Replication Block by an Enediyne Drug−DNA Deoxyribose Adduct†

Lizzy S. Kappen and Irving H. Goldberg*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Received September 10, 1998; Revised Manuscript Received October 28, 1998

ABSTRACT: Under anaerobic conditions neocarzinostatin chromophore, an enediyne antibiotic, forms a covalent drug−DNA adduct on the 5′ carbon of deoxyribose at a specific single site in a 2-nucleotide bulge, rather than strand cleavage, by a mechanism involving general base-catalyzed intramolecular drug activation to a reactive radical species. We have taken advantage of the selectivity of this reaction to prepare a single-stranded oligonucleotide containing a single drug adduct at a T residue and to study its effect on the template properties of the oligonucleotide in replicative synthesis, as followed by 5′-^{32}P-labeled primer extension by several DNA polymerases. With the Klenow fragment of *Escherichia coli* DNA polymerase I, synthesis stops at the base immediately 3′ to the adduct. The same enzyme, but lacking 3′ to 5′ exonuclease activity, permits synthesis to proceed by one additional nucleotide. This effect is enhanced when Mn^{2+} is substituted for Mg^{2+}. T4, herpes simplex virus, and cytomegalovirus DNA polymerases all act like Klenow polymerase. Sequenase (exo-minus T7 DNA polymerase) is qualitatively similar to exo-minus Klenow polymerase but is more efficient in inserting a nucleotide opposite the lesion. With the small-gap-filling human DNA polymerase β, which lacks intrinsic exonucleolytic activity, primer extension proceeds to the nucleotide opposite the lesion. However, when a gap was created opposite the lesion, polymerase β adds as many as two additional nucleotides 5′ to the adduct site. The fidelity of base incorporation opposite the lesion was not impaired, in contrast with adducts on DNA bases.

NCS−chrom (**1**, Scheme 1), the biologically active enediyne component of the antitumor antibiotic neocarzinostatin (reviewed in ref *1*), induces site-specific cleavage selectively at a DNA bulge in a folded single-stranded or duplex DNA in a reaction that requires oxygen but not thiol (*2*−*4*), which is normally required as an activator for the drug in duplex DNA damage. Drug activation in the DNA bulge reaction is by a novel mechanism (Scheme 1), which involves a base-catalyzed intramolecular Michael addition of the phenol enolate at C1″ to C12 to give cumulene **2**, which by a Bergman-type rearrangement is converted to the biradical **3** (*5*, *6*). The abstraction by the biradical of the C-5′ hydrogen atom from the deoxyribose of the target nucleotide ultimately results, in the presence of oxygen, in a strand break having a PO_4 at the 3′ end and a nucleoside 5′-aldehyde at the 5′ terminus. In addition, the generation of a new drug product **5**, dependent on a competent bulged substrate and concomitant with strand cleavage, implicates the bulged DNA in the drug activation process by a mechanism involving exclusion of radical-quenching solvent from the tight-binding bulge pocket and possibly by promotion of a conformational change in the active drug intermediate so as to enable intramolecular quenching of the radical center at C2 and carbon−carbon bond formation (**3a**) (*2*, *5*). The solution structure of a complex of a bulged DNA substrate and a stable analogue of the proposed NCS−chrom biradical has been elucidated (*7*).

In the absence of dioxygen there is no significant strand cleavage at the bulge; instead the drug forms an adduct at the single target T of the two-base bulge in a single-stranded 31-mer folded to form a hairpin structure (Figure 1) or in an oligonucleotide duplex containing the same bulge (*2*, *3*, *8*), as also found with thiol-activated NCS−chrom on duplex DNA lacking a bulge (*9*). Recent work has shown that the adduct at the DNA bulge, which is the only lesion formed under hypoxic conditions, can be stabilized by reduction and that it is a covalent monoadduct, probably linked via its C6 position to the 5′ carbon of the target nucleotide (*10*). In virtually all of the extensively studied drug−DNA adducts, such as those formed by mitomycin C (*11*), cis-diamminedichloroplatinum (*12*), psoralen (*13*, *14*), and benzo[*a*]pyrene (*15*), ligand adduction is at a DNA base. The potential lethal and/or mutagenic effects of a lesion will depend not only on the chemical structure of the adduct but also upon how well the enzymes involved in DNA repair are able to process it. Several studies have employed purified DNA polymerases in in vitro systems to assess their ability to extend a primer on DNA templates containing a variety of site-specifically positioned drug adducts on the DNA base (*15*−*21*). Despite their structural differences, a common feature shared by many drug−DNA adducts is their ability to impair replication by arresting the synthesis at or near the adduct site.

