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Sequence effect on alkali-sensitive sites in UV-irradiated SV40 DNA

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ABSTRACT

Ultraviolet light at 254 nm induces various kinds of DNA damage. We have located and quantified the pyrimidine (6-4) pyrimidone photoproducts along three hundred and forty two nucleotides of SV40 DNA. The level of photoproduct induction varies greatly according to the position on the DNA, but unlike what happens with pyrimidine dimers, the very adjacent nucleotides do not play a major role in the frequency of formation. A new alkali-sensitive site has been found on the ACA sequence after UV irradiation. This complex lesion is insensitive to the T4 endonuclease V and the E. coli photolyase, and may be involved with mutagenesis.

INTRODUCTION

Ultraviolet-light (UV) induces various kinds of DNA damage which are subject to repair by proficient cells. In the absence of an error-free complete repair, DNA lesions at specific genomic sites can lead to mutations and activation of some proto-oncogenes which are implicated in the initiation of tumorigenesis. This is probably the case for the hereditary disease, xeroderma pigmentosum, which exhibits a defect in the excision repair of UV-induced lesions, leading to a high incidence of skin tumors (1,2).

Pyrimidine dimers (PyPy) and pyrimidine (6-4) pyrimidone photoproducts (Py(6-4)Py) are the main UV (254 nm) induced DNA lesions, the former being the more frequent. Brash and Haseltine (3), Wood (4) working on lambda phage, promote Py(6-4)Py as the mutagenic lesion, whereas for Kunz and Glickman (5) who studied the F factor gene, PyPy is the mutagenic lesion. On the other hand, Brash et al. (6) using a supF tRNA gene transfected in human cells concluded that mutation hot spots are primarily determined by DNA structural features. Different biological

systems have been used in these studies which may explain the reported discrepancies. Obviously, more genetic loci should be looked at in order to get a clearer picture of the true mutagenic lesion.

Using the phenotypic reversion of temperature-sensitive mutants of SV40, it has been previously shown that UV-induced mutations, produced in monkey cells, always consisted of base substitutions localized opposite potential UV-induced lesions, essentially the T-T site (7). Although a reversion system makes difficult a direct correlation between mutations and lesion sites, it is important to know the location and the quantification of UV-induced DNA lesions in the SV40 target gene. The amount of pyrimidine dimers varies as following TT > TC > CT > CC (8) and depends upon the adjacent nucleotides (e.g.: a pyrimidine in 5' position enhances the TT dimer formation) as already described (9,10). The probability of formation of the different DNA lesions is therefore a function of the DNA sequence. Consequently, it is necessary to span a large DNA fragment to get all possible adjacent nucleotide distribution for a given type of lesion.

We report the location, the quantification, the dose dependence and the influence of the adjacent sequences on PyPy and Py(6-4)Py formation on a fragment spanning 342 nucleotides of the SV40 large T antigen gene around the tsA58 mutation. Unlike PyPy, the Py(6-4)Py formation frequencies appear to be independent of the adjacent nucleotides but may depend upon the nucleotide environment or the local structure of the DNA. This paper describes some characteristics of a new alkali-sensitive site produced by UV on the ACA sequence.

MATERIALS AND METHODS

Virus and viral DNA

The temperature-sensitive mutant of the early transcription unit of SV40, tsA58 (11), was used. It was propagated on the CV1P line of African green monkey kidney cells at the permissive temperature of 33°C. After two weeks, the viruses were extracted by three freeze-thaw cycles and purified on isopycnic CsCl gradient (viral density = 1.38). After dialysis against 10 mM

Tris pH 7.8, 1mM EDTA (TE), the supercoiled viral DNA was prepared from the purified virus by three phenol-chloroform extractions followed by ethanol precipitation.

