Sites of termination of *in vitro* DNA synthesis on psoralen phototreated single-stranded templates

JACQUES PIETTE† and JOHN HEARST

Department of Chemistry, University of California,
Berkeley, CA 94720, U.S.A.

(Received 6 December 1984; revision received 11 February 1985;
accepted 18 February 1985)

Single-stranded DNA has been photochemically induced to react with 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) and used as substrate for DNA replication with *E. coli* DNA polymerase I large fragment. By using the dideoxy sequencing procedure, it is possible to map the termination sites on the template photoreacted with HMT. These sites occur at the nucleotides preceding each thymine residue (and a few cytosine residues), emphasizing the fact that in a single-stranded stretch of DNA, HMT reacts with each thymine residue without any specificity regarding the flanking base sequence of the thymine residues. In addition, termination of DNA synthesis due to psoralen-adducted thymine is not influenced by the efficiency of the 3'-5' exonuclease proof-reading activity of the DNA polymerase.

Indexing terms: single-stranded DNA, psoralen, termination sites of DNA synthesis.

1. Introduction

Cycloadducts between psoralens and pyrimidine bases are formed by irradiation of a complex between DNA and psoralen with 365 nm light (Musajo and Rodighiero 1972, Isaacs *et al.* 1977). Although active excision and recombination repair mechanisms exist in the cells (see Hanawalt *et al.* 1979, Song and Tapley 1979) which restore the normal base sequence, a psoralen cycloadduct can be encountered by the replicating machinery during DNA synthesis. However, nothing is known about how DNA polymerase deals with psoralen adducts, but one would expect such a bulky lesion to be non-instructive and to be a block to DNA synthesis. It has been shown, however, that psoralens which possess the ability to cross-link two DNA strands inhibit DNA replication to a much greater extent than those exclusively forming monoadducts (Bordin *et al.* 1975). On the other hand, there is evidence (Averbeck and Moustacchi 1980, Drake and McGuire 1967) that the psoralen photoreaction can lead to mutation. Since the establishment of mutant clones require a viable organism, mutagenesis implies that mechanisms exist which permit bypass of the block to replication (Radman *et al.* 1977, Chanet *et al.* 1983) and/or repair of the lesion by an error prone pathway (Wikin 1976). The molecular mechanisms that control termination of replication or pausing at specific sites on a template (Huang and Hearst 1980) remain to be elucidated.

In this study, we have followed DNA synthesis, initiated at a single point on the

† Present address: Laboratory of General and Medical Microbiology, Institute of Pathology (B23), University of Liège, B4000 Liège, Belgium.

psoralen-photoreacted genome of single-stranded bacteriophages, by looking at the length of the synthesized polynucleotides and using the dideoxy DNA sequencing method as a standard. We provide clear evidence that psoralen adducts cause termination of DNA synthesis exactly one base before the thymine cycloadduct, irrespective of the efficiency of the proof-reading activity of the enzyme used for the replication.

2. Materials and methods

2.1. Nucleotides and other chemicals

Unlabelled deoxynucleoside triphosphates and dideoxynucleoside triphosphates were purchased from P.L. Biochemicals; α -[^{32}P]dATP was from Amersham. All other chemicals were reagent grade.

2.2. Enzymes

E. coli DNA polymerase I large fragment and T_4 DNA polymerase were purchased from BRL. Avian myeloblastosis virus (AMV) reverse transcriptase was provided by J. W. Beard (Life Science Center, U.S.A.). Restriction endonuclease Hae III was from BRL.

2.3. Preparation of single-stranded ϕ X174 and M13 mp10 DNAs

ϕ X174 single-stranded DNA was prepared from the purified virions according to the technique described by Piette and Moore (1982). Following phenol extractions, ϕ X174 DNA was extracted three times with ether and then precipitated with ethanol. The ϕ X174 DNA concentration was estimated on the basis of a molar extinction coefficient for DNA phosphorus of 8250 at 260 nm in 0.2 mol dm^{-3} NaCl. The double-stranded DNA of ϕ X174 (ϕ XRFI DNA) was purchased from BRL; it was digested with restriction endonuclease Hae III and the restriction fragments were recovered from the agarose gel by electroelution. They were then cleaned up by extraction, once with phenol and once with chloroform-isoamyl alcohol (24/1, v/v), twice with ether and then ethanol precipitated. The DNA was resuspended in 10 mol dm^{-3} Tris-HCl (pH 7.5) 0.1 mmol dm^{-3} EDTA and spin chromatographed through Sephadex G-50. All the DNA materials were checked for purity by analytical electrophoresis on agarose gels. To be used as template the ϕ X174 DNA was annealed with a purified denatured restriction fragment as described by Piette and Moore (1982). M13 mp10 single-stranded DNA was prepared according to Messing (1983) and annealed at 55°C for 5 min with the 15-base universal sequencing primer (P.L. Biochemicals).

