to a slightly lesser extent, the trans-syn photoproducts are highly instructive lesions because they direct the incorporation of two A's with a much higher frequency than seen for abasic sites, the prototypical non-instructional lesion. On the basis of the work of Lawrence and co-workers, the (6−4) product is also instructive by these criteria as it primarily directs the incorporation of G opposite the pyrimidone ring rather than an A that would have been predicted to be primarily incorporated opposite a noninstructional lesion (LeClerc et al., 1991). In contrast, the Dewar product appears to be the least instructive of the photoproducts with the Dewar valence isomer of the 3′-pyrimidone subunit behaving more like an abasic site. What is known from these limited studies, however, is the extent to which the mutation spectrum of an individual photoproduct depends on sequence. Herein, we report the mutation spectra for M13 clones containing site-specific cis-syn, trans-syn-I, (6−4), and Dewar photoproducts of TT in two different sequence contexts in *E. coli* under SOS conditions. We show that though the mutations induced are similar to those previously observed in different sequence contexts, there are some differences that may have important mechanistic implications.

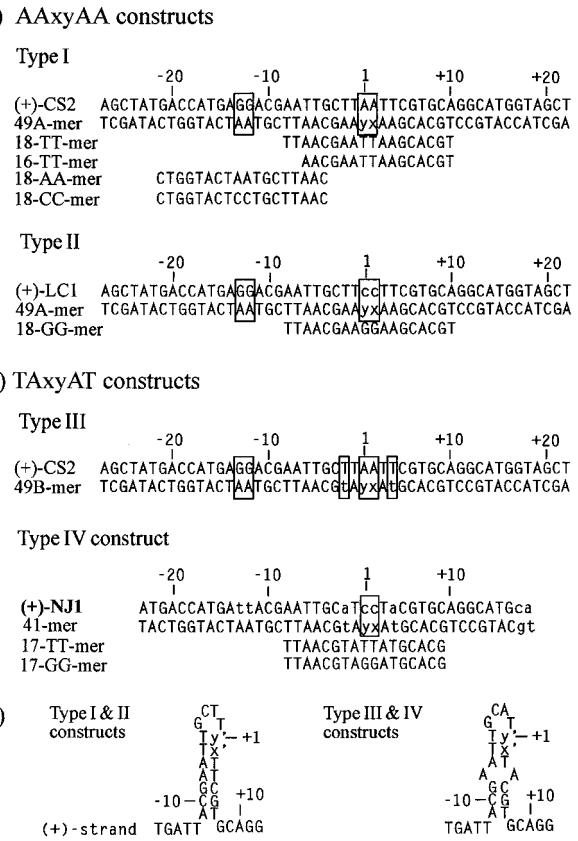

FIGURE 2: Oligonucleotides and vectors used in this study where (+) refers to the (+) strand of the M13mp18 clone used as the template in the synthesis of the heteroduplex RF bacteriophage DNA and xy represents the dipyrimidine photoproduct in the oligonucleotide used to prime the synthesis of the (−) strand. (A) Constructs of the AAxyAA sequence context, (B) constructs of the TAxyAT sequence context, and (C) Potential hairpin site in the (+) strand of the progeny phage of the two sequence contexts. The (+) strand is written 5′→3′, and all other oligonucleotides are written 3′→5′ where nucleotides in lower case are those that are different from those in the type I construct and where mismatched sites are enclosed in boxes.

MATERIALS AND METHODS

Cis-syn, (6−4), and Dewar photoproduct-containing 49A-mers of >95% purity were prepared as previously described (Smith & Taylor, 1993). The trans-syn-I 49A-mer was prepared from d(AAT[t,s-I]TAA) that was purified by HPLC from the sensitized irradiation products of the parent oligonucleotide and characterized by NMR spectroscopy. The (6−4) and Dewar 49B-mers were prepared in a similar fashion from d(GTAxyATG) (Zhao et al., 1995). The trans-syn-I dimer-containing 49B-mer was prepared from d(GTAT[t,s-I]TATG) that was synthesized with the trans-syn-I building block (Taylor & Brockie, 1988). All other materials and methods used are as previously described (Jiang & Taylor, 1993) unless otherwise indicated. Because of the methods used to prepare and purify the photoproduct containing oligonucleotides, none of the samples used in mutagenesis experiments contain the undamaged parent oligonucleotide. The <5% impurities in the (6−4) and Dewar samples are expected to be each other and co-eluting photoproducts, and, in the cyclobutane dimer samples, they are expected to be other cyclobutane dimers.

Preparation of the M13 Vector. The 26-mer duplex d(AATTGCTTAATTCGTGCAGGCATGGT)·d(AGCTACCATGCCTGCACGAATTAAGC) was cloned into *Eco*RI and *Hin*dIII cleaved M13mp18, and the desired clones were identified by colony hybridization with radiolabeled 18-AA-mer (Figure 2A), plaque purified, and sequenced. Mutation of the TT site at −13 and −12 to GG was accomplished via Kunkel's method for oligonucleotide-directed mutagenesis (Kunkel, 1985) utilizing the 18-CC-mer (Figure 2A) to prime (−) strand synthesis on the uracil-containing single-stranded (+)-TT bacteriophage that was isolated from the *dut*⁻ *ung*⁻ *E. coli* strain CJ236. The desired clones were identified by colony hybridization to radiolabeled 18-CC-mer, plaque purified, and sequenced. Single-stranded uracil-containing bacteriophage DNA, U-(+)-CS2, was then prepared by transfection and isolation from CJ236.