Purification and labelling of SV40 restriction fragments

Supercoiled SV40 DNA was digested with Hinc II and Hind III or with Hinf I restriction enzymes in appropriate buffers. Restriction fragments were then labelled at the 5' or 3' ends. The 5'-labelling was performed by the 5'-phosphate exchange reaction with γ^{32}P ATP (NEN, 3000 Ci/mM, 250 μCi) using 20 units of T4 polynucleotide kinase. The 3'-labelling was carried out by filling in the 3' ends of the restriction fragment with α^{32}P dXTP (NEN, 3000 Ci/mM, 20 μCi) using the large fragment of the E. coli DNA polymerase I (12). The labelled fragments were then loaded on a 4 % polyacrylamide gel. The 267 and 259 nucleotide fragments (H and I) of the Hinc II plus Hind III digest and the 237 nucleotide fragment (F) of the Hinf I digest were purified by electroelution and ethanol precipitated after cutting out polyacrylamide pieces containing DNA revealed by autoradiography. Since the fragments were labelled at each 5' or 3' ends, except for the Hinc II end (when labelling was made in 3'), further digestion with MboI or Pvu II for the I fragment, Rsa I for the H fragment, Hind III for the F fragment, followed by electrophoresis on a 6 % polyacrylamide gel, gave rise to purified fragments labelled at only one 5' or 3' end.

UV-irradiation

The labelled DNA fragments dissolved in TE were irradiated on ice by UV light at 2 kJ/m^2, 10 kJ/m^2, 25 kJ/m^2 or 50 kJ/m^2 from a germicidal lamp (mainly at 254 nm) with a dose rate of 7 W/m^2 as measured with a digital dosimeter (J-260 ; UV-photoproducts, Inc.).

Detection and quantification of UV-induced lesions

A) Pyrimidine dimers. DNA was incubated for 20 min at 37°C with an excess of the T4 endonuclease V in a buffer containing: 100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 8. The T4 endonuclease V, purified as already described (13), was a generous gift of Dr. P.C. Seawell and Dr. E. Vos (Stanford University, USA).

B) Py(6-4)Py. DNA was incubated in 1 M piperidine for 30 min at 90° C as described by Lippke et al. (14).

Labelled DNA, treated with the T4 endonuclease V or 1M piperidine, was lyophilized and then loaded on DNA sequencing polyacrylamide gels (12 % polyacrylamide, 8 M urea, 100 mM tris-borate, pH 8.3). DNA sequence ladders obtained by the Maxam and Gilbert method (12) were loaded on the same gel. After autoradiography, the radioactivity in the observed bands was computed by scanning the gel autoradiographs by optical densitometry using a microdensitometer (Joyce, model MK III C), the peak area on the scan being proportional to the amount of DNA in each band, as we ensured after previous calibration. The frequency of incision for the lesion is computed as described by Gordon and Haseltine (9).

Photoreactivating enzyme treatment

The restriction fragments of SV40 DNA have been purified as described above. After UV irradiation at 2 kJ/m^2, the DNA was treated by the E. coli photoreactivating enzyme (a generous gift of Dr. A. Sancar, University of North Carolina, USA). One unit per µg DNA was used in 100 mM NaCl, 10 mM Tris HCl pH 8, 10 mM EDTA for 10 min at 37°C in the dark, then the mixture was

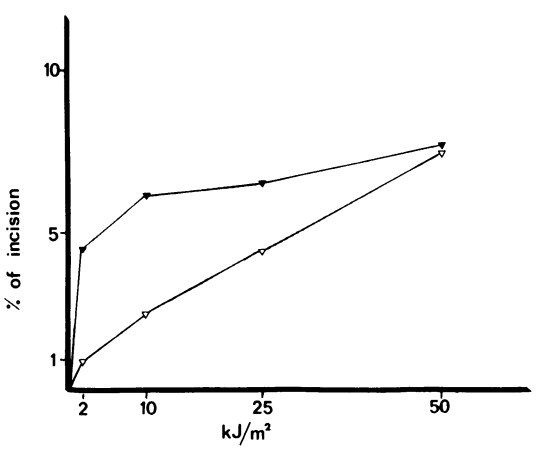

Figure 1. Percentage of incision at PyPy by the T4 endonuclease V (▼) or at Py(6-4)Py by hot alkali treatment (▽) of 342 nucleotides of SV40 DNA submitted to increasing doses of UV light. These results represent the mean of incision values from sixty five pyrimidine-pyrimidine sites.

illuminated with 30 kJ/m^2 of UVA (365 nm) at 37°C for 20 min (15).