2.4. Photoreaction

Solutions of $50 \mu\text{l}$ containing $0.1 \mu\text{g}$ DNA/ml in 10 mmol dm^{-3} Tris-HCl (pH 8.0) 1 mmol dm^{-3} EDTA were photoreacted with [^3H]HMT (4'-hydroxymethyl-4,5',8-trimethyl-psoralen; HRI Associated Inc, Emeryville, CA, U.S.A.) using an initial ratio of one HMT molecule per ten bases in either 5 or 50 mmol dm^{-3} NaCl. The DNA-HMT mixtures were irradiated at 10°C (1) with two 100 sA General Electric mercury vapour lamps and the irradiation chamber contained a cobaltous nitrate solution (40 per cent, w/w) acting as an optical filter which removed light outside the range of 340–380 nm; and (2) with a XBO-150 sA lamp (Osram) coupled with a WG320 filter (Schott, F.R. Germany) removing light below 300 nm.

The photoreacted DNAs were taken from the irradiation chamber and the non-covalently bound drugs were removed from DNA molecules by two chloroform and two ether extractions followed by the ethanol precipitation.

2.5. Synthesis of the complementary strand

Standard polymerization for sequencing gel analysis was carried out as described by Piette and Moore (1982) and Merville *et al.* (1984), using 10 μ l reaction mixtures containing α -[32 P]dATP (1 μ Ci), 50 ng photoreacted annealed DNA and one of the following enzymes: the large fragment of *E. coli* DNA polymerase I (Pol I) (0.2 units); T₄ DNA polymerase (T₄ DNA Pol) (2 units), or AMV reverse transcriptase (2.5 units). The amount of each polymerase used was selected to give maximum incorporation of label. The polymerization reactions were carried out in the buffers recommended by the enzyme suppliers. Incubations were carried out at room temperature for Pol I, and at 37°C for T₄ DNA Pol and AMV reverse transcriptase. After 15 min, 12 μ l of 0.5 mmol dm⁻³ stock of each of the four dNTPs were added and incubations were continued for an additional 15 min. When the polymerization was done on ϕ X174, the synthesized strand was cleaved from the primer by 45 min restriction at 37°C with 1.0 unit of Hae III. The DNAs were then ethanol precipitated in the presence of 0.5 per cent SDS, 200 mmol dm⁻³ NaCl, 20 mmol dm⁻³ Tris-HCl (pH 7.2), 10 mmol dm⁻³ EDTA. On unirradiated template, the sequencing of the DNA complementary chain was performed following the technique described by Sanger *et al.* (1977). The synthesized products were denatured in 98 per cent formamide containing 0.3 per cent bromophenol blue, 0.3 per cent xylene cyanol and 0.1 mmol dm⁻³ EDTA and separated by high voltage electrophoresis on polyacrylamide-urea gels.

3. Results

3.1. Mapping of the M13 mp10 replication termination sites induced by HMT photoreaction

The photoreaction of M13 mp10 DNA with HMT during various periods of time gave rise to a linear increase of the amount of HMT covalently bound to DNA. These conditions yielded up to 20 HMT covalent additions per M13 mp10 genome (figure 1(a)). The DNA was used as template for DNA replication by Pol I and using polyacrylamide gel electrophoresis run in denaturing conditions, the products of the replication can be separated according to their length (figure 1(b)). The potential blocks of synthesis due to polymerase halts on the photoreacted templates were precisely determined by running the synthesized products alongside with a dideoxy nucleotide chain terminating procedure using the corresponding primed non-photoreacted template. Figure 1(b) shows the analysis of the synthesis products and reveals numerous bands of increased intensities with irradiation time. These bands did not show up when the polymerization was carried out on unphototreated M13 mp10 DNA. The polymerization stops occurred mainly one base before adenine residues in the synthesized strand, thus corresponding to a modification by psoralen of thymine residues in the template strand. In a few cases, polymerization stopped one base before guanine residues corresponding to psoralen adducts on cytosine residues in the template (figure 1(c)). From these data, it appeared that all the thymine residues acted as a block for DNA replication and the polymerase stopped exactly one base before the HMT adduct to thymine. Moreover, all the thymine residues had undergone photoreaction with psoralen; thus in single-

