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IN VITRO DNA SYNTHESIS BY DNA POLYMERASE I AND DNA POLYMERASE α ON SINGLE-STRANDED DNA CONTAINING EITHER PURINE OR PYRIMIDINE MONOADDUCTS

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Abstract—The capacity of the large fragment of DNA polymerase I from *Escherichia coli* and of DNA polymerase α from *Drosophila* embryo to replicate single-stranded M13mp10 DNA containing either purine or pyrimidine monoadducts was compared. The monoadducts were respectively induced by cisplatinum and by furocoumarin photoaddition. For both types of lesions, it is observed that the eukaryotic enzyme is more inhibited than the prokaryotic one. By mapping the arrest sites produced by furocoumarin monoadducts on the synthesis catalysed by DNA polymerase α, we show that, in contrast with the photoreaction observed with double-stranded DNA, these compounds do not show a strong sequence specificity in reacting with single-stranded DNA.

The antitumoral drug *cis*-diamminedichloroplatinum(II) (or *cis*-DDP‡) is known to initially react with the N(7) position of guanine on DNA. In a second step, this monoadduct chelates with another purine base on the same or on the opposite DNA strand leading to intrastrand or interstrand cross-links respectively [1–3]. Intrastrand bifunctional lesions induced in single-stranded M13 phage DNA by *cis*-DDP appear to block completely *in vitro* DNA synthesis by both prokaryotic DNA polymerase I and eukaryotic DNA polymerase α [4]. Thus, it has been suggested that these biadducts are responsible for cytotoxicity by *cis*-DDP [5, 6]. When the majority of the lesions are monoadducts, DNA synthesis by DNA polymerase α is affected to a lesser extent than by biadducts. Furthermore, DNA polymerase I appears to bypass purines monoadducts efficiently whereas DNA polymerase α is more inhibited by these lesions [4].

In combination with UVA (near-UV light, 320–400 nm), furocoumarins, a class of tricyclic hydrocarbons used in photodermatology of psoriasis and other skin diseases, also form monoadducts and interstrand cross-links on DNA. In this case, however, only pyrimidine bases are involved in the photoreaction (for recent reviews see Refs 7 and 8). The photoreaction with furocoumarins can be easily controlled by either using monofunctional (trimethylangelicin, TMA) and bifunctional (trimethylpsoralen, TMP) molecules. Also by changing the incident wavelength, mainly monoadducts (at 405 nm) or monoadducts plus cross-links (at 365 nm) can be induced using the same bifunctional molecules (TMP) [9].

Such photoreactions have been produced on M13 DNA in parallel to *cis*-DDP treatment leading to a majority of monoadducts, and subsequently these substrates were used in an *in vitro* replication system. The aim of the work was to examine to what extent the differences previously observed between DNA polymerase I and DNA polymerase α for purine adducts holds for pyrimidine photoadducts. This may allow us, in relation to the accumulated genotoxicity data in prokaryotes and eukaryotes (for review see Ref. 10), to establish the possible role of the bypass process during DNA synthesis.

MATERIALS AND METHODS

Reagents and enzymes

cis-DDP was synthetized following published methods [11] and provided by Dr N. P. Johnson. TMP was from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and TMA was a gift from Prof. F. Dall'Acqua (University of Padova, Italy). M13 universal sequencing primer (17 mer), unlabelled dNTPs and dideoxy NTPs were from Pharmacia LKB Biotechnologies Inc.; α-^{32}P-labelled dATP was from Amersham (Amersham, U.K.). DNA polymerase I (Klenow fragment) was from Pharmacia; one unit of DNA polymerase I activity is the amount that catalyses the incorporation of 10 nmol of dNTPs into acid insoluble material in 30 min at 37°. DNA polymerase α from *Drosophila* embryos, fraction VI [12], was a gift from Dr I. R. Lehman (Stanford University). One unit of DNA polymerase α activity is defined as the amount which catalyses the incorporation of 1 nmol of dNTP into acid insoluble material in 60 min at 37°.

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‡ Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum (II); TMA, 6,4,4′-trimethylangelicin; TMP, 4,5′,8-trimethylpsoralen; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; UVA, near-UV light (320–400 nm).

