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PYRIMIDINE DIMER DEPENDENT CLEAVAGE OF SINGLE-STRANDED DNA BY T4 UV ENDONUCLEASE

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SUMMARY: T4 UV endonuclease cleaves double- and single-stranded DNA with equal specificity for photo-pyrimidine dimers. Thus, the enzyme can be used for mapping and quantifying pyrimidine dimers in single-stranded DNA as well as in double-stranded DNA.

Mapping of pyrimidine dimers shows that rates of UV-dimerization are not only affected by 5', 3' adjacent bases, but also by position within pyrimidine tracts. Di-pyrimidines at 3' ends of tracts are more photoreactive than those at 5'ends.

Survival studies with E. coli and UV irradiated bacteriophage ØX174 showed that the host cells repaired UV damaged double stranded (ds) DNA but not single stranded (ss) DNA (1,2,3). The reason for this difference became evident when it was found that this repair mechanism would excise a short segment of a pyrimidine dimer containing DNA strand and fill in the resulting single-strand gap by repair replication (4,5). Completion of this process is obviously dependent on ds-DNA substrates. Whether the steps that initiate excision repair, such as substrate recognition and cleavage of glycosidic and phospho-deoxyribose ester bonds would be also restricted to ds-DNA remained unknown.

Pyrimidine dimer specific endonucleases such as T4 UV endonuclease (6), in conjunction with DNA end-labeling and high resolution gel electrophoresis techniques (7), have become important tools in localizing pyrimidine dimers on ds-DNA (8). To extend this type of analysis to UV irradiated ss-DNA, we tested whether T4 UV endonuclease would act on UV damaged ss-DNA. Here we compare the cleavage of ds- and ss-DNA by T4 UV endonuclease and measure effects of neighboring bases on pyrimidine photo-dimerizations.

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METHODS

Preparation and [^{32}P] end labeling of DNA The 75 bp HindIII/EcoRI fragment of simian virus 40 (SV40) (nt. 1626-1700, ref. 9) was cloned into plasmid pUC12 (10) and 5'-[^{32}P] end labeled as follows: a) To label one strand only, the recombinant plasmid was cleaved with either EcoRI or HindIII, dephosphorylated with bacterial alkaline phosphatase (Bethesda Research Laboratories, BRL, Bethesda, MD) and incubated with T4 polynucleotide kinase (BRL) in the presence of [^{32}P]- γ-ATP (ICN, Irvine, CA) (ref. 7). Subsequently, the 75 bp SV40 HindIII/EcoRI fragment was cleaved off the vector with either HindIII or EcoRI and isolated following electrophoresis on polyacrylamide gels.

UV irradiation The 5'-[^{32}P] end-labeled DNA fragments were UV irradiated at an effective dose of 1,000 Jxm^{-2} using a low pressure mercury vapor lamp emitting predominantly at 254 nm. DNA's were double stranded during irradiation and, when required, denatured subsequently.

DNA strand separation The 75 bp fragment, which had been UV irradiated and 5'-[^{32}P] end-labeled at the EcoRI site, was denatured by heating. The strands were separated and isolated by preparative electrophoresis on urea/-polyacrylamide gels as described (7).

