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Mutagenicity of a unique thymine–thymine dimer or thymine–thymine pyrimidine pyrimidone (6-4) photoproduct in mammalian cells

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ABSTRACT

The mutagenic properties of UV-induced photoproducts, both the *cis-syn* thymine–thymine dimer (TT) and the thymine–thymine pyrimidine pyrimidone (6-4) photoproduct [T(6-4)T] were studied in mammalian cells using shuttle vectors. A shuttle vector able to replicate in both mammalian cells and bacteria was produced in its single-stranded DNA form. A unique photoproduct was inserted at a single restriction site and after recircularization of the single-stranded DNA vector, this latter was transfected into simian COS7 cells. After DNA replication the vector was extracted from cells and used to transform bacteria. Amplified DNA was finally analyzed without any selective screening, DNA from randomly picked bacterial colonies being directly sequenced. Our results show clearly that both lesions are mutagenic, but at different levels. Mutation frequencies of 2 and 60% respectively were observed with the TT dimer and the T(6-4)T. With the TT dimer the mutations were targeted on the 3′-T. With the T(6-4)T a large variety of mutations were observed. A majority of G→T transversions were semi-targeted to the base before the 5′-T of the photoproduct. These kinds of mutations were not observed when the same plasmid was transfected directly into SOS-induced JM105 bacteria or when the T(6-4)T oligonucleotide inserted in a different plasmid was replicated in SOS-induced SMH10 *Escherichia coli* bacteria. These semi-targeted mutations are therefore the specific result of bypass of the T(6-4)T lesion in COS7 cells by one of the eukaryotic DNA polymerases.

INTRODUCTION

The mutagenic potency of UV light has been clearly shown both *in vitro* and *in vivo* using a variety of experimental systems in mammalian cells as well as in bacteria (1–3). Numerous lesions are induced by UV light, usually implicating a doublet of adjacent pyrimidines, as in the pyrimidine dimers and the pyrimidine (6-4) pyrimidones (4). Other minor lesions are also produced that concern pyrimidines and purines (5–7). We designed experiments to characterize the mutagenicity induced by two of the main UV-induced lesions, the *cis-syn* thymine–thymine cyclobutane dimer (TT) and the thymine–thymine pyrimidine pyrimidone (6-4) photoproduct [(T(6-4)T)]. Studies have been carried out concerning the mutagenic properties of these two adducts in prokaryotes and in yeast (8), but no data are available in mammalian cells. We therefore constructed a single-stranded DNA shuttle vector carrying a unique photoproduct able to replicate in both mammalian cells and bacteria. This vector was then replicated in mammalian cells and mutations were analyzed by DNA sequencing of rescued plasmids after individualization and amplification of DNA molecules in bacteria, without any screening.

The main interest of using a single-stranded DNA plasmid is the fact that the lesion cannot be a substrate for the excision/resynthesis DNA repair mechanism and that survival of the plasmid is subordinated to replication of its genome across the photoproduct. Using a single-stranded shuttle vector carrying a unique DNA lesion also removes any ambiguity about the strand position of the lesion and on the targeting of the mutation. Moreover, using the same DNA sequence for insertion of one of the two lesions under analysis eliminates any potential effect of DNA structural features that have been shown to play an important role in the production of mutational hot spots (9). This modified DNA sequence is the same as the one used in *Escherichia coli* and yeast studies (10,11).

MATERIALS AND METHODS

Plasmid

The plasmid that has been used is pS189, a generous gift from Dr Seidman. As described previously (12), it possesses the origins of SV40 and of πAN7 which allow its replication in mammalian cells and in bacteria. Single-stranded DNA was produced using the M13 phage replication origin present on the plasmid and the M13K07 helper phage according to the procedure described previously in Analects (Pharmacia). Bacteria carrying the vector were selected by their ampicillin resistance.

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Cells and bacteria

Monkey COS7 cells which constitutively express the SV40 T antigen were used in these experiments. They were grown in Dulbecco's modified Eagle's medium in 90 mm Petri dishes supplemented with 7% fetal calf serum in a water-saturated 5% CO_2 atmosphere.