Photoproduct Mutation Spectra. The undamaged and photoproduct-containing 49-mers were used to prime (−) strand synthesis on uracil-containing (+)-strands with Sequenase Version 1.0 in place of T4 polymerase as previously described (Jiang & Taylor, 1993). The crude reaction mixtures were then used to transfect competent repair deficient (*phr*-1, *uvr*A6) CSRO6F′ *E. coli* hosts. SOS-induced hosts were prepared by exposing to 254 nm radiation prior to being made competent. The cells transfected with

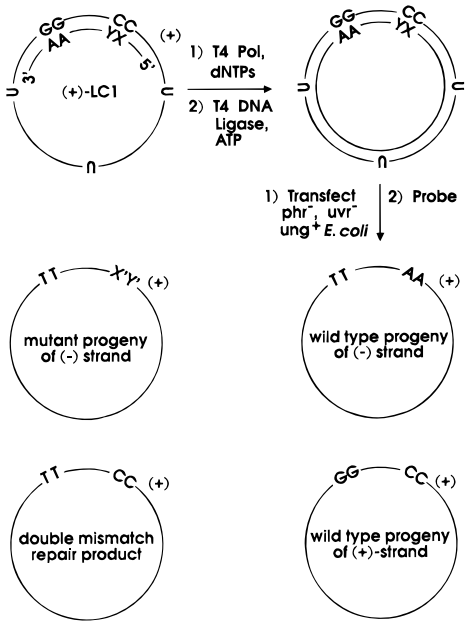

FIGURE 3: Scheme used for obtaining the mutation spectra of the type II constructs. The progeny of the (+) and (−) strands were distinguished by probing with 18-GG-mer and 18-AA-mer. The (−) strand directed double mismatch repair product was identified by its ability to hybridize to both probes. Attempts to distinguish wild-type and mutant progeny of the (−) strand by probing with either the 16-TT-mer or 18-TT-mer failed, possibly due to the formation of a hairpin (see Figure 2C), and therefore randomly selected (−) strand progeny were sequenced to obtain the mutation spectra. Mutation spectra of the other constructs were obtained by a similar strategy, except that repair products could not be identified in the type I constructs.

the type I constructs were plated, and the plaques were screened for progeny of the (+) strand by hybridization with 18-CC-mer and for progeny of the photoproduct-containing (−) strand by hybridization with 18-AA-mer (Figure 2A). For the type II constructs, progeny of the (+) strand were identified by hybridization to the 18-GG-mer. Selected progeny of both strands were plaque purified and sequenced by the dideoxy termination method. A similar strategy was used for the type III and type IV constructs (Figure 2B). The mutation spectra for the trans-syn-I dimer containing 41-mer was obtained as previously described for the cis-syn dimers of TT and TU sites (Jiang & Taylor, 1993).

Statistical Analysis. The chi-square (χ^2) test was used to evaluate the significance of differences in mutation spectra by sorting mutations into categories for which the expected values were 5 or greater to avoid significant errors in the analysis.

RESULTS

General Strategy. The *in vivo* mutation spectra of the photoproduct-containing 49-mers were obtained by a general method previously described by us for obtaining the mutation spectra of the cis-syn photodimers of TU and TT (Jiang & Taylor, 1993). Our method (Figure 3) is an adaptation of an efficient method for site-directed mutagenesis (Kunkel, 1985; Kunkel et al., 1987) that relies on the rapid degradation of the uracil-containing (+) strand of a heteroduplex replicative form (RF) bacteriophage DNA by a uracil glycosylase active (*ung*$^+$) *E. coli* host to favor replication of the (−) strand. A hybrid RF DNA containing a site-specific photoproduct is easily and rapidly prepared by primer extension of a photoproduct-containing primer with a double mismatch on a uracil-containing (+) strand and is then transfected into an *ung*$^+$, photoproduct repair deficient (*phr*$^-$, *uvr*$^-$) host. The progeny arising from the replication of the (+) and (−) strands are easily distinguished by hybridization probes for the double mismatches. Mutants resulting from bypass of a photoproduct are identified by probing the (−) strand progeny with a wild-type probe under relaxed and stringent conditions and then sequencing any weakly hybridizing colonies.

In the present case, we have obtained mutation spectra of the cis-syn, trans-syn-I, (6−4), and Dewar photoproducts in two different sequence contexts by way of four different constructs (Figure 2). In the type I, II, and III constructs, a (+)-GG/(−)-AA double mismatch was introduced 12 and 13 nucleotides to the 3′-side of the photoproduct site as a genetic marker for the (+) and (−) strand progeny. In the type I construct, the AA that would naturally occur opposite the TT photoproduct was left intact because we felt that this might be important for future studies aimed at studying the sequence specificity of photoproduct repair, which would require the native sequence. The type II construct was the same as the type I construct, except that a CC was introduced in place of the AA opposite the photoproduct to assay for competitive repair of the flanking double mismatch site (Figure 3). In the type III and IV constructs, the photoproducts were embedded in the sequence context TAxyAT instead of AAxyAA. Because the type III construct was prepared from the same (+) strand as used for the type I construct, two T•T mismatches flanked the photoproduct site. The type IV construct was the same as that previously used to obtain the mutation spectra of the cis-syn dimers of TT and TU and had two C's in the (+) strand directly opposite the dimer site.