RESULTS

1) Location and quantification of Py(6-4)Py

a) Dose response. While the level of incision at pyrimidine dimers reached a plateau between 2 and 10 kJ/m^2, the total number of Py(6-4)Py photoproducts increased from 0 to 50 kJ/m^2. At 2 kJ/m^2 UV, the dose at which most of the SV40 mutation spectra have been carried out (7), the average number of PyPy lesions was about five times greater than the average number of Py(6-4)Py as already described (10, 14) (Fig. 1). Results appeared different, however, if one looked at specific pyrimidine-pyrimidine sequences. With the SV40 DNA, as for other DNA (8,9), we found that the order of preference to produce PyPy lesions was as follows: TT > TC > CT > CC. For the Py(6-4)Py, the T(6-4)C lesions were formed more frequently than the others (Fig. 2). The high UV dose, we used, allows us to detect the

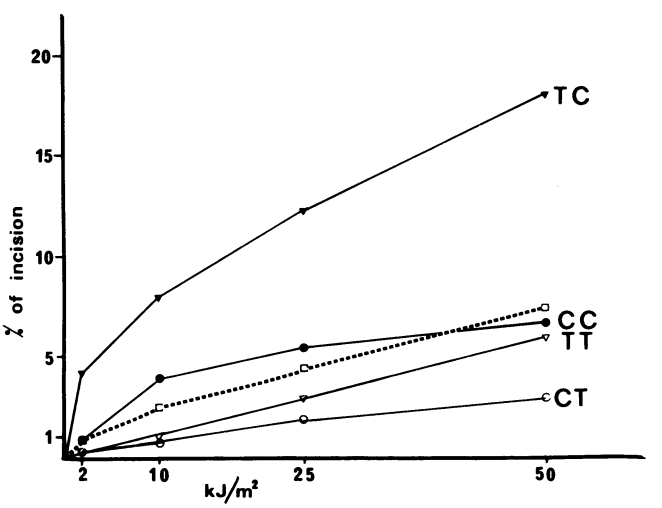

Figure 2. Percentage of incision at Py(6-4)Py by hot alkali treatment of a part of SV40 DNA submitted to increasing doses of UV irradiation at 254 nm. Twelve TC, thirty TT, seventeen CT and six CC different sites were computed for each point of the curves respectively. Dotted line shows the average of incision at each dose calculated from sixty five pyrimidine-pyrimidine sites and is identical to the one reported in Fig. 1.