$DH_5\alpha$ were utilized to detect mutants and JM105 or XL1 Blue to produce the single-stranded DNA plasmid.

SMH10 and JM105 bacteria were SOS induced as described previously (13), before being transformed with the damaged vector.

Oligonucleotides

A control oligonucleotide (GCAAGTTGGAG) or a modified oligonucleotide carrying either the *cis-syn* TT dimer or the T(6-4)T photoproduct were synthesized and purified as described previously (14,15). Other oligonucleotides necessary for vector construction were prepared in our laboratory by the solid phosphoramidite method using an Applied Biosystems 380B DNA synthesizer.

Construction of the vector

An adaptation of the method described by Banerjee *et al.* (14) and Leclerc *et al.* (15) was used (16). The single-stranded DNA vector was linearized at the unique *Bgl*II site after hybridization of a 20mer oligonucleotide, creating a double-stranded structure at this restriction site. The oligonucleotide helper was removed by heating at 80°C for 2 min then by cooling to 0°C and filtration through a Sephacryl S-400 column (Pharmacia). The linearized molecule was then re-circularized with a 50mer scaffold oligonucleotide, the sequence of its central part corresponding to the single-stranded region of the gapped molecule thus formed being complementary to the oligonucleotide carrying the unique photolesion. This latter was then inserted in the gap and ligation was performed using 400 U T4 DNA ligase at 15°C for 30 min. The 50mer scaffold was finally removed as described above for the 20mer. Finally, in order to remove any partially double-stranded intermediate molecules which may have been left, digestions with *Hin*cII and *Xba*I enzymes, whose restrictions sites are located at each site of the 50mer, were performed (16).

Experimental protocol

COS7 cells were transfected with the plasmid DNA using the DEAE–Dextran method (17). After 3 days, replicated extrachromosomal DNA was extracted from cells using an alkaline lysis method (18). This extracted DNA was used to transform DH5αrecA$^-$ bacteria by Hanahan's procedure (19) in order to isolate and amplify DNA molecules. The ampicillin resistance conferred by the plasmid allowed selection of transformants. Vector DNA was then prepared by alkaline lysis and DNA sequencing of the plasmid DNA containing the inserted oligonucleotide was performed by the chain elongation termination method using Sequenase II kits (Amersham).

RESULTS AND DISCUSSION

The mutagenic properties of TT dimers and T(6-4)T in mammalian cells are shown in Table 1. The mutagenicity of TT dimers is relatively low. A mutation frequency of only 2% has been observed. This mutagenicity is targeted opposite the photolesion, leading to T→C and T→A substitutions. Two transitions T→C and one transversion T→A have been observed on the 3′-T implicated in the dimer.

As far as the T(6-4)T is concerned a very elevated mutation frequency was observed. This frequency reaches 60% in the plasmid DNA progeny. Among these mutations a large majority of semi-targeted transversions was obtained. Eighty percent of G→T transversions occurred in the immediate vicinity 5′ of the photolesion. Very few mutations are targeted directly opposite the lesion (5%) and a few frameshifts are recorded near the original lesion, particularly a –1 deletion of the G residue corresponding to the hot spot of G→T substitutions of the 5′ border of the lesion. No mutants were found when no photolesion was present (Table 1).

Results obtained with the *cis-syn* TT dimer are not surprising when compared with its mutagenic potency in bakers' yeast, *Saccharomyces cerevisiae.* Indeed in this lower eukaryote, the mutation frequency of this lesion was <1% (10). In SOS-induced bacteria the error rate was higher and the mutation frequency reached ~7%. Although we detected only a few mutants, the mutations we observed are those expected from the observations in bacteria, that is to say, a base substitution at the 3′-T of the photoproduct. As argued by Banerjee *et al.* (14) for bacteria, it seems that the low level of mutation induced by the TT dimer in mammalian cells is most probably due to the fact that the dimer retains enough correct base pairing information for polymerase bypass, rather than a bias of adenine incorporation opposite a non-instructive lesion (20). In this latter case, the mutagenic potency of the photoadduct should be greatly elevated, since the A insertion frequency is ~60–80% in the case of non-instructive apurinic sites (21). A mutation frequency of 20–40% should therefore be observed when TT dimers are replicated, if the 'A rule' should apply.

