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Efficient Synthesis of a Supercoiled M13 DNA Molecule Containing a Site Specifically Placed Psoralen Adduct and Its Use as a Substrate for DNA Replication†

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ABSTRACT: We report a simple method for the in vitro synthesis of large quantities of site specifically modified DNA. The protocol involves extension of an oligonucleotide primer annealed to M13 single-stranded DNA using part of the T4 DNA polymerase holoenzyme. The resulting nicked double-stranded circles are ligated and supercoiled in the same tube, producing good yields of form I DNA. When the oligonucleotide primer is chemically modified, the resultant product contains a site-specific lesion. In this study, we report the synthesis of an M13 mp19 form I DNA which contains a psoralen monoadduct or cross-link at the *Kpn*I site. We demonstrate the utility of these modified substrates by assessing the ability of the bacteriophage T4 DNA replication complex to bypass the damage and show that the psoralen monoadduct poses a severe block to the holoenzyme when attached to the template strand.

There is considerable interest in the mechanism by which various structural modifications of DNA influence aspects of nucleic acid metabolism such as replication, transcription, and repair. These include lesions due to radiation, chemical carcinogens, and certain drugs. In vitro enzymatic studies have been hampered by the difficulty of producing workable quantities of DNA modified at a unique position. Such substrates would allow far more detailed questions to be asked and would yield more easily interpretable results than experiments using randomly modified nucleic acids. We report here a simple method for the in vitro synthesis of large quantities of supercoiled M13 double-stranded DNA containing a uniquely placed psoralen monoadduct or cross-link. The method is general and should be amendable to the synthesis of substrates with many other types of structural modifications.

Psoralens are a class of linear, tricyclic, aromatic molecules which readily intercalate into double-stranded nucleic acid. In the presence of near-ultraviolet light, these compounds react to form monoadducts and interstrand cross-links primarily with thymidine in DNA and with uridine in RNA [reviewed by Cimino et al. (1985)]. This activity has made them useful as chemotherapeutic agents in the treatment of several dermatological disorders including psoriasis and vitilago [for review, see Fitzpatrick et al. (1982) and Parrish et al. (1982)]. As part of an effort to better understand the biological consequences of these compounds, we have investigated the ability of the "core" T4 replication complex to synthesize past a psoralen monoadduct using a site specifically modified M13 substrate. We found the synthesis, which was initiated from a unique strand-specific nick, to be blocked by the monoadduct.

Materials and Methods

Preparation of Proteins and DNA. The phage T4 43, 44/62, and 45 proteins were purified by using the protocols developed by W. Konigsberg and co-workers, Yale University (personal communication). T4 DNA ligase was purchased from Bethesda Research Laboratories. DNA gyrase was a gift from N. Cozzarelli. Phage fd gene II protein was purified by the literature procedure (Dotto et al., 1981). M13 mp 19 single-stranded and double-stranded DNAs were prepared by the procedures described by Messing (1983).

Preparation of 4-(Hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT)-Monoadducted 13-mer. The 13-mer 5′-GCTCGGTACCCGG-3′ was synthesized on a Biosearch instrument using phosphotriester chemistry. Following deprotection, full-length product was isolated by electrophoesis on a 7 M urea–20% polyacrylamide gel. The desired band was detected by placing the gel over a thin-layer chromatography plate impregnated with a fluorophore and briefly illuminating in the dark with a shortwave ultraviolet lamp. After the band was excised, the 13-mer was extracted into 10 mM NaCl–1 mM ethylenediaminetetraacetic acid (EDTA), ethanol precipitated, and dissolved in 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl)–1 mM EDTA (TE).

Photochemical modification of the 13-mer with 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) was carried out by using the two-step procedure described by Van Houten et al. (1986) and Gamper et al. (1987) or, more recently, by a simpler one-step procedure. Using the latter procedure, we obtained preparative amounts of psoralen-monoadducted ol-