In Vivo Mutation Spectra. The various constructs were prepared by using the site-specifically photodamaged 49A-mer, 49B-mer, or 41-mer to prime replication of a uracil-containing (+) single-strand bacteriophage DNA, CS2, LC1, or NJ1, by Sequenase Version 1.0 and dNTPs followed by ligation with T4 DNA ligase and ATP. The uracil-containing (+) single strands were obtained from CJ236 (*ung*$^-$,*dut*$^-$) that was grown in the presence of uridine. Reaction mixtures resulting from primer-extension and ligation were used to transfect *E. coli* CSRO6F′ cells according to a standard technique (Hanahan, 1985). This strain was chosen because it is deficient in both photorepair (*phr*-1) and excision repair (*uvr*A6) of cyclobutane dimers and is infectable by M13 phage (F′) (Jiang & Taylor, 1993). The SOS response of CSRO6F′ was induced by exposing cells to 3.5−4 J m^{-2} of 254 nm light and incubating at 37 °C for 0.5 h immediately before the cells were made competent to insure sufficient time for the production of SOS proteins. Following transfection and plating, plaques started to appear 4−5 h later and were fully developed after 8−10 h. The number of plaque forming units/fmol are given in Table 1 and serve as a semiquantitative guide to the transfection efficiency of the various constructs. The transfection efficiency of the nondimer and dimer-containing vectors could not be directly compared because the efficiency of *in vitro* DNA synthesis for each primer was not quantified. No plaques were

Table 1: Results of Transfection Experiments of Photoproducts in the AAxyAA Sequence Context for the Type I Construct unless Otherwise Noted[a]

cells	substrate	pfu	(−) progeny	(−) mutants	(+) mutants	(+) GG→TT
SOS−	undamaged	80	34/100 (34)	0/15 (0)	0/5 (0)	na[b]
	cis-syn	30	10/100 (10)	0/10 (0)	0/1 (0)	na
	trans-syn-I (type II)	20	0/79 (0)	0/0 (0)	nd[c]	20/79 (25)
	(6−4)	30	10/200 (5)	0/10 (0)	0/6 (0)	na
	(6−4) (type II)	40	0/150	0/0	nd	15/150 (10)
	Dewar	7	11/200 (6)	0/11 (0)	0/2 (0)	na
SOS+	undamaged	140	163/450 (35)	0/72 (0)	0/10 (0)	
	cis-syn	110	58/306 (19)	0/58 (0)	0/12 (0)	
	trans-syn-I (type II)	60	188/468 (40)	1/83 (1)	nd	53/280 (19)
	(6−4)	60	94/550 (17)	20/60 (33)	0/20 (0)	
	(6−4) (type II)	30	54/144 (38)	34/54 (63)	nd	12/96 (13)
	Dewar	20	107/550 (19)	18/66 (27)	0/14 (0)	
	Dewar (type II)	45	42/400 (11)	14/42 (33)	nd	13/358 (4)

[a] Percentages are given in parentheses. [b] Not applicable as repair could not be assayed in this construct. [c] Not determined.

Table 2: Results of Transfection Experiments with Photoproducts in the TAxyAT Sequence Context in the Type IV Construct unless Otherwise Noted[a]

cells	substrate	(−) progeny	(−) mutants	(+) GG→TT
SOS−	undamaged[b]	99/199 (50)	0/99 (0)	na[c]
	cis-syn[b]	10/517 (2)	0/10 (0)	na
	trans-syn-I	0/354 (0)	0/0 (0)	na
	(6−4) (type III)	nd[d]	nd	nd
	Dewar (type III)	nd	nd	nd
SOS+	undamaged[b]	237/369 (64)	0/132	na
	cis-syn[b]	134/344 (39)	3/134 (2)	na
	trans-syn-I	102/691 (15)	9/102 (9)	na
	(6−4) (type III)	51/200 (26)	37/51 (73)	3/149 (2)
	Dewar (type III)	32/150 (21)	5/32 (16)	4/118 (3)

[a] Percentages are given in parentheses. [b] From Jiang and Taylor (1993). [c] Not applicable as repair could not be assayed in this construct. [d] Not determined.

observed in transfections with uracil-containing (+) strands by themselves.

Wild-type progeny of the (+) strand of the type I construct, and those that would have resulted from the (−) strand following (+) strand-directed double mismatch repair, could be readily identified by selective hybridization to the 18-CC-mer probe at either 37 or 50 °C. Progeny of the photoproduct-containing (−) strand and those that would have resulted from the (+) strand following (−) strand-directed double mismatch repair could be identified by selective hybridization to the 18-AA-mer probe (Figure 2A). Progeny of the (+) strands of the type II, III, and IV constructs could be distinguished from progeny of the photoproduct-containing (−) strands by probing with the 18-GG-mer, 18-TT-mer, and 17-GG-mer, respectively. Progeny resulting from the (+) strand following (−) strand-directed repair of the double mismatch in the type II construct could be identified as those hybridizing to both the 18-AA-mer and the 18-GG-mer. Likewise, progeny resulting from the (+) strand following (−) strand-directed repair of the double mismatch in the type III construct could be identified as those hybridizing to both the 18-AA-mer and the 18-TT-mer. Whereas few of the progeny of the photoproduct-containing strand were observed under non-SOS conditions, their numbers increased substantially under SOS conditions (Tables 1 and 2). Mutation spectra were obtained by sequencing the progeny of the photoproduct-containing (−) strands (Tables 3−6).