Mutations observed after replication of T(6-4)T in COS7 cells are more difficult to interpret in view of what has been observed in bacteria and in yeast (11,15). Indeed a large variety of mutations was observed and, above all, a hot spot located in the immediate vicinity of the 5′-T implicated in the photolesion. Moreover, it has to be noted that in five cases (almost 8% of the mutants) this base 5′ of the lesion has been deleted. It appears therefore that the nucleotide adjacent to the 5′-T of the photolesion is a privileged target under our experimental conditions. In order to test if some impurities in the modified oligonucleotides could explain this result, we used the same batch of modified oligonucleotides to transfect SOS-induced bacteria as described previously (14). The T(6-4)T and TT photoproduct-carrying oligonucleotides were inserted into the single-stranded DNA vector M13mpL1 (13). This DNA was then transfected directly into competent SOS-induced SMH10 bacteria, which is a uvr A6 derivative of the AB1157 strain. As in experiments carried out in COS7 cells, no selection was applied, as colonies were picked and sequenced at random. As shown in Table 2, all the mutations were targeted. However we have no explanation for the higher frequency of targeted deletions found in these experiments as compared with that observed previously (15). Mutagenesis induced by the *cys-syn* dimer in SOS-induced bacteria was totally as expected, i.e. a low mutation frequency (9.6%) and all mutants being targeted at the 3′-T of the dimer (Table 2). It therefore demonstrates that the semi-targeted mutations observed using the COS7 cells are not artefacts due to the T(6-4)T-carrying oligonucleotide. Another hypothesis to explain our results in COS7 cells could be that our vector exhibits

specific secondary structure at the site of the T(6-4)T lesions due to the DNA sequence present around it. This putative structure could be a substrate for a specific type of error-prone replication at that particular site 5′ of the T(6-4)T. Indeed, it has been shown in experiments in which UV DNA lesions were randomly induced that hot spots of mutagenesis could be the consequence of particular DNA structure features. The complete construction of pS189 carrying the unique DNA lesion used to transfect COS7 cells was thus used to transform SOS-induced JM105 bacteria. Table 3 shows that mutations are mainly those expected according to the results observed previously (15). Only one mutation was identical to the semi-targeted mutational hot spot observed in mammalian cells, while the vast majority (80%) of mutations are targeted at the lesion. In conclusion, the specific mutational hot spot found in COS7 cells is really due to replication of the lesion by the cells and not to a defect in the oligonucleotide or to a conformational structure of the pS189 plasmid DNA.

Table 1. Mutagenesis of TT *cis-syn* cyclobutane dimer and T(6-4)T in mammalian cells

Sequences observed	TT *cis-syn* dimer			T(6-4)T			TT control
	Number of sequences analyzed	Mutation frequency (%)	Relative mutation frequency (%)	Number of sequences analyzed	Mutation frequency (%)	Relative mutation frequency (%)	Number of sequences analyzed	Mutation frequency (%)
5′-GCAAGTTGGAG-3′[a]	120			41			102	
GCAA**T**TTGGAG[b]				51		80.9		
GCAAGT**C**GGAG	2		66.6	2		3.2		
GCAAG**G**TGGAG				1		1.6		
GCAAGTTGG**G**G				2		3.2		
GCAAGTT**C**GAG				1		1.6		
GCAA(Δ**G**)TT(**+C**)GGAG				5		7.9		
GCAAGTTGG(ΔA)G				1		1.6		
GCAAGT**A**GGAG	1		33.1					
Total	123	2.4		104	60.5		102	<1%

The photoadduct was located at the T-T site in the sequence 5′-GCAAGTTGGAG-3′.
[a]Non-mutated sequence.
[b]Mutations are underlined.

Table 2. Mutagenesis of TT *cic-syn* cyclobutane dimer and T(6-4)T in SOS-induced SMH10 *E.coli* bacteria

Sequences observed	Photolesion			
	TT *cis-syn* dimer		T(6-4)T	
	Number of sequences	Mutation frequency (%)	Number of sequences	Mutation frequency (%)
5′-GCAAGTTGGAG-3′[a]	188		3	
GCAAGT**A**GGAG[b]	18			
GCAAGT**C**GGAG	2		28	
GCAAG**C**TGGAG			1	
GCAAG(Δ**T**)TGGAG			27	
Total	208	9.6	59	95

The photolesion was located at the T-T site in the sequence 5′-GCAAGTTGGAG-3′.
[a]Non-mutated sequence.
[b]Mutations are underlined.