Conditions for dimer dependent cleavage of ds- and ss- UV irradiated DNA by T4 UV endonuclease T4 UV endonuclease was kindly provided by Dr. Kathleen Dixon (National Institutes of Health, Bethesda, MD) and used as described (11). Briefly, 100 ng of DNA, equivalent to 8,000 - 10,000 dpm [^{32}P], were exposed for 40 min at 37C to T4 UV endonuclease in 50 μl reaction volumes. Enzyme concentration and reaction condition were chosen to digest to completion 100 ng of UV irradiated (1,000 Jxm^{-2}) 75 bp DNA within 15 min. The reaction was terminated and the DNA precipitated by the addition of 150 μl of 10 mM Tris, pH 8.0; 0.35 M NaCl; 7 M urea; 10 mM EDTA; 0.5% SDS; and 5 μg carrier tRNA followed by 450 μl of cold ethanol. The precipitated DNA was denatured, electrophoresed on 8% polyacrylamide/urea gels, and autoradiographed as described (7). To map T4 UV endonuclease cleavage sites, the same but unirradiated DNA was chemically cleaved (A>G and G>A reactions, ref. 7) and electrophoresed in adjacent lanes. To control for the dependence of the T4 UV endonuclease cleavage reaction on the presence of UV photoproducts, unirradiated DNA was reacted under the same conditions as irradiated DNA and also applied to the same gel. The termini of the cleavage products of the T4 UV endonuclease and the Maxam-Gilbert reactions differ (11), resulting in lower electrophoretic mobilities for the T4 UV endonuclease cleaved fragments. The nucleotide assignment for the T4 UV endonuclease cleavage tracks in figures 2 and 4 was chosen to give the positions of the 3' pyrimidine in the dimers, not the actual cleavage sites.

Pyrimidine dimer dependence of the T4 UV endonuclease
To verify the specificity of the enzyme for UV irradiated DNA and the absence of contaminating endonucleases, UV irradiated and unirradiated, negatively supercoiled, covalently closed circular (ccc) pBR322 DNA was incubated with the enzyme preparation under reaction conditions (see above). The UV dose to the plasmid DNA was 50 Jxm^{-2} which produces about four pyrimidine dimers per molecule.

RESULTS

Specificity of T4 UV endonuclease for UV irradiated DNA Figure 1 shows that irradiated DNA is almost completely converted from supercoiled ccc-DNA to a relaxed, circular form within ten minutes of incubation with T4 UV

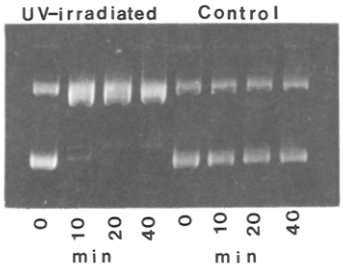

Figure 1. Digestion of pBR322 DNA by T4 UV endonuclease.
Left: UV irradiated DNA (50 Jxm^{-2}) reacted with T4 UV endonuclease.
Right: Unirradiated DNA reacted with T4 UV endonuclease (control).
Pyrimidine dimer to enzyme ratio same as in subsequent experiments.

endonuclease, whereas unirradiated DNA is not converted within forty minutes. Thus, the enzyme relaxes only UV irradiated DNA. Furthermore, there is no conversion to a linear form demonstrating the absence of double-strand scissions.