Figure 1. (a) Kinetics of photoaddition of [^3H]HMT to M13 mp10 DNA. The amount of covalent bound drug per M13 mp10 genome is plotted against irradiation time (min). Aliquots were withdrawn during irradiation and are chloroform- and ether-extracted and ethanol precipitated before counting. (b) Denaturing polyacrylamide gel (8 per cent) analysis of the products synthesized by Pol I on M13 mp10 DNA photoreacted with HMT during 0, 5, 10, 20 and 30 min. Lanes G, A, T and C refer to the dideoxysequencing standard synthesized by Pol I on the untreated template. (c) Position of the chain termination sites on M13 mp10 DNA. On the synthesized strand, the arrows indicate the position of the various bands and the stars indicate the position of the adducted bases on the template strand.

Figure 2. Position of the chain termination sites for ϕ X174 DNA primed with restriction fragment 2. The arrows and the stars indicate the position of the bands and the adducted bases respectively.

stranded DNA, HMT did not seem to display some specificity regarding the flanking base sequences of thymine residues.

3.2. Sites of DNA synthesis termination of ϕ X174 template photoreacted by HMT

Restriction fragments were used in this study in order to prime the complementary strand at different places in the ϕ X174 genome. Fragments 1, 2, 3 and 4 obtained by ϕ X174 (ϕ RF₁ DNA) digestion with Hae III had their 3'OH end situated at nucleotide 1776 (gene F) for fragment 1, at 3129 (gene H) with 2, at 4949 (gene A) with 3 and a position 1173 (gene F) with 4. The same experimental approach as described above was used to extend to ϕ X174 DNA, the characterization of the exact termination position of DNA synthesis.

As was found with M13 mp10 DNA, DNA polymerization carried out on unphototreated ϕ X174 or ϕ X174 DNA irradiated alone led to the formation of high molecular weight polynucleotides remaining at the top of the gel. Similarly, the visualization of several enzyme pausing sites is probably due to the secondary structure in the single-stranded template. A similar pattern was also observed when the polymerization was carried out on ϕ X174 DNA photoreacted with HMT. Figure 2 shows the sequencing data obtained when the photoreacted ϕ X174 DNA was primed with fragment 2. Similar patterns were also obtained when the polymerization was initiated at other places, reinforcing the idea that the HMT photoaddition on thymine residues was absolutely non-sequence specific on the single-stranded DNA template. This study was also extended to other replicating enzymes which had different exonuclease activities. In fact, T₄ DNA Pol did not display 5'→3' exonuclease activity but did have an extraordinarily active 3'→5' exonuclease which was 250 times more active than the Pol I proof-reading activity. On the other hand, AMV reverse transcriptase did not have a detectable exonuclease activity and is known as a polymerase making high levels of misincorporation when copying DNA (Kornberg 1980). Replication was thus performed with these two enzymes using photoreacted ϕ X174 DNA primed with fragment 1. The polymerization products were analysed by denaturing polyacrylamide gel electrophoresis and an identical termination pattern as described in figure 2 was obtained with these two enzymes.

4. Discussion

Psoralens are nucleic acid photoreagents which form mono- and diadducts to pyrimidine bases upon irradiation with long wavelength ultraviolet light (Isaacs *et al.* 1982). In single-stranded DNA and RNA molecules, psoralens can only form monoadducts in the single-stranded stretch of the molecule, but they can be involved in the formation of both monoadducts and interstrand bridges in the intramolecular helical regions of secondary structures. Regions exhibiting secondary structures are

often sites of important biological functions acting, for example, as origins of replication in DNA synthesis (Geider and Kornberg 1974), as initiation sites for transcription (Schaller *et al.* 1975) and as sites for RNA processing (Bass and Cech 1984). One of the conclusions from the results described above is that Pol I is stopped exactly one base before a HMT monoadduct in single-stranded regions of ϕ X174 and M13 DNA. Among the 700 nucleotides sequenced, all the bands observed reflected stops occurring one base before psoralen-modified thymine residues. Although, psoralen can form monoadducts in DNA either on the 5' or 3' side of thymine residues thus corresponding to the two possible diastereoisomeric photocycloadducts (Straub *et al.* 1981), the termination of the synthesis always occurs one base before the psoralen thymine adduct. Pol I, which copies the template strand in the 3'→5' direction, cannot insert a nucleotide opposite the psoralen modified base even if the adduct is on the 5' side of the base in the template strand. Similar results have been reported in the case of single-stranded DNA modified with *N*-acetoxy-*N*-acetyl-2-aminofluorene (*N*-AcO-AAF) which can form a guanosyl-*N*-AcO-AAF adduct (Kriek 1974) and with DNA reacted with benzo(α)pyrene (Moore and Strauss 1979).