M13mp10 DNA preparation and treatment with cis-DDP

M13mp10 DNA was prepared as described previously [13]. Synthetic oligonucleotide was annealed to the template by heating the phage DNA with primer (2 ng of primer/μg of DNA) at 60° for 1 hr. *cis*-DDP was dissolved at 1 mg/mL in 0.9% KCl 1 hr prior to the reaction. Primed DNA in 1 mM sodium phosphate, 3 mM NaCl, pH 7.3, was incubated with *cis*-DDP at 37° for 2.5 or 5 min, at a molar ratio (*ri*) of platinum/nucleotide equal to 1. The reaction was stopped by adding NaCl, to 0.5 M final concentration, and placing the sample immediately on ice. Platinated template was dialysed against 3 mM sodium phosphate, 0.1 mM EDTA, 0.5 M NaCl, pH 6.5, in order to remove unreacted drug. Using this procedure M13mp10 DNA containing a majority of cisplatinum monoadducts was obtained [4]. The high salt concentration used throughout the experiments reported here (500 mM NaCl in the dialysis buffer and 50 mM in the DNA replication buffer) should prevent the evolution of monoadducts into biadducts.

Photochemical modification of the M13mp10 DNA with TMP and TMA

Aliquots of 5 μg of primed single stranded M13mp10 DNA in TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) plus 10% dimethyl sulfoxide were incubated for 30 min in the dark in the presence of TMP or TMA at a molar ratio of furocoumarin/nucleotide of 3 and 0.33, respectively. Irradiation with UVA light at 365 or 405 nm was performed on ice as described previously [14]. The unreacted furocoumarin was extracted twice with chloroform/isoamylalcohol (19 : 1, v/v) and the DNA was ethanol precipitated and resuspended in TE buffer. Controls for DNA synthesis represent DNA samples treated with TMP or TMA in the absence of UVA irradiation.

DNA synthesis

DNA polymerase I assay. The reaction mixture (10 μL) containing 50 mM Tris–HCl, pH 7.5, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM NaCl, 25 μM dCTP, dGTP and dTTP, 2.5 μM [^{32}P]dATP (10,000 cpm/pmol), 400 ng of modified or unmodified primed DNA and 1 U of DNA polymerase I was incubated at 37° for 30 min. Then, samples were placed on ice, aliquots were spotted on Whatman glass filters (GF/C) and the remaining samples were immediately frozen in liquid nitrogen and stored at −20° for subsequent DNA sequencing. Filters were washed with ice-cold 5% trichloroacetic acid, 1% sodium pyrophosphate, then with ethanol and the radioactivity was determined.

Drosophila embryos DNA polymerase α assay. The reaction mixture (10 μL) containing 50 mM Tris–HCl, pH 8.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 20 mM ammonium sulfate, 2.5 μg of bovine serum albumin, 50 mM NaCl, 25 μM dCTP, dGTP and dTTP, 2.5 μM [^{32}P]dATP (10,000 cpm/pmol), 400 ng of modified or unmodified primed DNA, and 3 U of DNA polymerase α was incubated at 37° for 30 min. Samples were then processed as above.

Electrophoretic analysis of synthesized DNA

Electrophoresis was carried out not longer than 24 hr after DNA synthesis. One volume of deionized formamide containing 0.3% xylene cyanol, 0.3% bromophenol blue, and 0.3% EDTA was added to DNA samples which had the same amount of radioactivity; samples were boiled 5 min and then subjected to electrophoresis at 1500 V on a 8% polyacrylamide, 7 M urea denaturating gel. Autoradiography of the gels was performed using Trimax 3 M film at −70°. Dideoxy sequencing reactions [15] were carried out on untreated M13mp10 DNA.

Computer prediction of secondary structure

We have previously searched for locally stable secondary structure interactions in the 150 nucleotides of the M13mp10 single stranded DNA, immediately downstream of the 3'OH of the primer [16]. This analysis has been performed using Zuker and Stiegler's RNA fold program [17] in conjunction with the thermodynamic parameters determined by Freier *et al.* [18]. In a first step this entire region has been globally folded, then a scan was performed with an 80 nucleotides window and a 20 nucleotides step to emphasize short-range interactions.