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igomer by photoreacting 158 μg of kinased 13-mer (containing trace amounts of ^{32}P) with 104 μg of a complementary 8-mer (5′-GGGTACCG-3′; unkinased) and 45 μg of HMT (HRI Associates, Berkeley, CA) in 1.5 mL of 20 mM Tris-HCl (pH 7.0), 100 mM NaCl, 10 mM $MgCl_2$, and 1% ethanol. This solution was irradiated at 4 °C for 40 min with 380–400-nm light followed by a second irradiation after the addition of a fresh aliquot of HMT. The desired broad-band light was obtained from a 500-W elliptically focused Xe arc lamp whose output was passed through a Pyrex filter, an aqueous 1.7% cobaltous nitrate–2% sodium chloride filter, and a 380-nm cutoff filter. Irradiations were conducted in a 1-cm quartz cuvette placed within a temperature-regulated holder. Following irradiation, the DNA was precipitated with ethanol, dissolved in loading buffer, and electrophoresed on a 0.4-mm-thick 20% sequencing gel. Three bands, corresponding to unmodified 13-mer, HMT-monoadducted 13-mer, and HMT-cross-linked 13-mer/8-mer hybrid, were detected by autoradiography. The monoadducted material was extracted, precipitated with ethanol, and dissolved in TE buffer as described above. On the basis of recovered counts, 35 μg of HMT-monoadducted 13-mer was obtained.

Construction of Modified DNA. Single-stranded circular M13 mp19 DNA (24 μg) was mixed with a 5-fold molar excess of HMT-modified or control (unmodified) 13-mer (215 ng) in 40 μL of replication buffer (0.5 mM spermine, 0.3 mM spermidine, 65 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, and 10 mM Tris–acetate, pH 7.4) and incubated at 37 °C for 20 min. Immediately prior to the start of synthesis, a separate solution was prepared consisting of 530 μL of H_2O, 145 μL of 6.7× replication buffer, 2 μL of 50 mg/mL bovine serum albumin, 55 μL of 37 mM rATP, 182 μL of dNTPs (2 mM each), 10 μL of 380 μg/mL gene 43 protein, 6.7 μL of 3.93 mg/mL gene 45 protein, and 17.8 μL of 1.42 mg/mL gene 44/62 protein. Care was taken to add the proteins last, after which the solution was mixed with the primed DNA and synthesis allowed to proceed for 60 min at 37 °C. The nicked double-stranded circular product was ligated during a 45-min incubation at 37 °C with 14.3 μL of T4 DNA ligase (1 unit/μL). When desired, gyration was carried out by preincubating 1.1 μL of subunit A (800 units/μL) with 5.0 μL of subunit B (160 units/μL) at 4 °C for 10 min. The gyrase complex was added to the ligated DNA, and the solution was incubated for 60 min at 37 °C. The enzymes were inactivated by adding 42 μL of 5 M NaCl, 50 μL of 0.4 M EDTA, and 10 μg/μL proteinase K and incubating for 30 min at 37 °C. Deproteinization was effected by equal-volume phenol, chloroform–isoamyl alcohol (19:1), and ether extractions. The DNA was ethanol precipitated and washed with 70% ethanol, and the pellet was dissolved in 200 μL of H_2O.

Covalently closed circular DNA was isolated by a modification of the procedure described by Gamper et al. (1985). After addition of 22 μL of 10× Exo III buffer (50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and 0.5 M Tris-HCl, pH 8.0), the DNA was incubated for 60 min at 37 °C with 5 μL of exonuclease III (65 units/μL). An aliquot of the solution was analyzed on a 1% agarose gel to verify that all nicked circular and linear DNA had been converted to single-stranded material. If the conversion was incomplete, incubation was continued with additional enzyme as necessary. Deproteinization of the DNA solution was found to improve the yield of form I DNA, and therefore equal-volume phenol, chloroform–isoamyl alcohol, and ether extractions were performed. The DNA was ethanol precipitated as usual and dissolved in 200 μL of a buffer containing 20 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1 M NaCl. To this solution was added 100 mg of BND-cellulose (benzoylated naphthoylated DEAE-cellulose; Sigma). The suspension was mixed vigorously and then centrifuged. The duplex-containing supernatant was saved, while the BND-cellulose pellet was washed twice with 200 μL of the same buffer. Form I DNA was recovered from the combined supernatants by precipitation with an equal volume of isopropyl alcohol.