Determination of T4 UV endonuclease cleavage sites on the UV irradiated 75 bp HindIII/EcoRI fragment of SV40 The SV40 75 bp fragment (nucleotide # 1626-1700) includes several pyrimidine tracts which can photo-dimerize. These tracts are over- or underlined below. The upper strand is the "sense"- the lower the "antisense"-strand of the SV40 late transcription region.

```
1         10         20         30         40         50         60         70
AGCTTAGCAGCTGAAAAACAGTTTACAGATGACTCTCCAGACAAAGAACAACTGCCCTTGCTACAGTGTGGCTAG
    ATCGTCGACTTTTTGTCAAATGTCTACTGAGAGGTCTGTTTCTTGTTGACGGGAACGATGTCACACCGATCTTAA
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Figure 2 is an autoradiograph of cleavage fragments of this DNA 5'-[^{32}P] end-labeled at the HindIII site (sense strand). The right panel shows nucleotides 3 to 20, the left panel nucleotides 20 to 75. Lanes 1, 2, 6, and 7 serve as size markers. Lane 4 is a T4 UV endonuclease digest of unirradiated DNA. Lanes 5 and 9 are T4 UV endonuclease digests of UV irradiated DNA cleaved by T4 UV endonuclease. Lanes 3 and 8 are UV irradiated DNA reacted with piperidine (8). Figure 2 shows that T4 UV endonuclease cleaves UV irradiated DNA within pyrimidine tracts and that cleavage frequencies differ greatly. Figure 3 is a densitometric tracing of Figure 2, lane 5. Frequen-

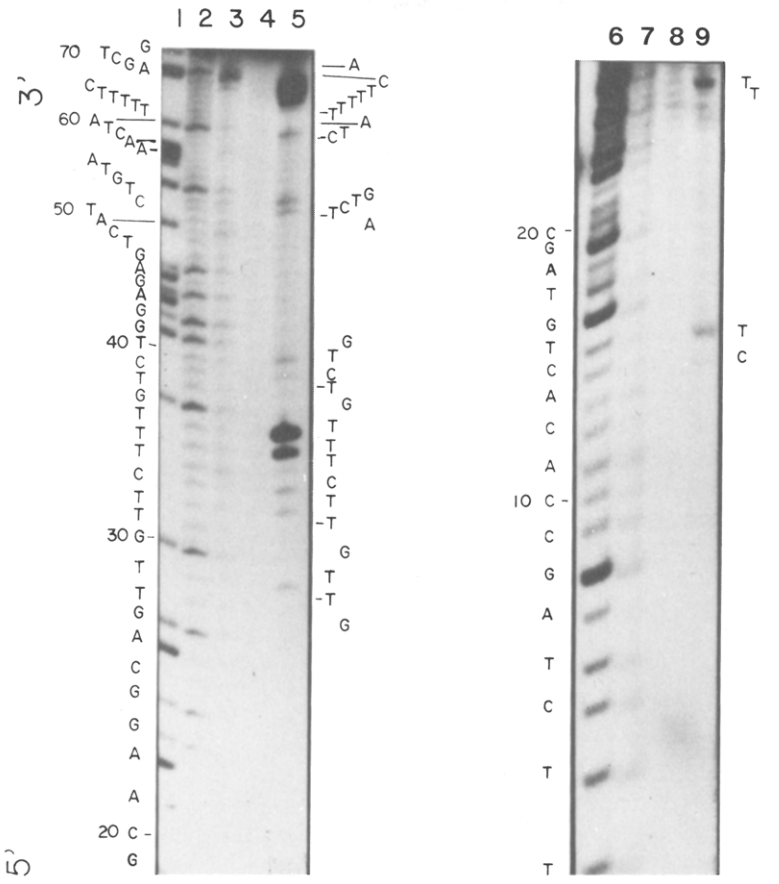

Figure 2. Autoradiograph of cleavage products of the 75 bp ds-DNA, 5'-[^{32}P] labeled at the antisense strand and resolved on an 8% polyacrylamide/urea gel.
Right panel: nucleotides 3-20; left panel: nucleotides 20-75.
Lanes 1, 6 & 2, 7: Hydrazine cleaved unirradiated DNA, A>G and G>A, respectively. Lanes 3 & 8: Piperidine cleaved UV irradiated DNA. Lane 4: Unirradiated DNA reacted with T4 UV endonuclease. Lanes 5 & 9: UV irradiated (1,000 Jxm^{-2}) DNA reacted with T4 UV endonuclease.

cies of T4 UV endonucleolytic cleavage were read from appropriately exposed autoradiographs from three independent experiments and compiled in Table 1.