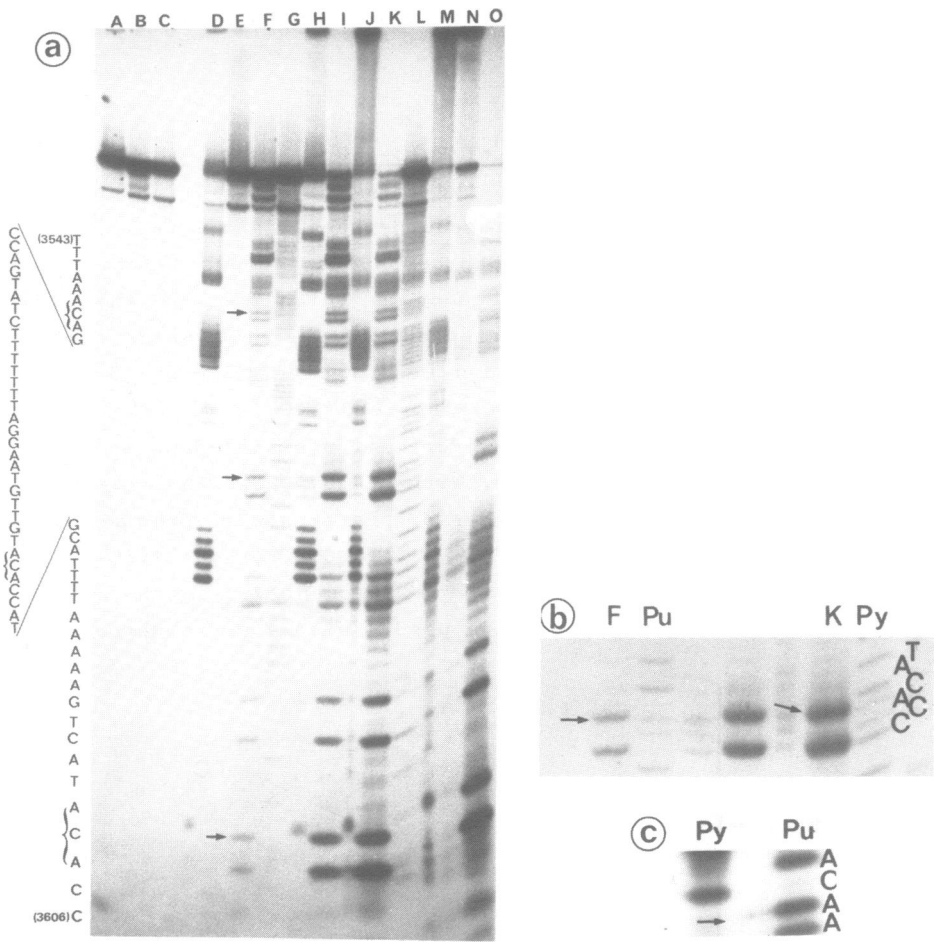

Figure 3. (a) Autoradiograph of a 12 % polyacrylamide sequencing gel of 3' end labelled SV40 DNA fragment. Unirradiated DNA was loaded on lane A, lane B (treated with T4 endonuclease V), lane C (hot alkali treated), lane G (purine sequence ladder), lane L (pyrimidine sequence ladder). 2 kJ/m^2 UV-irradiated DNA was loaded on lane D (treated with T4 endonuclease V), lane E, lane F (hot alkali treated). 10 kJ/m^2 UV-irradiated DNA was loaded on lane H (treated with T4 endonuclease V) and lane I (hot alkali treated). 25 kJ/m^2 UV-irradiated DNA was loaded on lane J (treated with T4 endonuclease V), lane K (hot alkali treated). 50 kJ/m^2 UV-irradiated DNA was loaded on lane M (treated with T4 endonuclease V), lane N, and lane O (hot alkali treated). The three ACA sequences, labelled with braces, correspond to the nucleotides 3549, 3579 and 3603 (for the C position) as indicated in Table 5. The arrows show the bands produced at ACA sequence with the hot alkali treatment.

(b) Enlargement of a part of the gel shown in (a) to exhibit the position of the ACA lesion (number 3579) as compared to the purine (Pu) and Pyrimidine (Py) sequence ladders. The lanes F and K are identical to those in (a) and the arrows show the bands corresponding to the ACA lesion incised with the hot alkali treatment. (c) The arrow indicates the band at ACA lesion (number 3506) induced by UV light (2 kJ/m^2) and produced with the hot alkali treatment of the 5' end labelled DNA. The Pu and Py lanes represent the sequence ladders.

C(6-4)T lesion, which was not previously described by Lippke et al. (14). We found a dose effect for the four species of Py(6-4)Py, but at 50 kJ/m^2 most of the DNA was cut by piperidine 1M at 90° C (Fig. 3a, lane 0). This indicated that the majority of the potential lesion sites were actually damaged and we found at this dose numerous sites sensitive to 1M piperidine at 90° C (see the following paragraphs) which were not located at pyrimidine-pyrimidine sequences.

b) Effect of adjacent nucleotides. A 342 bp region containing the tsA58 mutation of SV40 has been used to determine the effect of adjacent nucleotides on the frequency of formation of UV-induced lesions. This sequence is depicted in Fig. 4. A great variation in the frequency of Py(6-4)Py formation for a given pyrimidine-pyrimidine sequence was observed (Table 1). For example, for the T(6-4)C lesion produced at 10 kJ/m^2, the lowest efficiency of incision was 3.3 % (site n° 3716) and the highest efficiency was 16.2 % (site n° 3794).