Table 3: Mutation Spectra of the Cis-Syn Cyclobutane Photoproduct of TT in Different Sequence Contexts under SOS Conditions Shown in Order of the Nucleotides Inserted Opposite Each T of the Photoproduct during Bypass (See Figure 6)[a]

			cis-syn		
TT→	first opp. 3′-T	second opp. 5′-T	AA−AA[b] (type I)	TA−AT[c] (type IV)	AG−GG[d]
TT	A	A	58 (100)	131 (98)	2443 (94)
CT	A	**G**		1 (1)	6 (<1)
TC	**G**	A			28 (1)
TG	**C**	A		1 (1)	
TA	**T**	A		1 (1)	130 (5)
−T		−A			1 (<1)
		total	58	134	2608

[a] Mutation-causing insertions are in bold, and relative percentages of mutants are given in parentheses. [b] The mutation spectrum for this construct may be diluted by a small amount of TT progeny arising from (−) strand directed repair of the (+) strand. [c] From Jiang and Taylor (1993). [d] From Lawrence et al. (1990) in which the photoproducts were in the (+) single strand of M13mp7L1.

Table 4: Mutation Spectra of Trans-Syn Cyclobutane Dimers of TT in Different Sequence Contexts under SOS Conditions Shown in Order of the Nucleotide Inserted Opposite Each T of the Photoproduct during Bypass (See Figure 6)[a]

			trans-syn-I		
TT→	first opp. 3′-T	second opp. 5′-T	AA−AA (type II)	TA−AT[b] (type IV)	trans-syn AG−GG[d]
TT	A	A	82 (99)	93 (91)	536 (89)
CT	A	**G**		2 (2)	4 (1)
AT	A	**T**	1 (1)	6 (6)	16 (3)
TC	**G**	A			1 (<1)
TG	**C**	A		1 (1)	
TA	**T**	A			
−T		−A			33 (6)
		other			15 (3)
		total	83	102	605

[a] Mutation-causing insertions are in bold, and relative percentages of mutants are given in parentheses. [b] From Jiang and Taylor (1993). [c] The mutation spectrum for this construct may be diluted by a small amount of TT progeny arising from (−) strand directed repair of the (+) strand. [d] From Lawrence et al. (1990) in which the photoproducts were in the (+) single strand of M13mp7L1. [e] Along with a tandem G(−32)→T.

DISCUSSION

The *in vivo* mutation spectra of the four major photoproducts of the TT site in AATTAA (type I and II constructs) and TATTAT (type III and IV constructs) that were site-

Table 5: Mutation Spectra of the (6−4) Photoproduct of TT in Different Sequence Contexts under SOS Conditions Shown in Order of the Nucleotides Inserted Opposite Each T of the Photoproduct during Bypass (See Figure 6)[a]

			(6−4)			
TT→	first opp. 3′-T	second opp. 5′-T	AA−AA[b] (type I)	AA−AA (type II)	TA−AT (type III)	AG−GG[c]
TT	A	A	40[d] (68)	20 (37)	14 (27)	16 (9)
CT	A	**G**	1 (2)			2 (1)
GT	A	**C**				2 (1)
AT	A	**T**		2 (4)		
TC	**G**	A	18[e] (30)	31 (57)	35 (69)	158 (85)
CC	**G**	**G**				2 (1)
AC	**G**	**T**	1 (2)			3 (1.5)
TG	**C**	A			1 (2)	1 (0.5)
CG	**C**	**G**			1 (2)	
TA	**T**	A		1 (2)		
−T		−A				1 (0.5)
		total	60	54	51	185

[a] Mutation-causing insertions are in bold, and relative percentages of mutants are given in parentheses. [b] The mutation spectrum for this construct may be diluted by a significant amount of TT progeny arising from (−) strand directed repair of the (+) strand. [c] From LeClerc et al. (1991) in which the photoproducts were in the (+) single strand of M13mp7L1. [d] One had an untargeted −G4. [e] One had a tandem T(−24)→C.

Table 6: Mutation Spectra of the Dewar Photoproduct of TT in Different Sequence Contexts under SOS Conditions Shown in Order of the Nucleotides Inserted Opposite Each T of the Photoproduct during Bypass (See Figure 6)[a]

			Dewar			
TT→	first opp. 3′-T	second opp. 5′-T	AA−AA[b] (type I)	AA−AA (type II)	TA−AT (type III)	AG−GG[c] (sample 2)
TT	A	A	48[d] (73)	28 (67)	27 (85)	49 (58)
CT	A	**G**	2 (3)			7 (8)
GT	A	**C**				1 (1)
AT	A	**T**	3 (5)	4 (10)	2 (6)	4 (5)
TC	**G**	A	3 (5)	3 (7)	1 (3)	11 (13)
CC	**G**	**G**	1 (2)			2 (2)
GC	**G**	**C**	2 (3)	1 (2)		1 (1)
AC	**G**	**T**		1 (2)		4 (5)
TG	**C**	A	4 (6)	3 (7)	1 (3)	2 (2)
TA	**T**	A	3 (5)	2 (5)		4 (5)
CA	**T**	**G**			1 (3)	
		total	66	42	32	85