RESULTS

Comparison of the in vitro *synthesis by DNA polymerase I and DNA polymerase α on DNA containing monoadducted purines or pyrimidines*

Termination products resulting from the replication of unmodified and modified M13mp10 single-stranded DNA template by the prokaryotic DNA polymerase I and eukaryotic DNA polymerase α, as resolved on sequencing gels, are shown in Fig. 1. Different extents of modification of the templates were performed and a typical experiment is presented. On unmodified DNA template (lane 1), DNA polymerase I synthesizes products of high molecular mass found on top of the gel (see full size M13 position), whereas DNA polymerase α synthesizes shorter and discrete products (lane 2). It can be noticed that, in this last case, a strong pause site is present (lane 2, arrow level) and it persists when the DNA template is modified (even lanes on Fig. 1).

When the DNA template is modified by the presence of monoadducts on either purines, as after treatment with *cis*-DDP (lanes 13—16) or pyrimidines as after exposure to furocoumarins in combination with UVA (lanes 3–12), the replication by DNA polymerase α is clearly more inhibited than that by DNA polymerase I (compare odd and even lanes 3–16 in Fig. 1). Indeed, less radioactivity migrates as full size molecules when DNA polymerase α replicates damage templates, compared to DNA polymerase I (see upper part of autoradiogram). Also, after *cis*-DDP modification, a number of major termination sites appear after synthesis by DNA polymerase α (Fig. 1, lanes 14 and 16) in the high as well as in the low molecular mass range, whereas such termination sites are reduced and limited to the large size range for DNA polymerase I (Fig. 1,