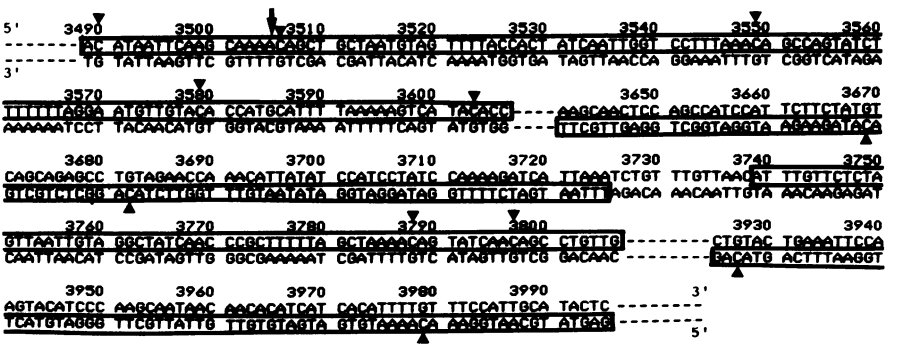

Figure 4: DNA sequence of a part of SV40 T-antigen gene containing the 342 base pairs (boxed sequences), we have used to locate and quantify the UV-induced lesions. The dotted lines correspond to nucleotides for which the quantification of lesions were impossible. The arrow indicates the site of the tsA58 mutation (7). The solid triangles show the ACA sequences studied in Table 5. The nucleotide numbers are given according to Tooze (16).

Table 1 : Percentage of alkali-induced incision at TC sites*

3' \ 5'	A	C	G	T
A	9 (3533) 15.6 (3767) 16.2 (3794)			7.7 (3497) 14.6 (3932)
C			3.5 (3541)	
T	4.9 (3559) 3.3 (3716)		5.2 (3748) 6.2 (3676)	6.5 (3746) 7.9 (3685)

Table 2 : Percentage of alkali-induced incision at TT sites*

3' \ 5'	A	C	G	T
A			0.3 (3753) 0.7 (3958)	0.4 (3723) 2.5 (3779) 0 (3524)
C	3.5 (3496)		0 (3686) 2.7 (3745)	0 (3933)
G	0.3 (3757) 5.8 (3537) 0.8 (3955)	0.8 (3951)	0.1 (3645) 1.4 (3805) 0.4 (3961)	1 (3712) 0 (3690) 0 (3742)
T	0.5 (3724) 0 (3741) 0.7 (3934)	1 (3714) 0.6 (3776)	0.3 (3691) 0.3 (3522)	0.3 (3713) 0 (3777) 2.3 (3778) 0 (3523)

Table 3 : Percentage of alkali-induced incision at CC sites*

3' \ 5'	A	C	G	T
A	3.4 (3527)		2.4 (3553)	
C	6.4 (3771)			
G		5.2 (3772)		
T			5.7 (3801)	2.5 (3542)

Table 4 : Percentage of alkali-induced incision at CT sites*

3' \ 5'	A	C	G	T
A	1.3 (3530)		0.3 (3764) 0.3 (3783) 0.4 (3513)	0 (3684) 1.7 (3749)
C			0 (3677)	0.4 (3747)
G		0 (3802)	0 (3672) 0.1 (3651) 0.6 (3510)	
T	0.8 (3941)	1.2 (3543)	0 (3775) 1.1 (3952)	1 (3715)

* 342 nucleotides of 10 kJ/m^2-UV-irradiated SV40 DNA were treated with 1M piperidine at 90° C for 30 min. The percentage of incision for each site was calculated as described in (9). The upper line shows the 5' flanking nucleotide and the vertical column shows the flanking nucleotide in 3' (no G nucleotide was present on the 3' of TC sites in the chosen sequence). The numbers in parenthesis indicate the position of the 3' nucleotide of the dinucleotide along the SV40 map according to Tooze (16) and depicted in Figure 4.