Figure 4 is an autoradiograph and a densitometric tracing of cleavage products of the antisense strand, [^{32}P] labeled at the EcoRI site. Lane 1 is chemically cleaved (A>G) to serve as size marker, lane 2 is unirradiated DNA reacted with T4 UV endonuclease, and lane 3 is UV irradiated DNA reacted with T4 UV endonuclease. The densitometric tracing is from lane 3. As with the sense strand, T4 UV endonucleolytic cleavage is confined to sites of

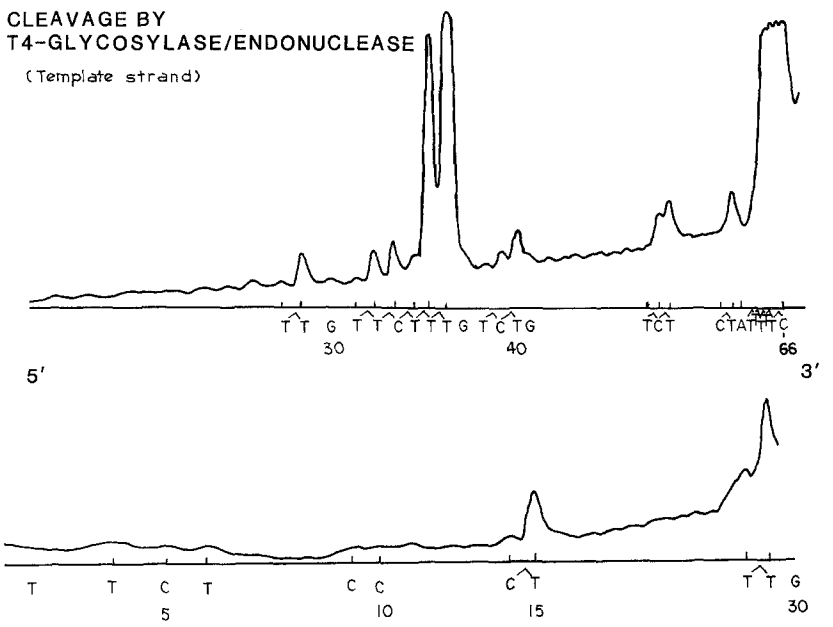

Figure 3. Densitometric tracing of figure 2, lanes 5 & 9 (UV irradiated DNA cleaved by T4 UV endonuclease).

possible pyrimidine dimers and cleavage frequencies differ considerably between sites.

A comparison of T4 UV endonuclease cleavage specificities and frequencies with UV irradiated ds- and ss-DNA substrates is shown in Figure 5. In

TABLE 1. RATES OF PHOTO-DIMERIZATION OF DI-PYRIMIDINE SEQUENCES AS A FUNCTION OF 5' AND 3' ADJACENT BASES

Strand* & nucleotide	5'	Py=Py**	3'	% ***	Strand & nucleotide	5'	Py=Py	3'	%
S 37/38	T	CC	A	0.0	A 47/48	G	TC	A	0.0
					A 38/39	G	TC	T	2.3
S 52/53	A	CT	G	0.6	A 50/51	A	TC	T	3.3
S 61/62	G	CT	A	0.7	S 36/37	C	TC	C	1.1
A 58/59	A	CT	A	3.9	S 34/35	C	TC	T	1.4
S 33/34	A	CT	C	1.1	A 32/33	T	TC	T	2.5
S 35/36	T	CT	C	1.8					
A 33/34	T	CT	T	1.9	A 28/29	G	TT	G	2.0
A 39/40	T	CT	G	3.0	S 22/23	G	TT	T	1.4
A 51/52	T	CT	G	4.0	A 31/32	G	TT	C	2.1
					S 58/59	C	TT	G	2.8
					S 23/24	T	TT	A	9.8
					A 34/35	C	TT	T	10.4
					A 35/36	T	TT	G	17.2

* Antisense strand (A) is the lower sequence of the print-out (see RESULTS). Nucleotide numbering is 5' to 3', i.e., right to left. Sense strand (S) is the upper sequence of the print-out. Nucleotide numbering is again 5' to 3' reading left to right.
** Py=Py denotes pyrimidine dimers.
*** % gives the percentage of cleavages at a given dimer; the sum of all cleavages on both strands equaling 100%.

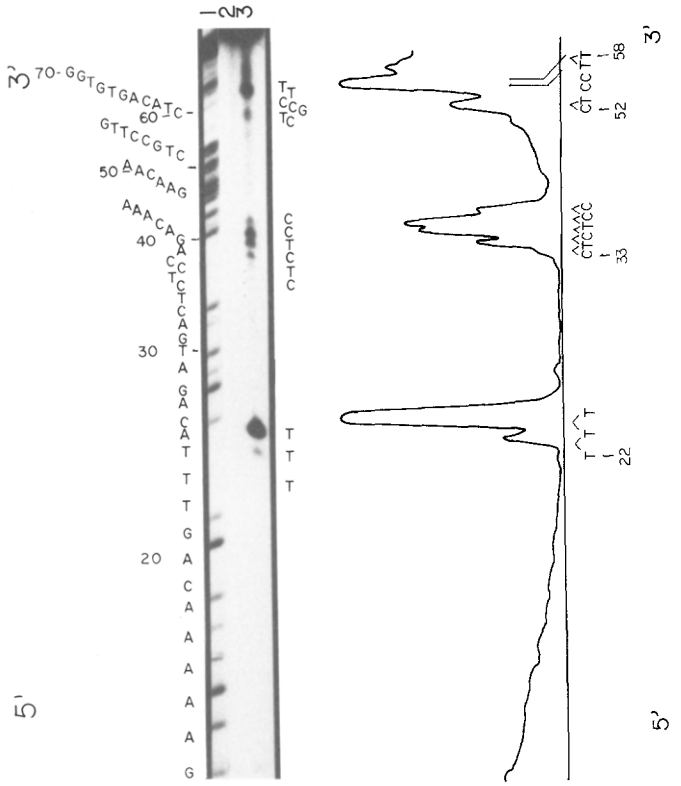

Figure 4. Autoradiograph and densitometric tracing of cleavage products of the 75 bp DNA 5'-[^{32}P] labeled at the sense strand. Lane 1: Hydrazine cleaved, A>G. Lane 2: Unirradiated DNA digested with T4 UV endonuclease. Lane 3: UV irradiated (1,000 Jxm^{-2}) DNA digested with T4 UV endonuclease. The densitometric tracing is of lane 3.

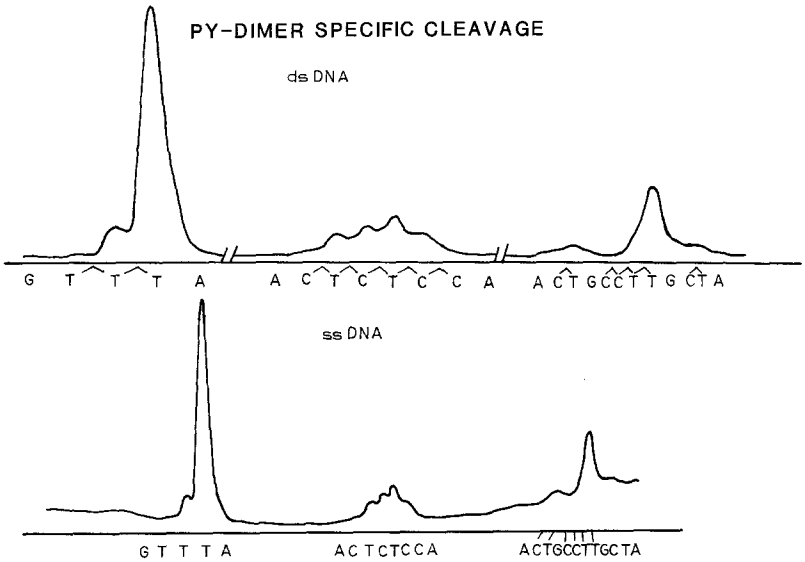