[a] Mutation-causing insertions are in bold, and relative percentages of mutants are given in parentheses. [b] The mutation spectrum for this construct may be diluted by a small amount of TT progeny arising from (−) strand directed repair of the (+) strand. [c] From LeClerc et al. (1991) in which the photoproducts were in the (+) single strand of M13mp7L1. [d] One had an untargeted C(−43)→T.

specifically embedded in the (−) strand of an M13-derived heteroduplex bacteriophage DNA were determined in *E. coli* under both SOS and non-SOS conditions. While the mutation spectra obtained are similar to those observed by Lawrence and co-workers for the corresponding photoproducts of the TT site in AGTTGG embedded in the single (−) strand of an M13-derived bacteriophage DNA (Lawrence et al., 1990; LeClerc et al., 1991), there are some interesting and possibly significant differences.

Replication under Non-SOS Conditions. No bypass of the trans-syn-I, (6−4), and Dewar photoproduct-containing (−) strands in the type II, III, and IV constructs took place under non-SOS conditions as judged by both pfu/fmol and by the fraction of (−) strand progeny (Tables 1 and 2). The results are similar to those that we had observed in our earlier *in vivo* study with the cis-syn dimer in the type IV construct in which <3% of the progeny were from the (−) strand (Jiang & Taylor, 1993). Lawrence and co-workers also observed little replication past the cis-syn, (6−4), and Dewar photoproducts in the (+) strand of single-stranded M13 vectors in uninduced cells. In contrast, a photoproduct that they produced by sensitized irradiation of d(GCAAGTTGGAG), which had properties that led them to assign it as a trans-syn dimer, was replicated with 14% efficiency compared to 1% for the cis-syn dimer and 100% for undamaged control DNA (Banerjee et al., 1990). The trans-syn dimer used in the type II construct was also prepared by sensitized irradiation and could be assigned as the trans-syn-I dimer by comparison of its NMR to that of the trans-syn-I dimer-containing oligonucleotide used in the type IV construct and which was produced by total synthesis from spectroscopically characterized intermediates (Taylor & Brockie, 1988). Why a trans-syn dimer in their system would be so much more easily bypassed is puzzling considering that a photoproduct prepared in the same way from d(GCAAGUUGGAG), in which the methyls on the T's are replaced by hydrogens, was less easily bypassed than the cis-syn dimer of either TT or UU (Gibbs & Lawrence, 1993).

In the case of the type I constructs, a significant fraction (5−10%) of the progeny appeared to arise from the photoproduct-containing (−) strand, and none of these progeny were mutants. Unfortunately, in the type I constructs, progeny arising from replication of the (+) strand following (−) strand-directed repair of the double mismatch cannot be distinguished, but they can in the type II and III constructs. When the same trans-syn-I or (6−4) product-containing 49-mers were used in the type II constructs, no progeny of the photoproduct-containing (−) strands were observed, and instead about 10−25% of the bacteriophage corresponded to progeny of (+) strand that had undergone (−) strand-directed repair of its GG mismatch (Table 1). The same repair process was observed to occur under SOS with the type II and III constructs (Tables 1 and 2), and under these conditions none of the (−) strand progeny were found to have had their AA double mismatch repaired. It is likely that repair of the double mismatch is exclusively directed to the (+) strand from nicks (Lahue et al., 1989) that result from enzymatic processing of the U's in the (−) strand. Thus, what appeared to be progeny of error-free bypass of the photoproducts in the type I constructs under non-SOS conditions were probably progeny of double mismatch repaired (+) strands. It seems that under both SOS and non-SOS conditions, in which many types of DNA damage present significant blocks to replication, repair and replication of the uracil containing (+) strand can compete with DNA damage bypass.

Replicative Bypass of Cyclobutane Dimers under SOS Conditions. SOS induction increased the number of progeny derived from the photoproduct-containing (−) strand as well as the number of mutants produced (Tables 1 and 2). Unfortunately, the 16- or 18-TT-mer hybridization probes could not be used to distinguish between mutant and wild-type progeny in the type I and II constructs, presumably because of a stable hairpin that had been inadvertently engineered into the sequence (Figure 2C). As a result, the mutation spectra for these constructs had to be obtained by direct sequencing of the progeny. Replicative bypass of the cis-syn cyclobutane dimer in the type I construct under SOS

conditions resulted in the highly specific, and nonmutagenic, introduction of two A's (100%), though this number may be inflated by the presence of progeny of the (+) strand resulting from double mismatch repair. A similar incorporation specificity for the cis-syn and trans-syn-I dimers in the type IV construct, for which the 17-TT-mer hybridization probe could be used to screen for about twice as many mutants, presumably because of less stable hairpin formation (Figure 2C). In this sequence context, 98% nonmutagenic bypass was observed for the cis-syn dimer (Jiang & Taylor, 1993) and 91% for the trans-syn-I dimer (Table 2). Even though it was only practical to sequence a limited number of progeny, the photoproduct mutation spectra obtained were quite similar those of Lawrence and co-workers, who observed 94% and 89% nonmutagenic bypass of the cis-syn and trans-syn dimers, respectively (Tables 3 and 4). The major difference was that the cis-syn dimer in their system led to 5% of a 3′ T·A→A·T transversion, whereas this mutation was not detected in the type I construct. Less than <1% of this mutation was detected in the type IV construct, but it occurred with a significantly lower frequency than in their system ($P < 0.025$) (Jiang & Taylor, 1993).