For preparation of the diadduct, HMT-modified M13 DNA was driven to the cross-linked form prior to the BND-cellulose extraction step, thereby utilizing this step to remove non-cross-linked DNA. Irradiation of the DNA was carried out for 5 min in 50 μL of H_2O at 4 °C with 400 mW/cm^2 of 320–400-nm light from an elliptically focused 500-W Hg/Xe arc lamp. Non-cross-linked form I DNA was irreversibly denatured by treatment with 100 μL of 0.3 M potassium phoshate, pH 13.2, for 7 min at 37 °C followed by neutralization with 75 μL of 1 M potassium phosphate, pH 4.0 (Pouwels et al., 1968; Kuhnlein et al., 1976). Prior to extraction with BND-cellulose as described above, phosphate was removed by dialysis, be gel filtration, or by washing in a Centricon-30 cartridge (Amicon). In this procedure, it is important to use phosphate buffers; in other buffers, non-cross-linked form I DNA may renature after transient exposure to high pH.

DNA Synthesis Reactions. Replication assays were done as described by Jongeneel et al. (1983). Briefly, 0.3 μg of form I DNA, either psoralenated or unmodified, was nicked in vitro with a 4-fold excess of purified gene II protein. After heat denaturation of the protein, the T4 43 (0.3 μg), 44/62 (2.5 μg), and 32 (10 μg) proteins, ATP (2 mM), and dATP, dGTP, and dTTP (0.15 mM each) were added. The reactions were incubated for 2 min at 37 °C in the absence of dCTP in order to allow all of the template DNA to acquire a holoenzyme complex (present in 40-fold molar excess over the template) and to synchronize the replication forks. [α-^{32}P]dCTP (1500 cpm/pmol) was then added to initiate the reaction. At various times, aliquots were removed and either spotted on a glass fiber disk for the determination of acid-insoluble counts or made 20 mM in EDTA and 30 mM in NaOH and electrophoresed through a 0.8% alkaline agarose gel. The gel was neutralized and dried onto DE-81 paper (Whatman), and the DNA bands were visualized by autoradiography.

RESULTS

Synthesis of Supercoiled DNA. Our method for the synthesis of supercoiled DNA in vitro consists of four simple steps, all of which are conducted in the same tube. First, an oligonucleotide primer is annealed to M13 single-stranded DNA. All four dNTPs and rATP are then added, as well as the purified products of the T4 genes 43, 44/62, and 45. These proteins constitute part of the T4 DNA polymerase holoenzyme that is active in T4-infected *Escherichia coli* cells. The 43 protein is the DNA polymerase. The 45 protein and the tight complex formed between the gene 44 and gene 62 products are accessory proteins; these species interact with the polymerase in an rATP-dependent manner to form a "sliding clamp", greatly increasing the processivity of DNA synthesis (Huang et al., 1981). As can be seen from Figure 1, this four-protein complex efficiently catalyzes synthesis all the way around the circle, providing a nearly quantitative yield of form II DNA. In the absence of the accessory proteins, a much poorer yield is obtained (data not shown), since most of the forks terminate at a few strong pause sites on the M13 template. A similar result is observed when the Klenow

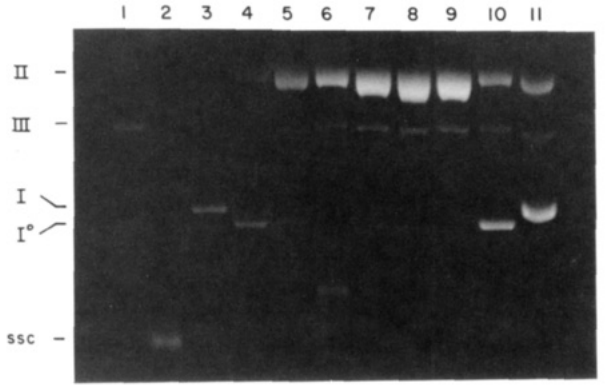

FIGURE 1: Enzymatic synthesis of supercoiled M13 DNA. Primer extension was carried out on circular M13 viral strand DNA as described under Materials and Methods. Aliquots were removed from the reaction mixture at each stage of synthesis and analyzed on a 0.8% agarose gel run for 18 h in the presence of 1 μg/mL ethidium bromide. Lanes 6–9 monitor the course of primer extension after 5, 15, 30, and 45 min of synthesis, respectively. Lanes 10 and 11 show the DNA product after ligation and subsequent gyration, respectively. M13 DNA standards include linear double strand (lane 1), single-strand circle (lane 2), supercoiled form I (lane 3), form I relaxed with T4 topoisomerase (lane 4), and form I nicked with fd gene II protein (lane 5).