Since it was previously reported that pyrimidines at the 5' position of a potential dimer site strongly enhanced the frequency of PyPy formation (9, 10), we considered the influence of the adjacent 5' and 3' nucleotides on the frequency of formation of the Py(6-4)Py. We analysed twelve TC sites, thirty TT sites, seventeen CT sites and six CC sites contained in the 342 nucleotides studied (Tables 1-4). Unlike the pyrimidine dimer formation, the study of the adjacent nucleotides is not sufficient to explain the large variation observed in the formation frequency of the Py(6-4)Py within a given subclass (Tables 1-4). For example, in the ATCA subclass, the highest value was found to be 16.2 % and the lowest 9 %. For the TTCA the highest value was 14.6 % and the lowest 7.7 %. Since the frequency of C(6-4)T formation is very low, the significance of the absolute values is difficult to ascertain, but the differences between reactive and non reactive sites are obvious (table 4). No relationship between the percentage of incision by 1M piperidine and the 5' and 3' adjacent nucleotides was obvious from the values reported in the tables 1, 2, 3 and 4. In these results, a UV dose of 10 kJ/m^2 was used, but we obtained similar results with the other UV fluences.

2) Formation of UV-induced cross-links

At dose of UV light greater than 2 kJ/m^2, DNA migrating slower than the purified fragment appeared at the top of the gel (Fig. 3a), at 50 kJ/m^2 this DNA represented 40 % of the total DNA. This DNA was insensitive to the T4 endonuclease V activity (Fig. 3a, lanes J and M) but disappeared after treatment with 1M piperidine (lanes K and O). We therefore concluded that this high molecular weight DNA was due to covalent cross-links, which have been calculated using the Poisson distribution. At 10 kJ/m^2, 9% of DNA fragments contained one cross-link, one cross-link was found in 19% and two cross-links in 2% of molecules irradiated at 25 kJ/m^2, while for 50 kJ/m^2 27% of molecules exhibit one cross-link and 5% exhibit two cross-links.

3) Other lesions induced by UV light

Lesions other than pyridimine dimers and pyrimidine (6-4) pyrimidones appeared on the DNA after UV irradiation. At the 50 kJ/m^2 dose, many bands at sites different from a