Lawrence and co-workers also reported that the product assigned as a trans-syn dimer caused 6% of a dimer-site-directed deletion of T·A, which is significantly different from that of no deletions observed for the type III ($P < 0.05$) and IV ($P < 0.025$) constructs containing the trans-syn-I dimer (Table 2). Instead, the major mutation in the type IV construct of the trans-syn-I dimer was 6% of a 5′-T·A→A·T transversion (Table 4), which differs significantly from that of 1% observed for the trans-syn-I dimer in the type II construct ($P < 0.025$). The extent to which the possible hairpin structures (Figure 2C) may be responsible for the differences in the mutation spectra of the trans-syn dimers is unknown. It is interesting to note, however, that Lawrence and co-workers found that a photoproduct assigned as a trans-syn dimer of UU in place of the trans-syn dimer of TT did not lead to any dimer site deletions of T·A, and instead led to 9% of a 5′-T·A→C·G as the major mutation (Gibbs & Lawrence, 1993).

Base Pairing with the Cis-Syn and Trans-Syn Dimers. The low mutagenicity of bypass of cyclobutane dimers of TT observed in all sequence contexts studied can be explained by standard Watson−Crick base pairing interactions (Figure 4), except for the 5′-T of the trans-syn-I isomer which appears from modeling studies to be incapable of hydrogen-bonding interactions due to interference from the methyl group (Taylor et al., 1990). It is possible that the high degree of preference for introduction of A opposite a T in a *syn* glycosyl conformation is due to favorable van der Waals interactions between the methyl group and the H2 of adenine. Hydrophobic interactions of this sort are not possible with any of the other nucleotides when in an *anti* glycosyl orientation. On the other hand, Lawrence and co-workers did not find a significant difference in the incorporation frequency of AA opposite the cis-syn and trans-syn isomers of UU which lack the C5 methyl group (Gibbs & Lawrence, 1993). It is surprising that the few mutations induced by the cis-syn dimer occur opposite the 3′-T of the dimer when NMR studies have suggested that the N3H of the 5′-T has the weakest hydrogen-bonding interactions of the two with adenine (Kemmink et al., 1987; Kim, J.-K., et al., 1995). Comparison of the chemical shifts of a cis-syn dimer

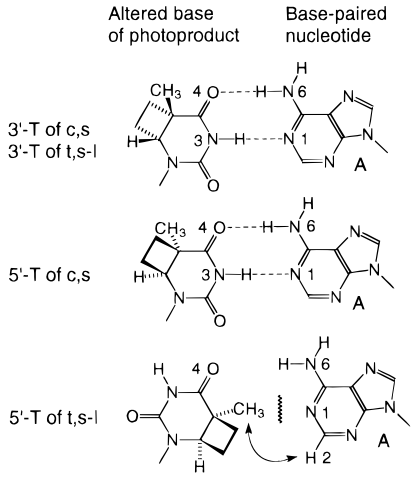

FIGURE 4: Possible base pairing interactions (dashed lines) and hydrophobic interactions (double-headed arrow) between the cis-syn, trans-syn-I, and trans-syn-II cyclobutane dimers of TpT and nucleotide triphosphates contributing to the selectivity of nucleotide incorporation.

containing duplex with the corresponding undamaged strand, however, show larger proton chemical shift differences for the section of duplex containing the 3′-T and its 3′-flanking neighbor (Taylor et al., 1990). These shift differences suggest that the 3′-end of the dimer may be distorted in such a way as to favor misincorporation in spite of favorable hydrogen bonding to an A.

Replicative Bypass of (6−4) and Dewar Products under SOS Conditions. In contrast to the low mutagenicity of the cyclobutane dimers, the (6−4) and Dewar photoproducts are much more mutagenic. The (6−4) product in the type II construct led to 57% 3′-T·A→C·G mutations which is significantly lower than that of 85% observed by Lawrence and co-workers for the same product flanked by G's instead of A's ($P < 0.005$) (Table 5). The frequency of 3′-T·A→C·G mutations in the type III construct (69%) is not significantly higher than in the type II construct ($P < 0.1$), but is still significantly lower than observed for Lawrence's construct ($P < 0.005$). In the type I construct, only 30% of the 3′-T·A→C·G mutation was observed, which can be attributed to about a 50% dilution of the progeny of the (6−4) product-containing strand by progeny of the (+) strand following (−) strand directed repair of the double mismatch in the (+) strand.