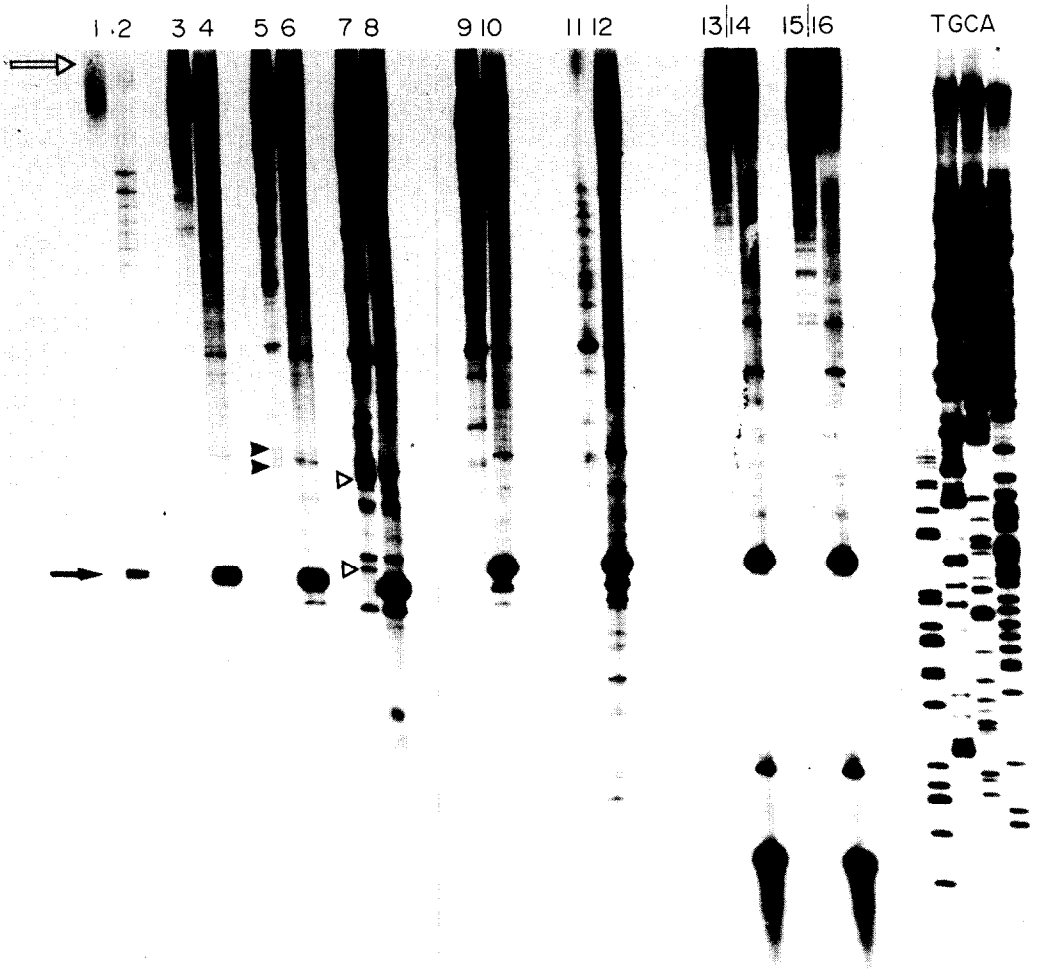

Fig. 1. Comparison of DNA replication by *E. coli* DNA polymerase I and *Drosophila* DNA polymerase α on furocoumarin- and *cis*-DDP-modified single-stranded DNA. DNA treatments are described in Materials and Methods. Even lanes represent the products of synthesis by DNA polymerase I, while odd lanes represent products of synthesis by DNA polymerase α. Lanes 1 and 2: untreated template; lanes 3 and 4: template treated with TMP and 500 kJ/m^2 of 405 nm UVA; lanes 5 and 6: template treated with TMP and 1000 kJ/m^2 of 405 nm UVA; lanes 7 and 8: template treated with TMP and 18 kJ/m^2 of 365 nm UVA; lanes 9 and 10: template treated with TMA and 3 kJ/m^2 of 365 nm UVA; lanes 11 and 12: template treated with TMA and 6 kJ/m^2 of 365 nm UVA; lanes 13 and 14: template treated with *cis*-DDP for 2.5 min at $ri = 1$; lanes 15 and 16: template treated with *cis*-DDP for 5 min at $ri = 1$. Open arrow indicates the migration position of full size M13 DNA. Closed arrow indicates a strong pausing site by *Drosophila* DNA polymerase α when copying undamaged template. Closed triangles indicate arrest sites by DNA polymerase I, not observed with DNA polymerase α, in the DNA synthesis on TMP-monoadducted template. Open triangles represent additional arrest sites by DNA polymerase I on template carrying TMP photoadducts (likely at interstrand cross-links). Note that less radioactivity was loaded in lanes 1, 2 and 11.

lanes 13 and 15), as previously reported [4]. As for *cis*-DDP monoadducts, DNA polymerase α is more inhibited than DNA polymerase I by furocoumarin monoadducts (compare odd and even lanes 3–12 in Fig. 1). As expected, the replication of template modified by furocoumarins plus UVA gives rise to a different pattern of bands, than does the replication of *cis*-DDP-modified template. It should be mentioned that a high extent of photoaddition on the template (DNA treated with TMA and UVA doses higher than 6 kJ/m^2) abolishes the difference in inhibition of replication between the two DNA polymerases (data not shown).

Inspection of different exposures of the autoradiogram shown in Fig. 1 and autoradiograms from different experiments revealed that, for the two enzymes, treatments known to induce only furocoumarin monoadducts (i.e. TMA plus 365 nm UVA and TMP plus 405 nm UVA) lead to similar band patterns with the exception of two more termination

sites at one position (indicated by closed triangles on lane 5) which are detected in the case of DNA polymerase I and are not seen with DNA polymerase α. When considering TMP photoaddition at 365 nm as opposed to 405 nm, two more termination sites of DNA polymerase I show up in the former case (see open triangles on lane 7) which may be due to interstrand biadducts (see below). At this point, a number of remarks can be made.

(i) According to quantification given in Miolo *et al.* [19] and in view of the low affinity of psoralen for single-stranded DNA, the number of photoadducts in the studied region of the template is inferior to one per fragment. This allows a good comparison of psoralen photoreactivity at each individual sites.