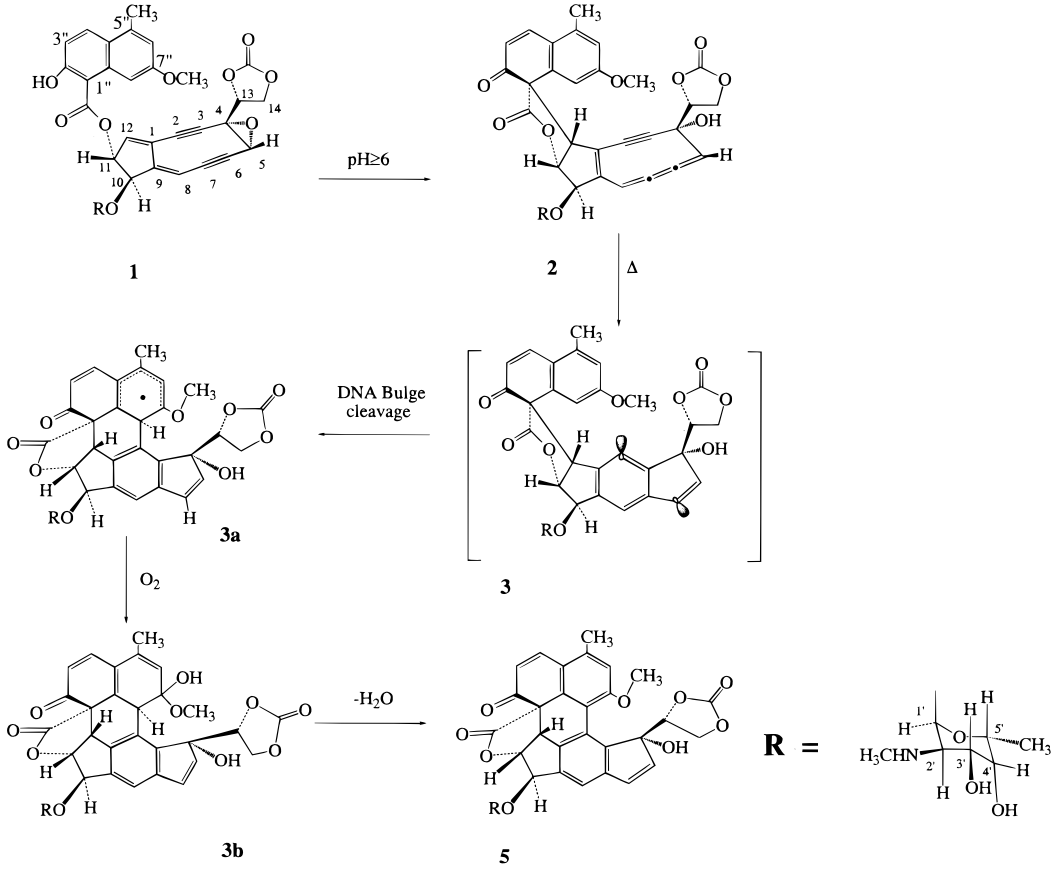
NCS−Chrom, with its enediyne core, is the prototype of a family of potent antitumor antibiotics that include calicheamicin (*22*), esperamicin (*23*), dynemicin (*24*), and C-1027 (*25*), all of which damage DNA by hydrogen

† This work was supported by U.S. Public Health Service Grant GM 53793 from the National Institutes of Health.

* To whom correspondence should be addressed.

10.1021/bi982185g CCC: $18.00
Published on Web 12/05/1998

Scheme 1: Proposed Mechanism for the Intramolecular Activation of NCS−Chrom at Basic pH in the Presence of Bulged DNA



abstraction from the deoxyribose moiety. However, NCS−chrom is unique in its ability to form an adduct specifically at a single nucleotide in a DNA bulge. In the present study we have taken advantage of this reaction to study the effect of the drug−DNA sugar adduct on the template function of oligonucleotides by several DNA polymerases.

MATERIALS AND METHODS

Nucleic Acid Substrates. Oligodeoxyribonucleotides were purchased from Midland Certified Reagent Co. or the Biopolymers Laboratory at Harvard Medical School. Radioactive materials were from New England Nuclear. Herpes simplex virus (HSV) DNA polymerase and cytomegalovirus (CMV) DNA polymerase were generous gifts from Dr. Donald Coen. T4 polynucleotide kinase, *Escherichia coli* DNA polymerase I, Klenow fragment of DNA polymerase I, and Klenow fragment lacking the 3′ to 5′ exonuclease activity were purchased from New England Biolabs. Sequenase version 2, a modified form of T7 DNA polymerase, was from U.S. Biochemical Corp. Recombinant human DNA polymerase β was a generous gift from Dr. Samuel Wilson.

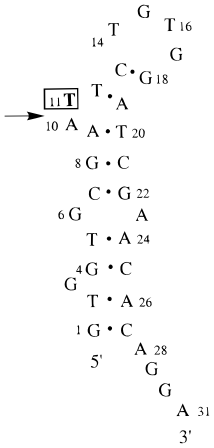


FIGURE 1: Sequence of the bulged 31-mer DNA substrate for NCS−chrom. The single target residue (T_{11}) is within the box. Arrow indicates the site of strand cleavage under aerobic conditions.

The primers were 5′-^{32}P-end-labeled with [γ-^{32}P] ATP and polynucleotide kinase by standard procedure (*26*). The labeled oligomers were purified by electrophoresis on a 15% sequencing gel. Neocarzinostatin powder (holo-NCS) was purchased from Kayaku Antibiotics (Tokyo). NCS−Chrom was extracted from the holoantibiotic by cold methanol containing 0.5 M acetic acid as described (*10*). The chromophore was stored at −70 °C, protected against light.

Anaerobic Drug Reaction and Isolation of the Adduct. The standard drug reaction contained 15 mM sodium acetate, pH 5.0, 100 mM Tris-HCl, pH 9.1, 60 μM hairpin 31-mer oligodeoxyribonucleotide containing a two-nucleotide bulge (Figure 1), and 142 μM NCS−chrom. Maximum methanol concentration from drug addition was 10%. Anaerobic reactions were performed in a vessel equipped with a side arm (*10*). This procedure permits the removal of oxygen from the reaction vessel under conditions where the drug will be stabilized by binding to the substrate at the low pH at which the damage is minimal or not at all. The substrate oligomer was placed in the main chamber in sodium acetate, and Tris-HCl was in the side arm. The vessel was cooled on ice prior