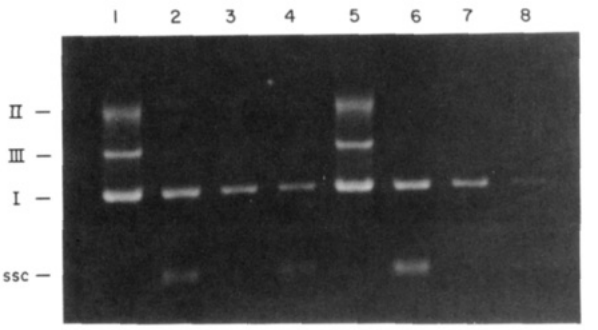

FIGURE 2: Enzymatic synthesis and purification of supercoiled M13 mp19 DNA containing a site-specific HMT monoadduct or cross-link. M13 mp19 viral strand was hybridized to the 13-mer primer and incubated in succession with the T4 replication proteins, T4 DNA ligase, and *E. coli* gyrase (lanes 1 and 5). The resultant supercoiled form I DNA was purified by digestion with exonuclease III (lanes 2 and 6) and extraction with BND-cellulose in 1 M NaCl (lanes 3 and 7). The DNA in lanes 1–3 was primed with unmodified 13-mer while the DNA in lanes 5–7 was primed with HMT-monoadducted 13-mer. HMT-cross-linked DNA (lane 8) was derived from the monoadducted DNA by irradiation with 320–400-nm light followed by transient alkaline denaturation and extraction in 1 M NaCl with BND-cellulose. M13 mp19 DNA standards were run in lane 4.

fragment is substituted for the T4 polymerase. The gene 32 helix-destabilizing protein, another component of the T4 holoenzyme in vivo, is omitted since its presence results in rolling circle replication (data not shown).

Once synthesis is complete, T4 DNA ligase is added to the reaction mixture, producing relaxed covalently closed circular DNA in 40–80% yield (Figure 1, lane 10). These molecules are then supercoiled by the addition of *E. coli* DNA gyrase (Figure 1, lane 11). The reaction is quenched by the addition of EDTA together with NaCl and sodium dodecyl sulfate (SDS), the proteins are removed by proteinase K digestion followed by phenol extraction, and the DNA is ethanol precipitated. To purify the desired supercoiled product, we employed the method of Gamper et al. (1985) which involves digestion of the impurities (mostly unligated material) with exonuclease III followed by batch removal of the resulting single-stranded DNA by benzoylated naphthoylated DEAE (BND) cellulose.

Preparation and Characterization of Double-Stranded M13 DNA Containing a Site-Specific Psoralen Adduct. The deoxyoligonucleotide 5′-GCTCGGTACCCGG-3′ was chemically synthesized, enzymatically phosphorylated at the 5′ end, and then photochemically modified in the presence of 380–400-nm light with the psoralen derivative 4′-(hydroxymethyl-4,5′,8-trimethylpsoralen (HMT). A complementary octamer was added to the irradiation mixture to take advantage of the marked reaction preference of psoralens for the 5,6 double bond of thymidines at double-stranded 5′-TpA-3′ sequences (Gamper et al., 1984; Sinden & Hagerman, 1984; Zhen et al., 1986a,b). Intercalation within this site leads to photoreaction on the 3′ face of the thymidine. With HMT, the cycloaddition reaction occurs primarily through the furan side double bond (Gamper et al., 1984). In the presence of 360-nm light, this adduct can undergo further reaction to form a cross-link; to minimize cross-linkage of the two oligomers, we have utilized 380–400-nm light in our reactions.

After irradiation, the reaction mixture was ethanol precipitated and electrophoresed through a 20% sequencing gel. Approximately 20% of the starting 13-mer contained an HMT monoadduct. Electrophoretic analysis of an aliquot of this material which had been irradiated with the complementary 8-mer utilizing 320–400-nm light indicated that 85% of the monoadducted 13-mer was cross-linkable and thus possessed a furan side HMT monoadduct on the 3′ face of the central thymidine. On the basis of previous studies (Gamper et al., 1984; Cimino et al., 1986; Van Houten et al., 1986), the remaining oligomers were assumed to be modified with a pyrone side HMT monoadduct to thymidine at the same TpA site.