Figure 5. Densitometric tracing of T4 UV endonuclease cleavage products of UV irradiated ds-DNA. Upper part: ds-DNA cleaved by T4 UV endonuclease. Lower part: ss-DNA cleaved by T4 UV endonuclease.

TABLE 2. T4 UV ENDONUCLEASE CLEAVAGE FREQUENCIES OF ds- AND ss-DNA *

Nucleotide position on sense strand:					
	22/23/24	33/34/35/36/37/38	51/52	58/59	61/62
	T =T =T	C =T =C =T =C =C	C =T	T =T	C =T
ds-DNA:	8.0; 56	6.2; 8.1; 10; 6.2; 2.2	3.5	16	4
ss-DNA:	8.4; 52	6.3; 8.3; 10; 7.0; 1.8	3.2	15	4

* Cleavage frequencies are represented by relative amounts of radioactivity for a given cleavage product averaged over three independent experiments. The experimental errors are < 12%.

this experiment, the 75 bp DNA was 5'-[^{32}P] end-labeled at the EcoRI site, isolated and irradiated with 1,000 Jxm^{-2}. One aliquot of ds-DNA was reacted with T4 UV endonuclease as described in METHODS, another aliquot was denatured, DNA strands separated and isolated by preparative urea/polyacrylamide gel electrophoresis, and the ss-DNA reacted with T4 UV endonuclease under the same conditions as the ds-DNA. Figure 5 shows densitometric tracings of irradiated ds- and ss-DNA cleaved by T4 UV endonuclease. From Figure 5 and Table 2, it can be seen that cleavage sites and frequencies are the same with the ds-DNA and the ss-DNA substrates.

DISCUSSION

Our studies confirm (8) that cleavage frequencies by T4 UV endonuclease at sites of possible pyrimidine dimerization differ greatly. They further show that T4 UV endonuclease cleaves UV irradiated ss-DNA and ds-DNA at the same sites and with the same frequencies.

The observed cleavage frequencies are likely to represent pyrimidine dimer distributions and not substrate preferences of the T4 UV endonuclease. Support for this assumption comes from the following observations. Dimer dependent endonuclease from M. luteus cleaves DNA at all possible dimers (T=T, C=T, T=C, C=C) and the combined action of this endonuclease and added exonucleases completely removes all dimers from UV irradiated DNA (12, 13). The cleavage patterns of UV irradiated DNA with T4 UV endonuclease and the M. luteus enzyme are the same (8). The cleavage times with T4 UV endo-