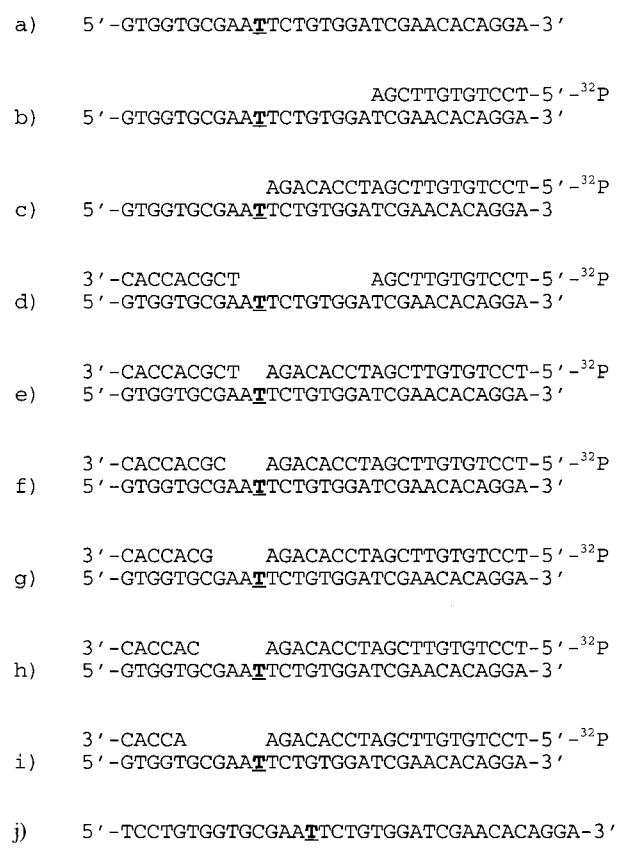


FIGURE 2: Substrates used in the polymerase assays. Panel a shows the 31-mer template containing NCS−chrom adduct. The site of drug adduction is $\mathbf{T}_{11}$, which is in boldface type and underlined in this and subsequent figures. The primers in panels b and c are ^{32}P-12-mer and ^{32}P-20-mer, respectively. Panels d−i represent gapped substrates, with ^{32}P-12-mer primer in panel d and ^{32}P-20-mer in panels e−i. The oligomers, which are complementary to the 5′ side of the 31-mer template in the gapped substrates, have a phosphate at their 5′ ends. Panel j shows the 35-mer template containing NCS−chrom adduct.

to the addition of NCS−chrom to the mixture in the main chamber. The contents of the flask were frozen and evacuated four times with intermittent thawing and freezing. The contents of the two chambers were then mixed and the reaction was allowed to proceed in the dark on ice for 1 h. The adduct was stabilized by reduction with borane−pyridine and isolated on a sequencing gel, as previously described (*10*).

DNA Polymerase Assays. 31-mer templates (control or NCS−chrom-modified) and the appropriate 5′-^{32}P-labeled primer (Figure 2) were annealed by heating in 25 mM Tris-HCl, pH 7.5, to 80 °C, followed by slow cooling to room temperature. For a 40 μL Klenow polymerase assay a mixture of 31-mer template (∼700 pmol) and 5′-^{32}P-labeled 12-mer primer (∼80 pmol) in 20 μL of 25 mM Tris-HCl, pH 7.5, was annealed. This was followed by the addition of Klenow buffer (final concentration of 35 mM Tris-HCl, pH 7.5), 5 mM $MgCl_2$, 7.5 mM DTT, and a mixture of dATP, dTTP, dGTP, and dCTP, each 0.75 mM (in some assays 1 mM) and 4 units of the enzyme. When Mg^{2+} was replaced by Mn^{2+}, the latter was present as 2 mM $MnCl_2$. The enzyme was added last. The incubation was at room temperature for times indicated in the figure legends. The reaction was terminated by adding excess of EDTA followed by heating at 75 °C for 10 min. In some cases the reaction products were precipitated with three volumes of ethanol after the addition of sodium acetate to 0.3 M. Isolation of a product from gel bands was done by soaking the gel slices overnight in 1M triethylammonium bicarbonate, pH 7.5, and recovering the product by a Sep-Pak cartridge (Waters Associates).

The assay conditions for 3′ to 5′ exonuclease-deficient Klenow polymerase and Sequenase were the same as for Klenow polymerase, except that in Sequenase assays the $MgCl_2$ concentration was 10 mM. DNA polymerase I and HSV and CMV polymerases (*27*) were assayed under conditions similar to those described for Klenow polymerase. HSV and CMV polymerase assays also contained 100 mM NaCl.

β Polymerase assay conditions were as described by Singhal and Wilson (*28*). Annealing mixture for a 30 μL reaction contained 25 mM Tris-HCl, pH 7.5, 31-mer template (∼100 pmol), ^{32}P-labeled 20-mer primer (∼6 pmol), and where a gap is to be created, a third 5′ phosphorylated oligomer (1.3 nmol) that is complementary to the 5′ end of the template (Figure 2). The enzyme reaction contained 33.3 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 2 mM DTT, 20 mM NaCl, 200 μg/mL bovine serum albumin, 0.5 mM each dATP, dTTP, dGTP, and dCTP, and the enzyme (0.17 μg/10 μL assay). Incubation was at room temperature for times indicated in the figure legends.

In all assays the amount of enzymes used was sufficient to replicate the unmodified 31-mer to full-length 31-mer. Some assays were also repeated with varying concentrations of deoxynucleotide triphosphates and the enzymes.

Products Analysis. The reaction mixtures were dried and the pellets were dissolved in 80% formamide containing 1 mM EDTA and marker dyes for analysis on a 15% polyacrylamide sequencing gel. The gel band intensities were quantitated on a PhosphorImager (Molecular Dynamics).

DNA Sequencing Analysis. Chemical cleavage reactions specific for T + C was done with hydrazine (15 min, 37 °C) as described by Maxam and Gilbert (*29*). Cleavage reactions specific for G + A and T with formic acid and potassium permanganate, respectively, were carried out as previously described (*10*). The cleavage products were analyzed on a 15% sequencing gel.