The Dewar product was less mutagenic than the (6−4) product, and in the type II construct two A's were incorporated opposite the Dewar photoproduct 67% of the time, whereas in the type III construct two A's were incorporated 85% of the time (Table 6). Though less mutagenic than the (6−4) product, the Dewar product caused a greater range of mutations than did the (6−4) product. Lawrence and co-workers had reported mutation spectra for two different samples of Dewar pyrimidone photoproduct, and our spectra most closely matches their sample 2, opposite which two A's were incorporated 58% of the time. Their sample 1 led to a much higher level of incorporation of G opposite the 3′-T of the (6−4) product than did their sample 2 (42% vs 13%), indicating that it may have contained a substantial amount of incompletely photoisomerized (6−4) product. If one assumes that their sample 2 was pure, then, in comparison, the Dewar product in the type II construct is not significantly different in mutagenicity ($P < 0.25$), whereas

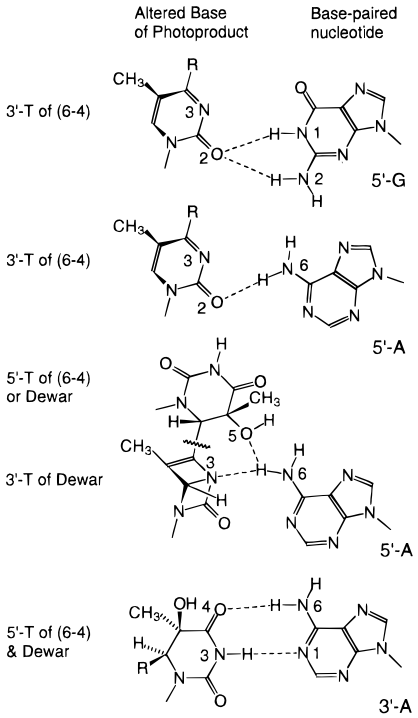

FIGURE 5: Possible hydrogen bonding interactions (dashed lines) between the (6−4) and Dewar pyrimidone photodimers of TT contributing to the selectivity of nucleotide incorporation.

the Dewar product in the type III construct appears to be significantly less mutagenic ($P < 0.005$).

Base Pairing with (6−4) and Dewar Photoproducts. There has been one reported molecular modeling study of duplexes containing the (6−4) photoproducts of all four dipyrimidine sites (Rao & Kollman, 1985). Unfortunately, the stereochemistry of C5 of the 5′-pyrimidine of the photoproducts was incorrect, and the pyrimidone subunit was much more bent than would seem reasonable from studies of the photoproduct of TpT (Taylor et al., 1988). Nonetheless, some of the features of the original modeling study are reproduced in our modeling experiments with (6−4) and Dewar products in octamers with the correct stereochemistry for C5 and a more planar pyrimidone ring in the (6−4) product. As in the original study, we find that the 5′-T seems to be able to maintain normal hydrogen bonding to A via standard Watson−Crick base pairing, which is supported by recent NMR studies of a decamer containing a (6−4) photoproduct (Kim et al., 1995). Likewise, the O2 carbonyl oxygen of the pyrimidone ring was found to be accessible for base pairing within standard Watson−Crick hydrogen-bonding distances, though with angles substantially less than the ideal value of 180°. In our models the O2 can hydrogen bond with the N1H or the N2H of an opposed G, and with less facility to the N6H of an opposed A (Figure 5). Base pairing between the pyrimidone and an A may be rather weak as suggested by the lack of evidence for hydrogen bonding between the two in the recent NMR study (Kim et al., 1995). In some of our molecular dynamics calculations, hydrogen bonding to the A opposite the pyrimidone ring was also observed to occur via the lone pair on the C5OH of the 5′-T, which would have been missed in the previous modeling study due to the incorrect stereochemistry at C5.

In our models, the lone pair on N3 of the 3′-pyrimidone ring is not very accessible for base pairing, in contrast to a

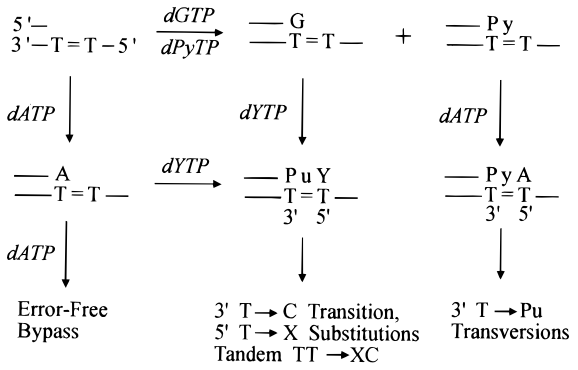

FIGURE 6: Mechanistic implications of the observed single and double substitution mutations opposite (6−4) and Dewar pyrimidone photoproducts. All arrows indicate the net specificity of elongation steps leading to complete bypass of the photoproducts and reflect the relative rates of incorporation and proofreading steps.

proposed base pairing scheme in which it is involved (LeClerc et al., 1991; Horsfall & Lawrence, 1994). When isomerized to the Dewar structure, however, the lone pair on N3 becomes more oriented towards the opposite strand, and hydrogen bonding to the N6H of A or the N2H of G now becomes geometrically feasible. The lone pair on the O2 carbonyl, however, becomes more oriented away from the opposite strand and is no longer accessible for hydrogen bonding to the G. These two changes could explain the increased preference for the incorporation of A over G opposite the 3′-T of the Dewar product when compared to the (6−4) product in *E. coli* under SOS conditions (Tables 5 and 6). The observation that only 12−20% G and 60−70% A was incorporated opposite the pyrimidone ring of the (6−4) product of TT in yeast suggests that the polymerase must also play an important role in determining the mutation spectrum of a photoproduct (Gibbs et al., 1995).