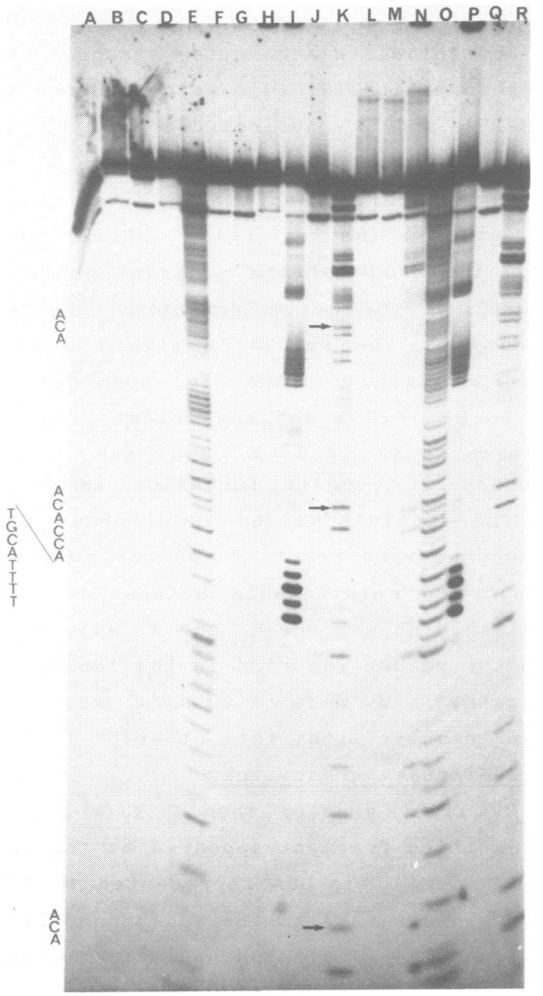

Figure 5. Autoradiograph of a 12 % polyacrylamide sequencing gel of 3' end labelled SV40 DNA fragment identical to that used in Fig. 3a. Lanes A, E, and O correspond to unirradiated DNA. Lane E is the purine sequence ladder and lane O is the pyrimidine sequence ladder. Lanes B, C, D, F, G and H correspond to UVA (365 nm) irradiated DNA. Lanes F, G and H correspond to DNA treated with photoreactivating enzyme of E. coli. DNA treated with T4 endonuclease was loaded on lanes B and F. Hot alkali treated DNA was loaded on lanes D and H. Lanes I, J, K, L, M, N, P, Q, R correspond to UV (254 nm) irradiated DNA (2 kJ/m^2). In lane I, DNA was then treated with T4 endonuclease V, in lane K the DNA was hot alakali treated. In lanes L, M and N, the DNA was pretreated with the photoreactivating enzyme of E. coli and irradiated with UVA (lane L the DNA was then treated with T4 endonuclease V and

lane N the DNA was then hot alkali treated). Lanes P, Q and R correspond to DNA irradiated with UVA, but not treated with the photoreactivating enzyme, before treatment with T4 endonuclease V (lane P) or hot alkali (lane R). The three ACA sequences, labelled with arrows, correspond to the nucleotides 3549, 3579 and 3603, for the C position, as indicated in Table 5.

pyrimidine-pyrimidine sequence, were detected in the lane corresponding to the treatment with 1 M piperidine (Fig. 3a, lane O). Most of these lesions were not abasic sites, because they were insensitive to the T4 enzyme, but they could result from hydration, oxidation or photoaddition (17). Some of these DNA damages could correspond to those recognized and excised by prokaryotic and eukaryotic redoxy endonucleases (18). Additional bands appeared sometimes in the lane corresponding to the irradiated DNA treated with T4 endonuclease V (Fig. 3a) in sites which were not pyrimidine-pyrimidine sequences. In the example of Fig. 3a (lane D), five bands were present in the sequence ATTTTA in which three pyrimidine dimers were possible. The supernumerary bands could correspond to purine-pyrimidine or pyrimidine-purine dimers as already reported (19, 20). These bands disappeared after the photoreactivating treatment (Fig. 5).

At all UV doses, the 1 M piperidine treatment gave rise to a strand incision at ACA sequences. This band was already detected at 2 kJ/m^2, but its intensity increased with higher UV doses (Fig. 3a, Table 5). Eleven of the twelve ACA sites, we observed, were detected with the piperidine treatment and the frequency of breakage was different for each site (Table 5). At the dose used for the SV40 mutation experiments (2 kJ/m^2), the percentages of incision at these sites were not negligible (Table 5). Fig. 3b shows the incision at one ACA site (arrow) on UV-irradiated DNA labelled at the 3' end. The migration of treated DNA on sequencing gels indicated that the incision occurred at the level of the 3'A of the ACA site (Fig. 3b). When the same experiment was carried out with 5'labelled DNA, the incision site was found to occur at the 5'A of the ACA site when compared to the Maxam and Gilbert sequencing ladders (Fig. 3c). The incision values were about five fold higher when measured on

Table 5 : Percentage of alkali-induced incision at ACA sites*.

UV-dose (kJ/m2) / Nucleotide Nb	2	10	25	50
3490	●	0.4	2.3	4.5
3506	0	0.1	0.4	1.3
3549	1.1	0.7	ND	ND
3549 **	3.2	5.2	10.7	10.1
3579 **	2.5	6.6	11.3	22.2
3603 **	1.7	5.2	7.3	12.5
3669	0.2	0.7	1.6	3.3
3682	0	0	0	0
3788	●	3.4	4.9	2.4
3797	ND	ND	ND	ND
3927	0.7	1.4	ND	ND
3979	●	0.4	1.3	3.6

* 342 nucleotides of SV40 DNA irradiated with the indicated doses of UV-light were treated with 1 M piperidine at 90° C for 30 min. ND corresponds to a non determined value due to technical limitations in our conditions (for example the position on the gel or a track of Py(6-4)Py very close). ● means that the lesion occurred but the value is very low (< 0,1 %). The nucleotide position corresponds to the position of the C of the sequence ACA on the SV40 genome according to Figure 4 . ** means that the DNA was labelled in the 3' extremity, while in the other cases the DNA was labelled in the 5' extremity.