The modified 13-mer was hybridized to M13 mp19 DNA at the complementary *Kpn*I site in the polylinker region and used as a primer for the synthesis of supercoiled DNA using both unmodified and HMT-adducted 13-mer primers. This demonstrates that the presence of the HMT monoadduct does not interfere with the annealing, synthesis, or ligation procedures. This was expected since a previous study had indicated that the DNA helix is slightly stabilized by an HMT furan side or pyrone side monoadduct (Shi & Hearst, 1986). In this particular synthesis, 40% of the input DNA was converted to supercoiled product, and of this 40%, approximately half was recovered after purification. The aliquots analyzed in Figure 2 were derived from equivalent fractions of the total DNA and thus indicate yield as well as purity of the form I DNA. Since this procedure can be easily scaled up (we routinely use 40 μg of single-stranded template), it provides easy access to large quantities of interesting DNA substrates.

Formation of the diadduct from the site specifically placed furan side HMT monoadduct was achieved by exposing the form I DNA to 320–400-nm light. Purification of the cross-linked product (Figure 2, lane 8) was based on its renaturability after transient exposure to alkaline pH. contaminating non-cross-linked DNA (primarily pyrone side HMT-monoadducted molecules) was irreversibly denatured by this treatment and removed by extraction with BND-cellulose in 1 M NaCl (data not shown). In our hands, this approach proved superior to an alternative methodology in which excess HMT-modified 13-mer was hybridized and cross-linked to single-stranded circular M13 DNA followed by separation of the hybrid from excess oligomer under denaturing conditions and subsequent primer extension.

In order to verify the presence of a psoralen adduct at the *Kpn*I recognition sequence, a restriction analysis was performed (Figure 3). Both the HMT-cross-linked and the mixed population of furan side and pyrone side HMT-monoadducted M13 DNAs were resistant to *Kpn*I restriction, thus confirming

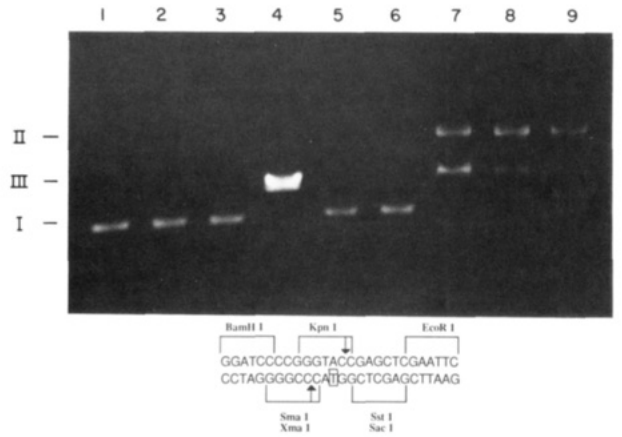

FIGURE 3: *Kpn*I and *Xma*I restriction of unmodified, HMT-monoadducted, and HMT-cross-linked M13 mp19 DNA. Supercoiled M13 mp19 DNA was analyzed on a 1% agarose gel run for 1 h in the presence of 1 μg/mL ethidium bromide after no treatment (lanes 1–3) or after 60-min restriction with *Kpn*I (lanes 4–6) or *Xma*I (lanes 7–9). Unmodified mp19 DNA was run in lanes 1, 4, and 7; HMT-monoadducted mp19 DNA was run in lanes 2, 5, and 8; and HMT-cross-linked mp19 DNA was run in lanes 3, 6, and 9. The unmodified mp19 DNA used as a control in this and other experiments was prepared by primer extension using the unmodified 13-mer as a primer; after synthesis, this DNA was irradiated with 320–400-nm light in a mock cross-linkage reaction. The HMT-modified thymidine (box) and the recognition sites of the various restriction endonucleases tested are depicted on the polylinker sequence. The arrows denote those phosphodiester linkages which are rendered resistant to enzymatic hydrolysis.

the presence of a psoralen adduct at the central 5′-TpA-3′ site in the *Kpn*I recognition sequence. Separate experiments with control DNA indicated that the slight amount of nicking observed with the modified substrates occurred outside the *Kpn*I sequence (data not shown). Other restriction enzymes whose recognition sites were nearby or adjacent to the modification site were also examined. Neither the monoadduct nor the cross-link had an effect on *Bam*HI-, *Eco*RI-, *Sst*I-, *Sac*I-, or *Sma*I-dependent linearization (data not shown). However, the rate of restriction by *Xma*I was reduced by the presence of an HMT adduct, with the cross-link having the greater effect. Interestingly, among these enzymes, only *Xma*I and *Kpn*I cleave a phosphodiester bond within two base pairs of the modified thymidine. Thus, our restriction data, in agreement with independent DNase I footprinting and two-dimensional NMR studies (Shi and Tomic, personal communications), indicate that the structural perturbation due to a psoralen monoadduct or cross-link is localized to within three base pairs on either side of the modified thymidine. With respect to *Xma*I digestion, only one of the two hydrolysis sites is found within this region, and therefore linearization is inhibited by the HMT adduct to a greater extent than nicking.