Mechanistic Implications of the Tandem Mutations Induced by the (6−4) and Dewar Pyrimidone Photoproducts. The only photoproducts that induced detectable amounts of targeted tandem double mutations were the (6−4) and Dewar photoproducts, and these mutations were only of the type TT·AA→NC·GN, with the exception of one TT·AA→CG·CG and one TT·AA→CA·TG mutation observed for the (6−4) and Dewar products, respectively, in the type III construct (Tables 5 and 6). These mutations, along with the observed single base pair substitutions, can be seen to originate from the net nucleotide incorporation specificity for each step in the bypass of the photoproducts (Figure 6). Examination of Table 5 reveals that, of the four nucleotides, there is a marked preference for insertion of the purine nucleotides A and G opposite the 3′-T of the (6−4) photoproduct and that these nucleotides are most frequently followed by incorporation of an A opposite the 5′-T and can be followed at a much lower frequency by C, G, or T. This contrasts with the Dewar product, for which A is incorporated opposite the 3′-T with the highest frequency and, when G is incorporated opposite the 3′-T, appears to be followed by A with less specificity.

The suppression of exonucleolytic proofreading during photoproduct bypass that is suggested to occur under SOS (Woodgate et al., 1987) could explain the formation of highly unusual single and tandem base substitutions induced by the (6−4) and Dewar products. In the case of a (6−4) product, there is a high preference for incorporation and elongation

of GA opposite the photoproduct and a low incidence of mutants which result from the incorporation and elongation of AC, AG, or AT (Table 5). Given that GA is the most complementary sequence to the (6−4) product in this case, the replication complex appears to have been able to elongate what amounts to double mismatches opposite the photoproduct. The formation of double mismatch bypass products is consistent with a two-step misincorporation−bypass model for mutagenesis proposed by Bridges and Woodgate in which proofreading by pol III is inhibited by RecA and polymerization past the photoproduct is facilitated by umuC and umuD′ proteins (Bridges & Woodgate, 1985a,b; Echols & Goodman, 1990).

Similar specificity trends were also observed for the Dewar product when flanked by either G or A. In these cases there is clearly a marked preference for incorporation and elongation of AA opposite the photoproduct, yet with low but significant frequency there were mutants resulting from the incorporation and elongation of GC, GG, and GT. Whereas A is almost exclusively incorporated and elongated following the incorporation of A opposite the 3′-base of the Dewar product, A appears to follow a G that is incorporated opposite the 3′-base with diminished preference. This diminished preference suggests that, unlike A, a G opposite the 3′-T lowers the subsequent selectivity of incorporation and suggests that a G opposite the pyrimidinone ring distorts the template−primer in such a way to lower the selectivity of subsequent nucleotide incorporation and elongation. The ability of flanking sequence to influence nucleotide insertion frequencies have been observed in DNA polymerase fidelity studies (Mendelman et al., 1989).

Implications for UV Mutagenesis. From the limited number of sequence contexts investigated, it would appear that the cis-syn and trans-syn dimers of a TT site in bacteriophage DNA cause the fewest mutations in *E. coli* under SOS conditions, whereas the Dewar and (6−4) products cause substantially more mutations. The relative contribution of these photoproducts to UV-induced mutations at TT sites in wild-type cells will depend, however, on the relative rates of induction, repair, and bypass of the photoproducts, some or all of which may show significant sequence dependence that would result in mutation hot spots. Cis-syn dimers are the major photoproducts of TT sites induced by 254 nm light in native DNA, whereas (6−4) products are produced at a relative rate of about 10% or less (Brash & Haseltine, 1982), trans-syn dimers less than 2% (Patrick & Rahn, 1976), and Dewar products not at all. In the presence of UVB light, (6−4) products are produced initially but are then converted to their Dewar valence isomers (Taylor & Cohrs, 1987). The formation of cis-syn dimers and (6−4) products has been found to show strong sequence dependence (Brash & Haseltine, 1982; Pfeifer et al., 1991), but nothing is known about the sequence dependence of trans-syn-I or Dewar product formation. Cis-syn dimers can also be repaired by *E. coli* photolyase (Kim & Sancar, A., 1993), whereas a trans-syn dimer is only repaired with very low efficiency (Kim et al., 1993) and the (6−4) and Dewar products not at all (Smith & Taylor, 1993). All of the photoproducts are substrates for the uvr(A)BC excinuclease of *E. coli*, which repairs the cis-syn, trans-syn, (6−4), and Dewar photoproducts in the duplex form of 49-mers used in our *in vivo* mutation studies with relative rates of approximately 1:6:9:9 (Svoboda et al., 1993). Thus, even though the (6−4) product is the most mutagenic of the four photoproducts of a TT site, it is produced at about a tenth the rate of the cis-syn dimer and repaired almost 10 times faster by uvr(A)BC, thereby greatly minimizing its contribution to UV-induced mutations. Repair of cis-syn dimers by both photolyase and uvr(A)BC has been shown to have little sequence dependence (Svoboda et al., 1993), but nothing as yet is known about the sequence specificity of repair of the other photoproducts or how sequence affects transcription-coupled repair (Mellon & Hanawalt, 1989; Selby & Sancar, 1993). Comparison of our mutation spectra of photoproducts flanked by A with those of Lawrence and co-workers which were flanked by G, while similar, suggest that the sequence flanking a photoproduct may influence the mutagenic consequences of replicative bypass of DNA photoproducts. To determine the true extent of sequence effects on the repair and mutagenicity of photoproducts, however, will require obtaining repair rates and mutation spectra of individual photoproducts as a function of flanking sequence under otherwise identical conditions, an undertaking that is entirely feasible and now in progress.

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