(ii) Furthermore, the number, the intensity and the localization of bands that show up on autoradiograms depend on the extent of photoaddition on the template.

(iii) Notably UVA fluences were chosen according to the low affinity of furocoumarins for single-stranded DNA, and their low absorption of 405 nm UVA light. For example, it is clear from the autoradiogram in Fig. 1 that the treatment of the template with TMP and 18 kJ/m^2 of 365 nm lead to more photoadditions than 1000 kJ/m^2 of 405 nm, as reflected by the presence of additional short termination sites (lane 7 vs lane 5). This is confirmed by the extent of inhibition of replication. Indeed, for DNA polymerase α, the extent of synthesis inhibition, determined by trichloroacetic acid precipitation of DNA (see Materials and Methods), is about 35, 50 and 65% for template treated with TMP plus 500 kJ/m^2, 1000 kJ/m^2 of 405 nm light, and 18 kJ/m^2 of 365 nm UVA light, respectively. For template treated with TMA plus 3 and 6 kJ/m^2 of 365 nm light, the inhibition of DNA synthesis by DNA polymerase α is 55 and 65%, respectively.

Distribution of termination sites at furocoumarins and cis-*DDP monoadducts in the DNA sequence*

Termination sites on *cis*-DDP and furocoumarins plus UVA-treated primed M13mp10 single-stranded DNA have been localized over a region of 150 nucleotides from the 3′ start of DNA synthesis and are reported in Fig. 2. The four main arrest sites on platinated template correspond to putative *cis*-DDP lesions at G in the following contexts GG, GAG or 5′GGA as already reported [4, 20]. Most of the arrest sites which were assigned after replication on TMA- or TMP-modified template can be interpreted as sites of potential furocoumarin photoadducts at pyrimidine positions. However, it has to be noted that, after examination of a number of autoradiograms, we reproducibly observed that DNA polymerase α terminates not only at potential sites of monoaddition, but also one nucleotide before. Elongation of the chain one or two nucleotides past a monoadduct was also found. Termination sites of DNA polymerase probably depends upon the sequence context. Our observed sites of photoaddition correspond to those produced by another furocoumarin, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT), plus UVA and revealed as termination sites of DNA polymerase I [21]. TMP and TMA exhibit very similar photobinding sites on double-stranded DNA as do HMT and other psoralen derivatives [19, 22]. It is worth mentioning that bands on autoradiograms corresponding to termination sites at TMP or TMA photoadducts show similar intensities (Fig. 1), indicating that neither compound exhibits preferential binding at specific sequences. This implies that, in contrast to the photoreaction with double-stranded DNA [19, 22], the photoreaction of TMP and TMA with single-stranded DNA do not show any strong sequence specificity. In contrast, in the case of *cis*-DDP, the same sequence specificity seems to exist for single and double stranded DNA [23, 24].

We previously investigated the existence of regions of secondary structure in the 150 nucleotides long sequence on which the arrest sites have been mapped [16]. The two major hairpin structures predicted on the basis of thermodynamic rules, one spanning from nucleotide 3 to 50 (stem A) and the other from