The likelihood of a psoralen adduct being introduced fortuitously at another site on the M13 genome is considered remote. Specificity in targeting the lesion resides in the base pairing of the modified 13-mer, which should bind to a unique site in a simple phage DNA. Furthermore, it has been demonstrated that the presence of a psoralen monoadduct does not affect the kinetics or the specificity of hybridization (Gamper et al., 1987). When this same HMT-modified 13-mer was annealed and photofixed to an M13 derivative which lacked the *Kpn*I sequence (M13 and mp8), no hybrid could be detected (Gamper et al., 1987).

DNA Synthesis Is Blocked by an HMT Monoadduct on the Coding Strand. With large quantities of modified DNA in hand, the effect of the bulky psoralen adduct on DNA synthesis by the phage T4 replication complex was determined.

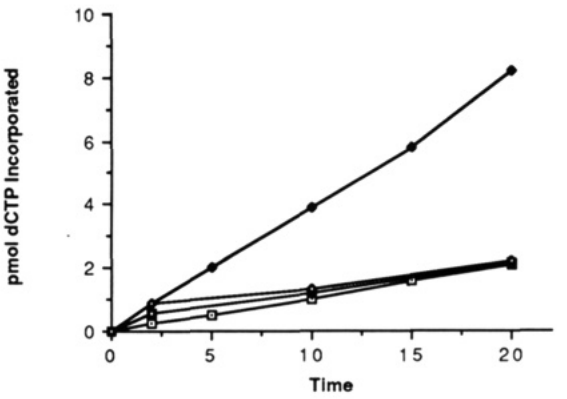

FIGURE 4: Extent of DNA synthesis catalyzed by the T4 replication proteins using unmodified and HMT-monoadducted M13 mp19 DNA. Each substrate was used as a template for DNA synthesis either with or without prior site-specific nicking with the fd gene II protein. Synthesis was monitored by the uptake of labeled dCTP into acid-insoluble materials. (♦) Control DNA cut with gpII. (◇) Control DNA not cut with gpII. (■) Psoralenated DNA not cut with gpII. (□) Psoralenated DNA cut with gpII.

Synthesis using the newly synthesized minus strand as a template was initiated from a site-specific nick, located at position 5671 on the viral (plus) strand introduced into the supercoiled M13 DNA by incubation with purified phage fd gene II protein (Dotto et al., 1981). This nick provided a unique 3′-hydroxyl primer for replication about 600 base pairs upstream from the HMT adduct. After heat inactivation of the gene II protein, leading strand synthesis by the five T4 replication proteins (43, 45, 44/62, and 32) was monitored radiometrically both by acid precipitation of reaction aliquots onto a glass fiber disk and by electrophoresis through a denaturing agarose gel. The nicked M13 template either was unmodified or contained an HMT monoadduct at the *Kpn*I restriction site.

Figure 4 compares the extent of synthesis supported by the unmodified and HMT-monoadducted substrates both with and without a gene II protein-induced nick. For the HMT-monoadducted DNA, a low level of synthesis is observed that is independent of whether the DNA has been nicked with the gene II protein. The similarity to the profile obtained with supercoiled control DNA suggests that this synthesis is "background" and reflects synthesis primed from randomly occurring nicks. Half of these nicks would be expected to be in the minus strand and therefore support the use of the unmodified plus strand as a template. These data are suggestive that an HMT monoadduct on the coding strand is a block to T4 replication, whereas the same monoadduct when on the noncoding strand is essentially silent.