RESULTS

NCS−Chrom Forms a Site-Specific Monoadduct on the DNA. In the present study we chose, as template, a single-stranded 31-mer DNA previously reported (*2*) to have a single site of attack for NCS−chrom at the T of its two base bulge in its folded form (Figure 1). The sequence at its bulge is also identical to that in the 31-mer used in the recent work where the product formed with NCS−chrom under anaerobic conditions has been chracterized as a covalent monoadduct (*10*). The drug-adducted 31-mer, generated by anaerobic reactions containing the 31-mer oligodeoxyribonucleotide (Figure 1) and NCS−chrom, was stabilized by reduction and purified on a sequencing gel. It had a slower mobility than the unmodified control 31-mer. The site of drug adduction, by analogy to the previous work and further supported by sequencing analysis (see later), is T_{11}. The drug-adducted 31-mer and the control 31-mer, which had been subjected to all the treatments as in the drug reaction but without the drug, served as templates. The templates were annealed to

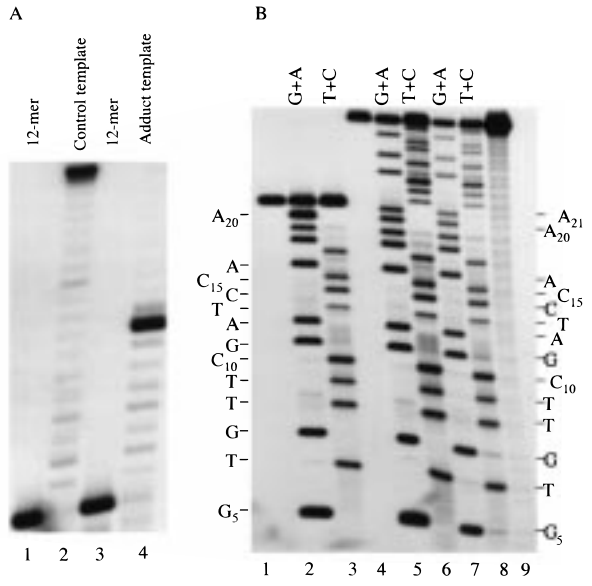


FIGURE 3: DNA replication block in primer extension by Klenow polymerase on NCS−chrom adduct-containing DNA template. 5′-^{32}P-labeled 12-mer extension (20 min) on control and drug-modified 31-mer template was assayed as described in Materials and Methods. (A) Lane 2, control template; lane 4, drug-modified template; lanes 1 and 3 have the 12-mer primer that had been annealed with the control and the drug-modified template, respectively. (B) Maxam−Gilbert sequencing analysis of the products of primer extension from panel A. Lanes 1−3, product from NCS−chrom-modified template; lanes 4−6, product from control template; lanes 7−9, chemically synthesized complementary strand of the 31-mer template. Lanes 1, 4, and 9 represent the respective samples without any treatment.

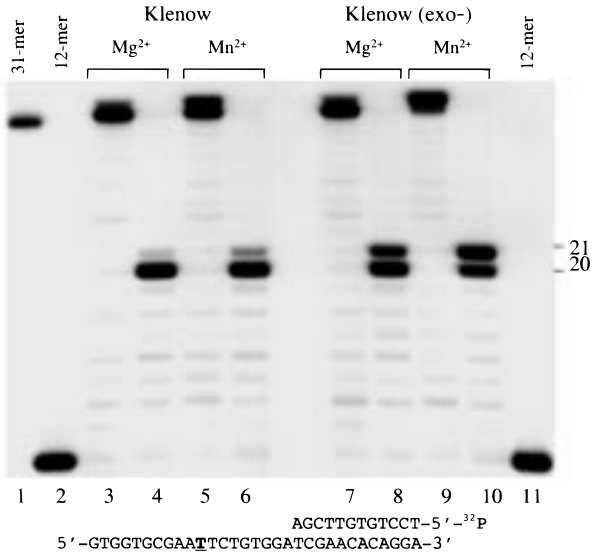


FIGURE 4: Comparison of Klenow polymerase and Klenow polymerase (exo$^-$) for DNA synthesis on NCS−chrom-adducted DNA template and the effect of Mn^{2+} substitution for Mg^{2+}. 5′-^{32}P-labeled 12-mer extension on control and drug-modified 31-mer template by Klenow polymerase (lanes 3−6) and Klenow polymerase (exo$^-$) (lanes 7−10) in the presence of Mg^{2+} or Mn^{2+} for 20 min was assayed as in Figure 3A. Lanes 4, 6, 8, and 10, products of synthesis on NCS−chrom-modified template; lanes 3, 5, 7, and 9, products of synthesis on control template; lane 1, 31-mer marker that, in this and subsequent figures, represents chemically synthesized 5′-^{32}P-end-labeled 31-mer that is complementary to the 31-mer template; lanes 2 and 11, 12-mer primer. Shown at the bottom is the template/primer.

the appropriate 5′-^{32}P-labeled primers (Figure 2) and the extension of the primers was followed in the polymerase assays. The products of the primer extension were analyzed on a denaturing polyacrylamide gel.

Effect of NCS−Chrom Adduct on DNA Synthesis by Klenow Polymerase. The primer/template shown in Figure 2b was the substrate. The single site of drug adduction on the 31-mer template is at position 11 (T_{11}) from the 5′ end and at 21 from the 3′ end. On the untreated 31-mer all the ^{32}P-labeled 12-mer primer was extended to full-length 31-mer, indicative of efficient replication (Figure 3A, lane 2). By contrast, the primer extension on the drug-adducted 31-mer was terminated prematurely (lane 4). Increasing the reaction time or the amount of enzyme did not show detectable translesion synthesis (data not shown). To identify the termination sites, the sequence of the synthesized products (lanes 2 and 4) was determined. As a control the complementary strand of the 31-mer template was chemically synthesized, 5′-^{32}P-labeled and also subjected to Maxam−Gilbert sequencing analysis. The data show (Figure 3B) that the sequence of the product made enzymatically on the control template (lanes 4−6) and that of the chemically synthesized 31-mer (lanes 7−9) are identical, confirming that the replicative system functioned normally. In the sequencing procedure, chemical modification of a base results ultimately in the removal of that nucleotide (*29*). Hence the band for cleavage at a particular base represents an oligomer shorter by one nucleotide and having a phosphate group at its 3′ end. The band for cleavage at A_{21} in the control (lanes 5 and 7) represents a 20-mer with a phosphate at its 3′ end. It can be seen that the termination product of primer extension on the NCS−chrom-modified template (lanes 1−3) has a mobility slightly slower than that of the A_{21} band in the control. This result is consistent with its being a 20-mer with a 3′ hydroxyl end, as expected of a product from enzymatic primer extension. Thus the termination of synthesis (Figure 3A) occurred at the base immediately 3′ to the drug-modified nucleotide T_{11}. If the enzyme recognizes the nucleotide opposite the adduct as spurious and excises it, it is then likely that Klenow polymerase lacking the 3′ to 5′ proofreading function would catalyze the chain extension beyond the block at T_{11}.