nuclease used in our experiments are three times the ones required for complete cleavage of the substrate (see METHODS) assuring complete cleavage in all cases.

Pyrimidine dimerization is a photoreversible process (14, 8) reaching steady states above 2,000 to 3,000 Jxm^{-2}. Thus, at the UV dose of 1,000 Jxm^{-2} used in our experiments, dimer frequencies approximate rates of dimerization. These rates are strongly influenced by three parameters: a) the nature of the pyrimidine base (T more reactive than C); b) the position of the base within a tract of several pyrimidines (3' more reactive than 5'); and c) the 5' and 3' adjacent base. The position effect for dimerization within oligo-pyrimidine tracts is pronounced at the T repeats of the sense strand position 22/23 vs 23/24 which contain 1.4% and 9.8% of total dimers and template strand position 34/35 vs. 35/36, which contain 10% and 17% of total dimers, respectively. The overall gradient of dimer frequency in antisense strand position 31 through 36 [(G)TTCTTT(G)] is 2, 2.5, 2, 10, and 17 percent of total dimers, respectively. Note that the 5' and 3' dithymidines differ in dimerization frequencies by a factor of 8. In addition to the inhibitory effects of adjoining purines (G more inhibitory than A, see Table 1), we observe a polar effect on dimerization rates within pyrimidine tracts. To what extent these differences are controlled by structural constraints, base stacking interactions, and excited state energy migration remains to be analyzed.

Up to five-fold differences in steady state levels of pyrimidine dimers were reported previously (8), demonstrating that photo-dimerization and photo-reversal of dimers are differently affected by adjoining bases and dipyrimidine positions.

The cleavage patterns of ds- and ss-DNAs are indistinguishable from one-another (Fig. 5; Table 2). This shows that T4 UV endonclease recognizes dimers and cleaves phospho-ester bonds in both types of DNA with equal fidelity. Therefore, T4 UV endonuclease is suitable for analysis of pyrimidine dimer distributions in ss-DNA.

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