The electrophoretic analysis in Figure 5 is consistent with this interpretation. With the HMT-monoadducted substrate (Figure 5B), a strong band appears immediately on the denaturing gel and persists throughout the reaction. The position of this band, at a slightly higher molecular weight than a linear M13 mp19 DNA standard, is that expected for a block a few hundred base pairs downstream of the start site. The band is dependent on the presence of the psoralen monoadduct and is not observed unless synthesis proceeds from the unique gene II nicking site. The distinct band observed upon synthesis on the gpII-nicked unmodified template is the same length as linear M13 mp19 DNA. This band results from a "chew back-fill in" reaction of linear double-stranded M13 mp19 DNA produced as a minor byproduct in the gpII nicking procedure (Figure 1, lane 5). This band is also present when gpII-nicked HMT-modified template is employed but is not as noticeable since the autoradiogram shown in Figure 5A was

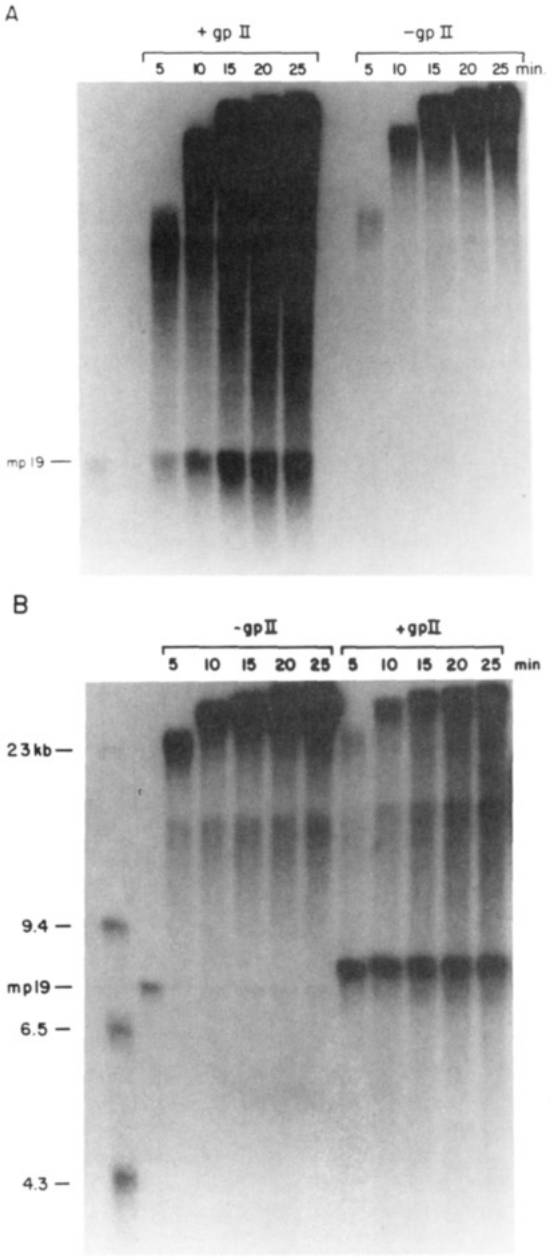

FIGURE 5: Alkaline agarose gel electrophoresis of DNA synthesis reactions catalyzed by the T4 replication proteins using (A) unmodified and (B) HMT-monoadducted M13 mp19 DNA. Each template was used either with or without a site-specific nick introduced by the fd gene II protein. Reaction aliquots were removed at the indicated times and analyzed on an alkaline 0.8% agarose gel. The gene II protein generates a small amount of form III DNA which in turn is labeled by replacement synthesis catalyzed by the T4 gene 43 protein; this accounts for the origin of the form III band obtained with the gpII-nicked DNA.

exposed for a longer period of time in order to detect even a weak band corresponding to a pause at the *Kpn*I site. We interpret these results to mean that the psoralen monoadduct functions as a strong block to the T4 replication fork. If the bulky lesion merely caused the polymerase holoenzyme to pause, rather than stop completely, the observed band should disappear with time since the synchronized population of replication forks, which move at a rate of 400–800 nucleotides per second, reaches this position in seconds. However, even after 20 min, the intensity of the band is not diminished. In addition, a "ladder" of bands of progressively higher molecular weight is not observed, though this would be the expected result of a pause site in rolling-circle synthesis. The high molecular weight DNA seen regardless of nicking by gene II protein is believed to represent unimpeded synthesis from random nicks using the unmodified plus strand as a template. Since incorporation of labeled dCTP is being monitored and this randomly primed synthesis will be of the rolling-circle type, only a small percentage of spurious forks are required to generate the intense high molecular weight signals observed on the gel. In experiments to be reported elsewhere, we have determined that a psoralen monoadduct on the noncoding strand has no effect on DNA synthesis while a psoralen cross-link functions as an absolute block (Kodadek, unpublished results).