In experiments shown in Figure 4, Klenow polymerase (lanes 3−6) and Klenow polymerase devoid of 3′ to 5′ exonuclease activity (lanes 7−10) are compared for their ability to extend the ^{32}P-labeled 12-mer on the 31-mer templates. In contrast to the absolute termination of Klenow polymerase-dependent synthesis on the drug-modified template at the nucleotide 3′ to the lesion (lane 4), its proofreading-deficient counterpart partially overcomes the block to generate a significant amount of the 21-mer, by inserting a nucleotide opposite the lesion at T_{11} (lane 8). Mn^{2+} has been shown to enhance translesional replication on damaged DNA templates (*19*, *30*). To find out if the replication arrest on NCS−chrom-damaged template is overcome by Mn^{2+}, primer extension by both the Klenow polymerases was also assayed in the presence of Mn^{2+}. With Klenow polymerase, substitution of Mn^{2+} made a small difference; synthesis was nearly quantitatively blocked at the same site as with Mg^{2+}, except for a very weak band of the 21-mer (compare lanes 4 and 6). On the other hand, Mn^{2+} substitution in the Klenow polymerase (exo$^-$) assay causes a significant increase in the 21-mer (compare lanes 8 and 10). Primer extension on the

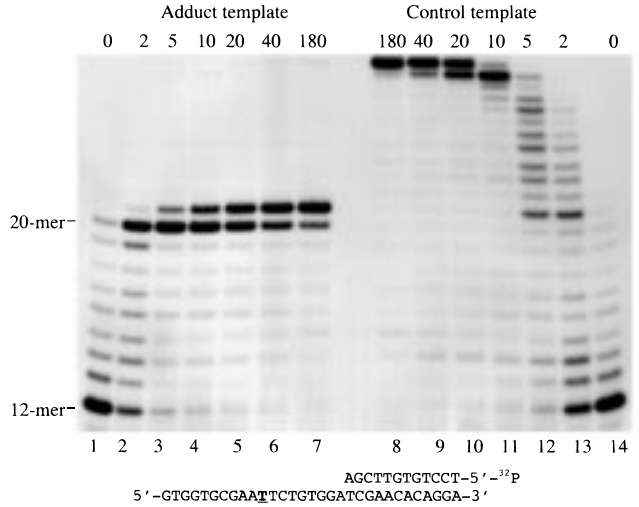


FIGURE 5: Time course of 5′-^{32}P-labeled 12-mer primer extension catalyzed by Klenow (3′ to 5′ exo$^-$) polymerase on NCS−chrom adduct-containing 31-mer template in the presence of Mn^{2+}. Assay conditions are as in Figure 4. At times (0−180 min) indicated, aliquots of the reaction mixture were withdrawn, and the reaction was terminated and analyzed on a sequencing gel as described in Materials and Methods. Shown at the bottom is the template/primer.

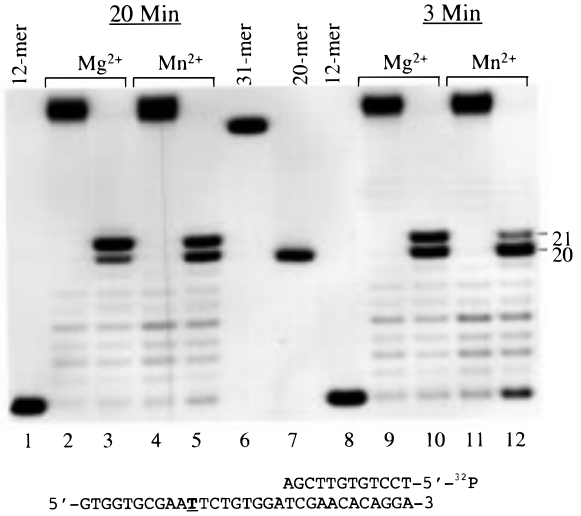


FIGURE 6: Arrest of synthesis on NCS−chrom adduct-containing DNA template catalyzed by Sequenase. 5′-^{32}P-labeled 12-mer primer extension on 31-mer template for 3 and 20 min was as described in Materials and Methods. Lanes 3, 5, 10, and 12, products of synthesis on NCS−chrom-modified template; lanes 2, 4, 9, and 11, products of synthesis on the control template; lanes 1 and 8, 12-mer primer; lane 6, 31-mer marker; lane 7, 20-mer marker. Shown at the bottom is the template/primer.

control template in the presence of Mg^{2+} proceeds to full-length 31-mer (lanes 3 and 7). Synthesis on the control template in the presence of Mn^{2+} produced a band with mobility slower than that of the 31-mer, presumably by addition of one extra nucleotide (lanes 5 and 9). In the Mg^{2+}-dependent assay such a band is very weak (lanes 3 and 7).

A time course of primer extension by Klenow polymerase (exo-) in the presence of Mn^{2+} shows that prolonged incubation is required to incorporate a nucleotide opposite the lesion (Figure 5, lanes 1−7). There were no extra bands for products longer than 21-mer. It should be noted that synthesis up to the site 3′ to the lesion, which results in product 20-mer, is not significantly affected. In 5 min of synthesis on the drug-modified template all the radioactivity of the primer is mainly in the 20-mer (lane 3), and with control template it is in a series of bands, all representing products longer than 20-mer (lane 12).