Table 3. Mutagenesis of the T(6-4)T photolesion inserted in pS189 replicated in SOS-induced JM105 bacteria

Sequences observed	Number of sequences	Mutation frequency (%)
5′-GCAAGTTGGAG-3′[a]	29	
GCAAGT**C**GGAG[b]	10	
GCAAG**C**TGGAG	3	
GCAA**T**TTGGAG	1	
GCAAGT**GCAGCC**	1	
GCAAGTTGG**C**G	1	
Total	45	35.5

The photolesion was located at the T-T site in the sequence 5′-GCAAGTTG-GAG-3′.
[a]Non-mutated sequence.
[b]Mutations are underlined.

When present on a single-stranded DNA vector the T(6-4)T photoproduct led to a mutation frequency of 91% after replication in uvr A6 bacteria (15) and all mutations were targeted opposite the lesion, with a great majority of them being 3′-T→C transitions. In mammalian cells a high mutagenic potency was also obtained (60%). For mammalian cells, studies reported in the literature concerning UV mutagenesis were mainly performed after random UV irradiation of the target. Numerous studies were carried out with single- or double-stranded DNA shuttle vectors, lacZ′ and supF tRNA for example being the mutagenesis targets. In these cases, mutations may only be correlated with putative UV-induced lesions. The specificity of the mutations observed under these experimental conditions is less striking, i.e. the number of mutations targeted opposite pyrimidine–pyrimidine sequences varied between 60 and 80% in normal cells. Although most of the mutations observed were targeted (22,23), frameshift and semi-targeted mutations were also reported (24). Indeed, using a single-stranded DNA shuttle vector a non-negligible number of semi-targeted mutations located at the 5′-side of the putative pyrimidine–pyrimidine lesion was found, these semi-targeted mutations being or not being associated with a mutation opposite the 3′ base of the pyrimidine doublet. The problem now is to understand how such a mutation may have occurred. Several hypotheses may be put forward, but up till now no mechanism that could explain this phenomenon has been demonstrated. It is clear that the T(6-4)T photoproduct induces an important distortion of the DNA strand and that this distortion may play a crucial role in the mutation spectrum observed, where point mutations and also deletions or insertions occurred all around the lesion. This suggests that some slippage phenomenon during replication may intervene leading to this kind of error. Moreover, it has to be recalled that the DNA replication mechanism of the single-stranded DNA vector transfected into mammalian cells is still largely unknown. However, comparing replication in *S.cerevisiae* of a gapped duplex DNA shuttle vector with replication of a single-stranded DNA, both carrying a unique T(6-4)T photoproduct, Gibbs *et al.* (11) got similar results, indicating that replication of a single-stranded vector is very close if not identical to that of a double-stranded one. Mutation frequencies and the kinds of mutations were different from those found in our work, as 60–70% of molecules were replicated accurately. Almost all the induced mutations were targeted to the T-T site, 10–20% of them being 3′-T→C substitutions. On the other hand, our results concerning the high mutagenic potency of T(6-4)T are in agreement with those obtained in Chinese hamster cells using the *hprt* gene as target (25,26). These studies showed that the T(6-4)T photoproduct was the main UV-induced mutagenic lesion and that no preferential repair of the lesion in the transcribed gene was found. It seems, therefore, that the mutagenic properties of the photoproduct is correlated with the organism and with the replication conditions.

In bacteria, replication of both lesions requires a pretreatment in order to induce SOS conditions in the cells. As in *S.cerevisiae* and unlike in *E.coli*, UV pretreatment is not necessary in mammalian cells to bypass the lesions. No pretreatment of any kind in mammalian cells was necessary to obtain a mutated plasmid progeny. However, we cannot eliminate the possibility of the induction of SOS-like conditions in COS7 cells by the transfection protocol itself.

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