Polymerization on HMT photoreacted ϕ X174 DNA has also been carried out using two other polymerases: (i) T₄ DNA Pol, which has a highly active proof-reading activity; and (ii) the viral AMV reverse transcriptase, which has a polymerizing function analogous to most known DNA polymerases but has no detectable exonuclease activity (Baltimore and Smoler 1971). On *N*-AcO-AAF-adduct DNA templates, Pol I and T₄ DNA Pol terminate immediately preceding the lesion, whereas polymerases without proof-reading activity (AMV reverse transcriptase and human DNA polymerase α) terminate at the site of the lesion, and thus can insert a nucleotide complementary to the adducted base (Moore *et al.* 1982), this nucleotide being removed out the growing chain by the 3'→5' exonuclease activity. The data presented above clearly show that all three enzymes used terminate before the adduct and demonstrate that the single-stranded DNA synthesis termination induced by psoralen photoreactions is a process that does not depend on the proof-reading activity associated with many polymerases, reinforcing the idea that the polymerases do not even insert a nucleotide opposite the psoralen base adduct.

It is obvious that DNA structure must affect enzyme behaviour. Kinetic experiments carried out in an earlier study on a double-stranded circular DNA photoreacted with psoralen showed that Pol I holoenzyme can bypass a psoralen monoadduct (Piette and Hearst 1983). Furthermore, the rate of synthesis of the enzyme is attenuated only when the adduct is situated on the coding strand (Piette and Hearst 1983).

In conclusion, psoralen monoadduct-terminated DNA synthesis can thus be pointed out as a lethal lesion for single-stranded DNA bacteriophages where the first event in their replication cycle is the synthesis of the complementary strand to the viral DNA. Psoralen adducts on thymine or cytosine residues are the only DNA lesions detected in these experiments and must be responsible for all the biological disorders consecutive to HMT photoreaction. In aerated aqueous solutions and in the presence of DNA, HMT does not seem to have a photodynamic effect because no guanine residues have been found to be able to block the complementary chain growth. In addition, it cannot be argued that polymerase bypasses photooxidized guanine residues because they were shown to act as replication blocks after single-stranded photosensitization mediated by proflavine (Piette *et al.* 1984).

Acknowledgments

We thank Dr. H. Gamper and S. Lipson for many helpful discussions and for reading the manuscript. Professor A. Van de Vorst's thoughtful comments have been appreciated. J. Piette is a research associate of the National Fund for Scientific Research (Brussels, Belgium).