Effect of NCS−Chrom Adduct on DNA Synthesis by Sequenase. Replication assays were performed with substrate b of Figure 2 and Sequenase, which also lacks 3′ to 5′ exonuclease function. Primer extension on the drug-modified template produced two bands corresponding to the 20-mer and the 21-mer (Figure 6, lane 3), indicative of termination of synthesis prior to and opposite the lesion, respectively. Even at 3 min incubation the two blocked products are present at a ratio of approximately 1:1 (lane 10). While these results are qualitatively similar to those obtained with Klenow polymerase (exo−), Sequenase appears more efficient in inserting a nucleotide opposite T_{11} and in so doing Mg^{2+} is more effective than Mn^{2+} (compare lanes 3 and 5 and lanes 10 and 12). Synthesis on the control template appears to have gone farther than the expected full-length 31-mer, presumably by an extra nucleotide (lanes 2, 4, 9, and 11).

Several Other DNA Polymerases Are Also Blocked by NCS−Chrom Adduct. *E. coli* DNA polymerase I holoenzyme, T4 DNA polymerase, HSV DNA polymerase, and CMV DNA polymerase were tested for their ability to extend the primer in assays containing the drug-modified or control 31-mer template and ^{32}P-labeled 12-mer primer. In all cases replication stopped at the nucleotide immediately 3′ to T_{11} (data not shown). Since these enzymes possess 3′ to 5′ exonuclease funtion, these results are not unexpected.

DNA Polymerase β Can Bypass NCS−Chrom Adduct. All the enzymes tested in the preceding experiments stopped replicative synthesis on the drug-modified template either at the nucleotide immediately 3′ to T_{11} or after incorporation of a nucleotide opposite T_{11} itself. There was no detectable chain extension beyond the adduct site. The work of Wilson and co-workers has shown that mammalian DNA polymerase *β*, which lacks intrinsic nuclease activity (*31*), can fill short gaps in DNA up to six nucleotides provided the gap has a phosphate group on its 5′ side (*28*, *32*). In Figure 7A is shown the extension by polymerase *β* of ^{32}P-labeled 12-mer primer on NCS−chrom-modified and control 31-mer template. The two bands obtained with the drug-modified template (lane 4) represent a 20-mer and 21-mer, and this corresponds to termination of synthesis at positions immediately 3′ to and opposite the lesion, respectively. To test if a gap across the lesion would promote translesional synthesis, a 10-nucleotide gap extending from one nucleotide 5′ to and eight nucleotides 3′ to the lesion was created (Figure 2d) by annealing the drug-modified 31-mer with 5′-^{32}P-end-labeled 12-mer primer and a second 5′-phosphorylated 9-mer that is complementary to the 31-mer template from its 5′ end. Primer extension on the gapped substrate (Figure 7A, lane 6) produces an extra band of a 22-mer at the expense of the 20-mer and 21-mer. This shows that in the gapped substrate the synthesis progressed past the lesion by one nucleotide to fill the gap. In controls without or with the gap the product was the full-length 31-mer (lanes 3 and 5), consistent with displacement synthesis at higher polymerase *β* levels (*28*). Since primer extension on the drug-modified template is not significantly affected up to the position 3′ to the lesion to generate the product 20-mer (lane 4), in subsequent experiments we used ^{32}P-labeled 20-mer as primer and 31-mer template without

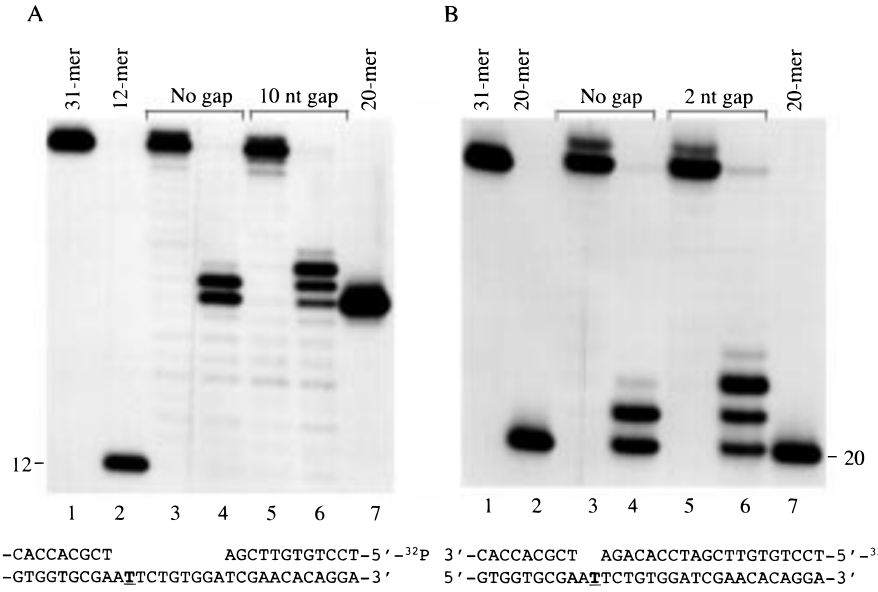


FIGURE 7: DNA synthesis on NCS−chrom-modified 31-mer template by polymerase β and effect of a gap across the lesion. (A) Extension of ^{32}P-12-mer was followed in a 2 h assay as described in Materials and Methods. Products of enzyme synthesis were from control template in lanes 3 and 5 and from drug-modified template in lanes 4 and 6. Markers: lane 1, 31-mer; lane 2, 12-mer primer; lane 7, 20-mer. (B) Reaction conditions were similar to those in panel A except for the difference in gap size and the ^{32}P-labeled primer, which in this case is the 20-mer. Products of synthesis were from control template in lanes 3 and 5 and from drug-modified template in lanes 4 and 6. Markers: lane 1, 31-mer; lanes 2 and 7, 20-mer primer. nt denotes nucleotide. Shown at the bottom are the gapped template/primers. The template/primers without the gap lack the unlabeled 9-mer as shown in Figure 2b,c.