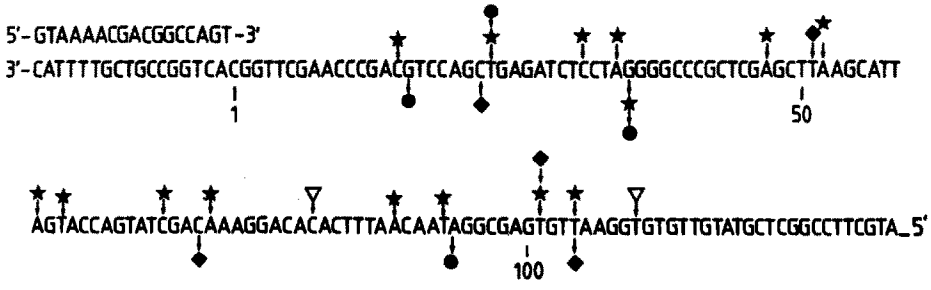

Fig. 2. Schematic diagram showing the portion of sequence (treated strand) used to monitor inhibition of DNA synthesis catalysed by DNA polymerases I and α. Nucleotide numbering is from the 3′OH end of the synthetic primer and arrest sites have been localized by comparing termination bands showing in Fig. 1 to the sequence of the intact primed single-stranded DNA determined by the dideoxy reaction procedure [21]. (●) Termination bands detected on platinated DNA; (◆) termination bands detected on TMP-treated DNA; (★) termination bands detected on TMA-treated DNA; (▽) additional arrest sites detected by DNA polymerase I on TMP photoadducts obtained at 365 nm likely at interstrand cross-links. For the sake of clarity, only a few arrest sites at TMP photoadducts are indicated.

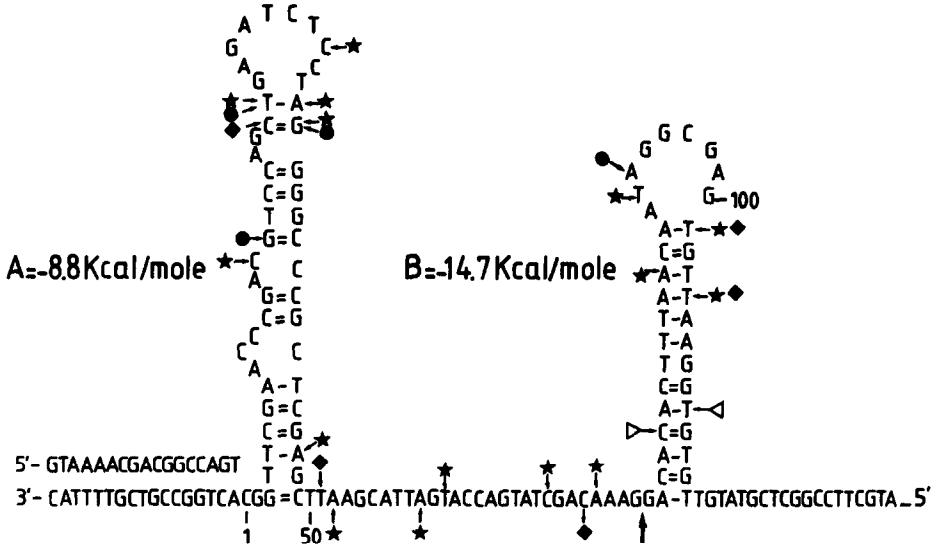

Fig. 3. Potential short-range secondary structure interactions. Along the 150 nucleotides of M13mp10 downstream the 3'OH end of the primer, the two stems which present a significant stability (free-energy $\Delta G^0 < -5$ kcal/mol) are identified by a capital letter (A and B) and their respective folding energy given. M13mp10 sequence is numbered from the 3'OH end of the primer. The arrow indicates the strong pausing site detected during the replication of this sequence by the eukaryotic α DNA polymerase. Symbols are as in Fig. 2.

nucleotide 79 to 113 (stem B) are shown in Fig. 3. These two hairpins show different stability, with only −8.8 kcal/mol for stem A, which presents several irregularities, and −14.7 kcal/mol for stem B. It is interesting to note that the GG sequence immediately preceding the more stable hairpin (see arrow of Fig. 3) is a very strong pause site observed during the replication of M13mp10 DNA template by the *Drosophila* DNA polymerase α (see Fig. 1, lane 2). The mode of replication of polymerase α is known to be particularly sensitive to the presence of secondary structure in the template [25, 26].

Combined treatment of nucleic acids with bifunctional furocoumarins (such as TMP) and irradiation at 365 nm, allow the formation of interstrand cross-links and is useful to investigate the presence of hairpin structures [27]. The first stem loop (A) does not exhibit any favorable site for the formation of TMP-photoinduced interstrand cross-links (i.e. existence of 5'TpA and 5'TpG on one of the complementary strand). On the contrary, the stem B contains three putative cross-linkable sites among which two are located at the foot of the hairpin. The additional arrest sites, produced by TMP plus 365 nm radiation and likely to occur at interstrand crosslinks, are observed at these two last positions (Fig. 3). This is an additional argument in favor of the existence and the stability of this hairpin (B). Also, the increased intensity of the pause site on photoadducted template is in favour of a stabilization of this hairpin by photoadducts. The configuration of the less stable hairpin would probably prevent stabilization by intercalated photoadducts.