Discussion

Until now, the preparation of site specifically damaged viral or plasmid DNA molecules has relied upon three relatively inefficient methodologies to incorporate a modified oligonucleotide into a larger self-replicating DNA. In most cases, small quantities of the desired covalently closed circular product are obtained, and usually this DNA is used directly without purification. The most widely used method involves hybridization of the modified oligomer to a gapped circular heteroduplex which contains a single-stranded gap complementary to and identical in length with the oligonucleotide. Ligation of the nicked product generates a form I DNA molecule. This approach has been used successfully to site specifically insert O^6-methylguanine (Green et al., 1984) and adducts of 2-aminofluorene (Johnson et al., 1986), *cis*-diamminedichloroplatinum (Pinto et al., 1986), and 4-aminobiphenyl (Lasko et al., 1987). The major limitation of this method is the requirement for a gapped circular heteroduplex. Preparation of the gapped circular DNA may require cloning of the oligonucleotide sequence or enzymatic processing of the gap itself. Furthermore, placement of the modification at alternative sites on the DNA necessitates the construction of new heteroduplexes. A second more direct procedure involves ligation of a modified double-stranded DNA oligonucleotide to a linearized plasmid or viral genome. In this manner, small quantities of circular DNA containing a site specifically placed psoralen cross-link (Zhen et al., 1986a,b) or 2-aminofluorene adduct (Mitchell & Stohrer, 1986) have been prepared for use in transformation studies. In the third method, a modified oligonucleotide is hybridized to a circular single-stranded DNA and enzymatically extended with the Klenow fragment of DNA polymerase I. Although this procedure is versatile and straightforward, in the presence of ligase yields of form I DNA are very low (Craik, 1985). Uninterrupted extension of the primer by Klenow fragment is inhibited by regions of secondary structure in the single-stranded template. Small amounts of DNA containing a uniquely positioned O^6-methylguanine have been prepared with this method (Chambers et al., 1985).

The approach described here utilizes a T4 replication complex formed from the 43, 44/62, and 45 proteins to carry out an extremely efficient primer extension reaction on a single-stranded circular DNA substrate. The processivity of this complex together with its ability to synthesize through secondary structure, yet not catalyze strand displacement synthesis, leads to high yields of covalently closed product in the presence of ligase. By utilization of a modified primer, a DNA adduct can be site specifically incorporated into the newly synthesized DNA strand, thereby providing a well-defined substrate for studying the structural perturbations and biological consequences of the modification. Although we have used a psoralen-monoadducted oligonucleotide, this technique should be applicable to most types of DNA damage. Adducts

which significantly perturb the DNA helix may necessitate the use of longer primers.

The T4 replication product can be processed to yield a variety of DNA substrates. Ligation in the presence of an intercalating agent or subsequent treatment with gyrase generates supercoiled DNA. Covalently closed circular DNA of known superhelical density will prove useful in trannsformation and transfection experiments and in studies of transcription, repair, and replication. Site specifically nicked DNA, as demonstrated here, will facilitate in vitro replication experiments. By omitting ligase treatment, the major product is a DNA molecule that contains a nick on the newly synthesized strand 5′ to the primer. Alternatively, treatment of supercoiled product with fd gene II protein introduces a site-specific nick into the template strand. Subsequent exonucleolytic digestion of this nicked DNA will produce a single-stranded circular DNA which retains the site-specific adduct.

Employing HMT-modified M13 DNA which contains a strand-specific nick, we have characterized the response of the "core" T4 replication complex to a psoralen monoadduct. Our results indicate that furan side and pyrone side HMT monoadducts to thymidine block replication when located on the template strand. This pattern of inhibition is identical with that observed for *E. coli* and T7 RNA polymerase by Shi (personal communication) and is consistent with the observation of Gruenert et al. (1985) that psoralen and angelicin monoadducts block replication in several human cell lines. The T4 replication complex, however, differs from the *E. coli* DNA polymerase I enzyme which is competent to replicate past monoadducts on the coding strand (Piette & Hearst, 1983). This activity could be mutagenic and may reflect the role of pol I in SOS repair as well as its weak 3′ editor function. Experiments are in progress to determine the cytotoxic and mutagenic potential of these site-specific lesions in several *E. coli* strains with defined repair deficiencies.

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