and with a two-nucleotide gap across positions opposite and one 5′ to the lesion (Figure 2c,e). The results show, as in Figure 7A, that in the absence of a gap about half of the primer is extended to the 21-mer by incorporation of a nucleotide opposite the lesion (Figure 7B, lane 4). On the other hand, with the gapped substrate the gap is filled in to produce two extra bands corresponding to the 21-mer and 22-mer (lane 6). Irrespective of the difference in gap size, the primer extension pattern in Figure 7B is identical to that obtained in Figure 7A. Since the adduct does not impede the extension of the ^{32}P-12-mer primer to the 20-mer (Figure 7A), both substrates are essentially equivalent with respect to the translesional bypass. On the control templates the synthesis progressed to completion (lanes 3 and 5), but in other experiments with much less enzyme ($^1/_{250}$) the two-nucleotide gap in the control was just filled, while synthesis on the drug-modified template did not proceed beyond the 21-mer (data not shown). These results show that DNA polymerase β, unlike the other polymerases, can promote synthesis past a NCS−chrom adduct and that presence of a gap opposite the lesion is a prerequisite to achieve this.

To further explore the influence of a gap on the translesional synthesis by β polymerase, the gap size was varied starting from the position opposite the adduct to its 5′ side by annealing the drug-modified 31-mer with 5′-^{32}P-end-labeled 20-mer primer and a second 5′-phosphorylated oligomer of different lengths, each being complementary to the 31-mer template from its 5′ end (Figure 2e−i). The results are shown in Figure 8. In the absence of a gap, synthesis on the drug-modified template is terminated after a partial (∼50%) extension of the primer to the 21-mer with the insertion of a nucleotide opposite T_{11} (lane 2). Translesional synthesis on the two nucleotide-gapped substrate gives two extra bands corresponding to the 21-mer and 22-mer (lane 3). With a three-nucleotide gap there is an additional, albeit

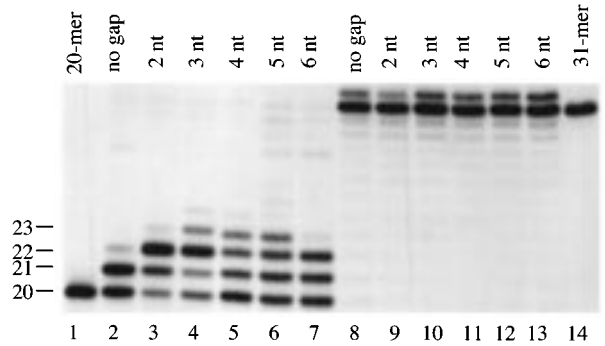


FIGURE 8: Effect of varying the gap size on translesional synthesis by polymerase β on NCS−chrom-modified and control 31-mer template. ^{32}P-labeled 20-mer extension on the 31-mer template with and without gaps across T_{11} was determined in a 5 h incubation under standard conditions as described in Materials and Methods. Gap sizes are indicated in nucleotides (nt). Lanes 2−7, products of synthesis on the drug-modified template; lanes 8−13, products of synthesis on the control template; lane 1, 20-mer primer; lane 14, 31-mer marker.

weak, third band indicative of chain extension by one more nucleotide (lane 4). Further increase in gap size by 4−6 nucleotides did not show a corresponding increase in chain length (lanes 5−7). With the six-nucleotide gap there are only two extra bands (lane 7). To rule out the possibility that the failure to fill in gaps longer than three nucleotides is due to the shortness of the duplex region 5′ to T_{11}, this region was lengthened by four additional base pairs with a 35-mer template (Figure 2j) and the appropriate 5′ phosphorylated oligomers. Experiments similar to those in Figure 8 using the 35-mer template containing NCS−chrom adduct and 2−6 nucleotide gaps across the adduct gave a gap-filling pattern identical to that obtained with the 31-mer (data not shown). These results suggest that there is a requirement for an optimum gap size (*28*, *32*), which in this case appears to be 2−3 nucleotides. Primer extension on the control template

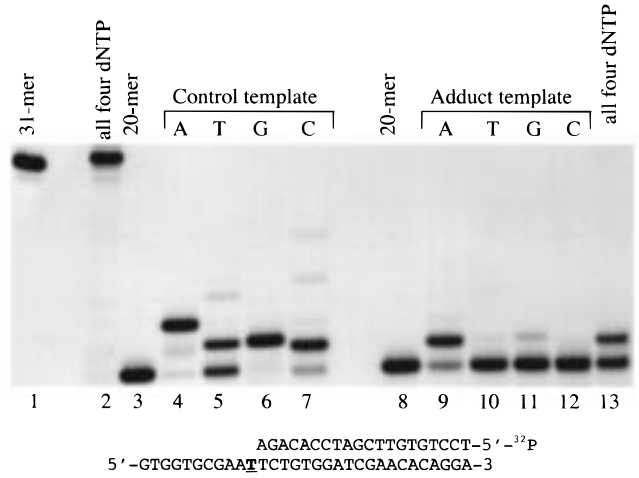


FIGURE 9: Fidelity of translesional DNA replication by proofreading-deficient Klenow polymerase on NCS−chrom-adducted and control DNA template. Insertion of a nucleotide opposite T_{11} of the 31-mer template was followed in assays (20 min) containing ^{32}P-labeled 20-mer primer and a single deoxyribonucleotide triphosphate. Lane 1, 31-mer marker; lanes 2 and 13, product synthesized on the control and the drug-modified template, respectively, in the presence of all four dNTPs; lanes 3 and 8, 20-mer primer; lanes 4−7, products from control template; lanes 9−12, products from drug-modified template. A, T, G, and C represent incorporation from dATP, dTTP, dGTP, and dCTP, respectively. Shown at the bottom is the template/primer.

proceeded to completion with or without a gap to generate the expected 31-mer (lanes 8−13).