DISCUSSION

The capacity of the prokaryotic DNA polymerase I Klenow fragment and of the eukaryotic DNA polymerase α to replicate *in vitro* single stranded DNA containing either purine or pyrimidine adducts, has been examined in a number of laboratories. As far as pyrimidine dimers are concerned, DNA synthesis by both enzymes appears to be essentially blocked at such a lesion [28]. However, in particular experimental conditions (i.e. high concentration of deoxyribonucleotides triphosphate and dNTPs bias) a certain extent of translesion synthesis is observed in the case of DNA polymerase I [29]. Similarly, intrastrand cross-links involving purines, such as those produced by *cis*-DDP, inhibit DNA polymerase I and DNA polymerase α to a large extent [22]. Therefore, lesions involving two bases, either purines or pyrimidines, appear to have a similar effect on the *in vitro* DNA replication of single stranded DNA by prokaryotic and eukaryotic enzymes. *cis*-DDP has been shown initially to form a monoadduct with a guanine on single stranded DNA. Such a lesion does not seem to arrest DNA synthesis by *E. coli* DNA polymerase I, while it appears to affect DNA synthesis by DNA polymerase α [4].

In order to establish if these observations hold for pyrimidine monoadducts, we compared the capacity of the two enzymes to replicate single stranded DNA containing furocoumarin monoadducts. We show that, as in the case of *cis*-DDP monoadducts, DNA polymerase α is more inhibited than DNA polymerase I by TMA and TMP monoadducts. In other words, it appears that, at least *in vitro*, the prokaryotic

DNA polymerase bypasses purines as well as pyrimidine monoadducts on replicating single-stranded DNA, more efficiently than the eukaryotic DNA polymerase. It is worth mentioning that bypass of HMT monoadducts on double-stranded DNA template has been observed *in vitro* using a nick-translation assay and the bacterial DNA polymerase I [30]. However, bypass of psoralen monoadducts in yeast cells, i.e. implying eukaryotic DNA polymerase(s), has been reported [31].

It is of interest to note that the two enzymes replicate the intact template in a different manner (see lanes 1 and 2 on Fig. 1), the eukaryotic one being less efficient in synthesizing high molecular mass products. Polymerization by DNA polymerase α is known to be more sensitive to the presence of secondary structures in single-stranded DNA [25, 26]. This may be the consequence of its fairly distributive mode of replication *in vitro*. This mode of synthesis might affect its behaviour toward monofunctional adducts, rendering this enzyme more sensitive to these lesions in comparison to DNA polymerase I. Indeed, the latter polymerase has been reported to be more processive in replicating single stranded template [25, 32]. A comparison of the capacity of replicating monoadducted single-stranded DNA between DNA polymerase α and highly processive DNA polymerases, such as DNA polymerases ε and δ plus proliferating cell nuclear antigen [33], will be necessary in order to verify this possibility.

A computer search for duplex stability into the first 150 nucleotides of the M13mp10 single stranded template used here suggested the existence of two regions of secondary structure [16]. An additional proof of the presence of these stem loops is the occurrence of the strong pause site immediately preceding the stem B, during the replication of M13mp10 DNA template by DNA polymerase α. Our data generated with TMP plus UVA (365 nm), i.e. arrest sites of DNA polymerase I possibly at interstrand cross-links, are in agreement with the existence of such secondary structures. The strong pause site of DNA polymerase α prior to the stem is likely to be responsible for the absence of arrest sites of DNA polymerase α at cross-links.

It is well established that monoadducts induced either by *cis*-DDP [34] or by photoaddition of monofunctional psoralens [10] are poorly mutagenic in both prokaryotes and eukaryotes. This may be related to a relatively efficient bypass of such lesions by the DNA polymerases, as reported here. Moreover, in this context, we have to assume a high fidelity of the bypass process.

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