UV-irradiated DNA labelled at its 3' extremity compared to DNA labelled at the 5' extremity (Table 5 and compared Fig. 3b and Fig. 3c). We don't have any clear explanation for this difference.

The nature of this lesion, insensitive to the T4 endonuclease is still unknown. Therefore, we used the photoreactivating enzyme to characterize further the ACA lesion. As previously described, the E. coli enzyme was able to monomerize pyrimidine dimers but unable to monomerize Py(6-4)Py (21). The ACA lesion did not appear to be photoreactivable by the E. coli enzyme (Fig. 5).

DISCUSSION

This paper reports the location, quantification and dose effect of UV induced lesions spanning 342 nucleotides of the tsA58 SV40 DNA. The pyrimidine-pyrimidine sequences (T-T, T-C, C-C and C-T) show different reactivities towards UV-light. As previously reported for pyrimidine dimers, the level of UV-induced lesions can be influenced by the adjacent nucleotides (9,10,14). The four classes of Py(6-4)Py are produced with different frequencies. The TC dinucleotide being the most reactive while the CT dinucleotide is the less reactive, but nevertheless the frequency of the C(6-4)T lesions is not negligible. If we consider a specific lesion (for example the T(6-4)C), its frequency of formation is very heterogeneous. Among the possible sites, the frequency of formation of a C(6-4)T at a given position can be more important that the T(6-4)C. This lack of rule makes necessary the location and quantification of UV-induced lesions directly on the target gene for the correlation studies with respective mutagenesis spectra. These measurements should be made or extrapolated at the UV dose used for mutation analysis. Indeed, the ratio PyPy versus Py(6-4)Py can vary considerably depending upon the delivered dose (Fig. 1) (14). The local structure of the DNA and/or the electronic group transfer along the DNA chain could explain the heterogeneity in the frequency of lesion formation.

Some lesions, detected with piperidine 1 M at 90° C, appear

in sites which are unable to form Py(6-4)Py or pyrimidine dimers. Some of these sites sensitive to the T4 endonuclease seem to be abasic sites, but we found a new lesion located at ACA sequences, which was not previously described (14,18). In the 342 nucleotides we studied, there are twelve ACA triplets. All of them, except the site at the 3682 position, are sensitive to UV irradiation as a function of the dose. Data obtained by labelling the UV-irradiated DNA either at the 3' or the 5' end seem to indicate that the ACA triplet is removed after the chemical treatment. Therefore, this new lesion appears to be complex, involving several bases. We searched in the literature for a mutation spot at the ACA sequence. Indeed, J.H. Miller (22) reported that 6 % of G to A transitions induced by UV light on the lacI gene are untargeted. Fifty seven per cent of these untargeted mutations occur at an ACA site. LeClerc et al. (23), studying UV mutagenesis in M13 lac Z' DNA, found that 30% of untargeted mutations are also at ACA sites. These two examples are suggestive of a mutagenic potency of these UV-induced DNA lesions.

In conclusion, this work shows the importance of studying the UV-induced lesions on a large DNA fragment. The formation frequency of PyPy and Py(6-4)Py lesions is clearly variable as a function of the sequence. The exact role of the nucleotidic environment is difficult to define due to the problem of analyzing all the possibilities of sequences in a piece of DNA. It is however clear that the frequency of formation of the Py(6-4)Py lesion is strongly dependent on the environment and not only on the immediately adjacent nucleotides. Studies on synthetic oligonucleotides and systematic analysis of UV-induced lesions on large pieces of DNA may elucidate the mechanisms involved in the induction of these various lesions.

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