Fidelity of Incorporation opposite the NCS−Chrom-Modified Nucleotide. To determine if the correct nucleotide is inserted opposite the drug-modified T_{11} in translesional synthesis, enzyme assays were performed with 5′-P^{32}-labeled 20-mer primer and 31-mer template (control or drug-modified, Figure 2c) in a reaction containing Mg^{2+} and only one dNTP at a time. Extension of the primer to the 21-mer will place a nucleotide opposite T_{11}. In the synthesis catalyzed by Klenow polymerase (exo−) on the control template, misincorporation of all four nucleotides occurred to varying extents and lengths (Figure 9, lanes 4−7), as has also been found by others (*19*, *33*). On the other hand, the correct nucleotide A is preferentially inserted opposite the drug-modified T_{11} (lanes 9−12). Furthermore, misincorporation of the other three nucleotides is also considerably reduced compared to that in the control (Table 1). A similar preference for incorporation of A opposite T_{11} and a significant decrease in misincorporation of the other three nucleotides, as compared to that on the control template, were also obtained in assays with Sequenase or polymerase β (data not shown). These results show that NCS−chrom adduct does not interfere with the normal base pairing at the site of its adduction. When Mn^{2+} was substituted for Mg^{2+} in the assays for Klenow polymerase (exo−) and Sequenase, there was extensive misincorporation of all four nucleotides with both the drug-modified and control 31-mer templates (data not shown).

DISCUSSION

To assess the biological consequences of a NCS−chrom monoadduct covalently bound to the DNA sugar (*10*), we used a single-stranded 31-mer DNA containing the adduct as a template for primer elongation in in vitro systems

Table 1: Fidelity of DNA Replication by Proofreading-Deficient Klenow Polymerase on NCS-Chrom-Modified DNA Template[a]

	Incorporation (%)			
template	A	T	G	C
control	94	54	93	79
adduct-modified	68	6	10	3

[a] Data come from quantitation of the 20-mer and 21-mer bands in Figure 9 (lanes 9−12) with drug-modified 31-mer template and ^{32}P-20-mer primer. The percent of the ^{32}P-20-mer extended to the 21-mer with each base, is given as percent incorporation. A, T, G, and C represent incorporation opposite T_{11} from dATP, dTTP, dGTP, and dCTP, respectively.

catalyzed by several polymerases including the most commonly used model enzyme, the Klenow fragment of *E. coli* DNA polymerase I. As has been found for many DNA adducts of diverse chemical structures (*16*−*21*), DNA synthesis is blocked on the template containing a NCS−chrom adduct (Figures 3−7). The site of synthesis arrest depends on the particular enzyme used. While primer extension by Klenow polymerase is totally arrested at the nucleotide 3′ to the adduct (Figure 3A), exonuclease-deficient Klenow polymerase and Sequenase partially overcome the block to incorporate a nucleotide opposite the lesion (Figures 4 and 6). As in the case of mitomycin C (*19*), substitution of Mn^{2+} for Mg^{2+} in the exonuclease-deficient Klenow polymerase assay enhanced the incorporation of a nucleotide opposite T_{11} (Figure 4). However, unlike the situation with mitomycin C, a significant amount of incorporation opposite T_{11} occurs with this enzyme and Sequenase in the presence of Mg^{2+} (Figures 4 and 6). A recent paper (*33*) on the effect of chemically synthesized selenophenyl and pivolyl adducts at the C4′ position of DNA deoxyribose on DNA replication is of particular interest in view of the fact that NCS−chrom adduction is also at the deoxyribose, although its linkage is via the C5′ position. In the case of the C4′ selenophenyl adduct the major block to synthesis by Klenow polymerase was at one position past the adduct, and for the pivolyl adduct it was immediately 3′ to and opposite the lesion, both in contrast to the absolute block at 3′ to the NCS−chrom lesion. Nevertheless, no full-length bypass synthesis products were produced (Figures 3 and 4), a finding similar to that obtained for C4′ adducts (*33*).

Human DNA polymerase β fills short gaps (*28*, *32*) and single-nucleotide gaps associated with base excision repair in DNA (*34*−*37*). It bypassed a cisplatin adduct in translesional synthesis to produce full-length products (*38*). Unlike in the case of cisplatin adduct, however, synthesis on a NCS−chrom-modified template by polymerase β is terminated at positions immediately 3′ to and opposite the lesion with no full-length products (Figure 7). With the introduction of a gap across the lesion, translesional synthesis occurs past the adduct, although it reaches a plateau after filling in 2−3 nucleotides (Figure 8), indicative of an optimum gap size (*28*, *32*). It is clear that synthesis by polymerase β and the other polymerases is not significantly affected up to the nucleotide 3′ to the adduct, but incorporation opposite and past the lesion is stalled, a finding similar to that reported for the bypass synthesis by T7 polymerase on a *N*-2-aminofluorene adduct (*39*).

The finding that base-pairing fidelity is maintained in the incorporation of a nucleotide opposite the drug-bound T

residue by Klenow polymerase (exo−) (Figure 9 and Table 1), Sequenase, and polymerase β suggests that the normal base pairing at T_{11} is not perturbed by drug adduction to deoxyribose, as was also found with C4′ adducts (*33*). This conclusion is consistent with an earlier analysis showing that the base specificity of adduct formation (mainly at T and A residues) does not reflect the base specificity of NCS-induced mutagenesis in λ phage systems, where mutagenesis, especially that occurring at G·C base pairs, can be attributed to abasic site formation (*9, 40*).

ACKNOWLEDGMENT

We thank Dr. Samuel Wilson for the generous gift of DNA polymerase β and Dr. Donald Coen for HSV and CMV DNA polymerases.

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BI982185G