References

- AVERBECK, D., and MOUSTACCHI, E., 1980, Decreased photo-induced mutagenicity of monofunctional as opposed to bifunctional furocoumarins in yeast. *Photochemistry and Photobiology*, **31**, 475-478.
- BALTIMORE, D., and SMOLER, D., 1971, Primer requirement and template specificity of the DNA polymerase of RNA tumor virus. *Proceedings of the National Academy of Sciences, U.S.A.*, **68**, 1507-1511.
- BASS, B. L., and CECI, T. R., 1984, Specific interaction between the self-splicing of RNA of Tetrahymena and its guanosine substrate: implications for biological catalysis by RNA. *Nature, London*, **308**, 820-826.
- BORDIN, F., MARCIANI, J., BACHICHETTI, F., DALL' ACQUA, F., and RODIGHIERO, G., 1975, Studies on the photosensitizing properties of Angelicin and angular Furocoumarins forming monofunctional adducts with pyrimidine bases of DNA. *Italian Journal of Biochemistry*, **24**, 258-267.
- CHANET, R., CASSIER, C., MAGAÑA-SCHENCKE, N., and MOUSTACCHI, E., 1983, Fate of photo-induced 8-methoxypsoralen monoadducts in yeast. Evidence for bypass of these lesions in the absence of excision repair. *Mutation Research*, **112**, 201-214.
- DRAKE, J. W., and MCGUIRE, J., 1967, Properties of r mutants of bacteriophage T₄ photodynamically induced in the presence of thiopyronine and psoralen. *Journal of Virology*, **1**, 260-265.
- GEIDER, R., and KORNBERG, A., 1974, Conversion of the M13 viral single-strand to the double stranded replicative form by purified proteins. *Journal of Biological Chemistry*, **249**, 3999-4005.
- HANAWALT, P. C., COOPER, P. K., GANESAN, A. K., and SMITH, C. A., 1979, DNA repair in bacteria and mammalian cells. *Annual Review of Biochemistry*, **48**, 783-836.
- HUANG, C. C., and HEARST, J. E., 1980, Pauses at positions of secondary structure during *in vitro* replication of single-stranded fd bacteriophage DNA by T₄ polymerase. *Analytical Biochemistry*, **103**, 127-139.
- ISAACS, S. T., SHEN, C. K. J., HEARST, J. E., and RAPOPORT, H., 1977, Synthesis and characterization of new psoralen derivatives with superior photoreactivity with DNA and RNA. *Biochemistry*, **16**, 1058-1064.
- ISAACS, S. T., CHUN, C., HYDE, J. E., RAPOPORT, H., and HEARST, J. E., 1982, A photochemical characterization of reactions of psoralen derivatives with DNA. *Trends in Photobiology*, edited by C. Hélène, M. Charlier, Th. Montenay-Garestier and G. Laustriat (New York: Plenum Press), pp. 279-294.
- KORNBERG, A., 1980, *DNA Replication*, edited by A. C. Bartlett (San Francisco: W. H. Freeman), pp. 167-201.
- KRIEK, E., 1974, Carcinogenesis by aromatic amines. *Biochimica et Biophysica Acta*, **355**, 171-203.
- MERVILLE, M. P., PIETTE, J., LOPEZ, M., DECUYPER, J., and VAN DE VORST, A., 1984, Termination sites in the *in vitro* DNA synthesis on single-stranded DNA photosensitized by promazines. *Journal of Biological Chemistry*, **259**, 15069-15077.
- MESSING, J., 1983, New M13 vectors for cloning. *Methods in Enzymology*, **101**, 20-89.
- MOORE, P. D., and STRAUSS, B. S., 1979, Sites of inhibition of *in vitro* DNA synthesis in carcinogen and UV-treated ϕ X174 DNA. *Nature, London*, **278**, 664-666.
- MOORE, P. D., RABKIN, S. D., OSBORN, A. L., KING, C. M., and STRAUSS, B. S., 1982, Effect of acetylated and deacetylated 2-aminofluorene adducts on *in vitro* DNA synthesis. *Proceedings of the National Academy of Sciences, U.S.A.*, **79**, 7166-7170.
- MUSAJO, L., and RODIGHIERO, G., 1972, Mode of photosensitizing action of furocoumarins. *Photophysiology*, **7**, 115-121.

- PIETTE, J., and MOORE, P. D., 1982, DNA synthesis on ϕ X174 template damaged by proflavine and light treatment. *Photochemistry and Photobiology*, **35**, 705–708.
- PIETTE, J., and HEARST, J. E., 1983, Termination sites of *in vitro* nick translation reaction on DNA that had photoreacted with psoralen. *Proceedings of the National Academy of Sciences, U.S.A.*, **80**, 5540–5544.
- PIETTE, J., CALBERG-BACQ, C. M., LOPEZ, M., and VAN DE VORST, A., 1984, Termination sites of DNA synthesis on 'proflavine and light' treated ϕ X174 single-stranded DNA. *Biochimica et Biophysica Acta*, **781**, 257–264.
- SANGER, F., NICKLEN, S., and COULSON, A. R., 1977, DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, U.S.A.*, **74**, 5463–5467.
- SCHALLER, H., GRAY, C., and HERMANN, H., 1975, Nucleotide sequence of an RNA polymerase binding site from the DNA of bacteriophage fd. *Proceedings of the National Academy of Sciences, U.S.A.*, **72**, 737–741.
- SONG, P. S., and TAPLEY, K. J., Jr, 1979, Photochemistry and photobiology of psoralens. *Photochemistry and Photobiology*, **29**, 1177–1197.
- STRAUB, R., KANNE, D., HEARST, J. E., and RAPOPORT, H., 1981, Isolation and characterisation of pyrimidine-psoralen photoadducts from DNA. *Journal of the American Chemical Society*, **103**, 2347–2355.
- RADMAN, M., VILLANI, G., BOITEUX, J., DEFAIS, M., CAILLET-FAUQUET, P., and SPADARI, S., 1977, On the mechanism and genetic control of mutagenesis induced by carcinogenic mutagens. *Origins of Human Cancer*, edited by H. H. Hiatt, J. D. Watson, and J. A. Winsten. Cold Spring Harbor conferences on cell proliferation, vol. 4, (New York: C.S.H.), pp. 903–922.
- WITKIN, E. M., 1976, Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*. *Bacteriological Reviews*, **40**, 869–907.