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Transcription by Eucaryotic and Procaryotic RNA Polymerases of DNA Modified at a d(GG) or a d(AG) Site by the Antitumor Drug *cis*-Diamminedichloroplatinum(II)†

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Received June 28, 1990; Revised Manuscript Received September 7, 1990

ABSTRACT: We have investigated whether DNA modified at a d(GG) or a d(AG) site by the chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP) can be used as template by wheat germ RNA polymerase II. The templates used in the present study were obtained by ligation of double-helical oligodeoxyribonucleotides, containing 18 pyrimidine bases and 2 central dG, or dA and dG, bases on one strand and 18 purine bases and 2 central dC, or dT and dC, bases on the complementary strand. Therefore, the *cis*-DDP adducts are only present on one strand of each of the two templates and are regularly spaced by 18 pyrimidine bases. These constructs allowed us to investigate the effect of *cis*-DDP on transcription of the platinated strand and of the complementary unplatinated sequence. Transcription experiments were carried out in the presence of dinucleotide primers and either a single triphosphate substrate (abortive elongation) or the full set of triphosphate substrates dictated by the template sequence (productive elongation). The results show that the eucaryotic RNA polymerase can catalyze dinucleotide-primed reactions on platinated DNA. However, the eucaryotic enzyme behaved very differently depending on which strand was transcribed. Thus, transcription elongation was completely blocked on the strand carrying the metal complex, whereas transcription elongation was not blocked on the complementary template strand. However, on this latter strand and with the platinated polymers, productive elongation was slightly inhibited. Furthermore, abortive elongation leading to dinucleotide-primed trinucleotide formation was enhanced on the template strand complementary to that carrying the *cis*-DDP adducts. Similar results were obtained in transcription of the platinated templates by *Escherichia coli* RNA polymerase, suggesting that the *cis*-DDP-induced effect is associated with modifications of the basic catalytic properties of the transcriptases.

cis-Diamminedichloroplatinum(II) (*cis*-DDP)[1] has been introduced by Rosenberg et al. (1969) as a powerful chemotherapeutic drug for the treatment of certain human cancers (Loehrer & Einhorn, 1984). Numerous studies suggest that the curative effect of the metal complex can be attributed to the attack of cellular DNA and to the formation of several types of adducts with DNA bases. In vivo and in vitro, *cis*-DDP reacts preferentially with adjacent purine residues, yielding two major adducts, *cis*-[$Pt(NH_3)_2${d(GpG)}](N7,N7) and *cis*-[$Pt(NH_3)_2${d(ApG)}](N7,N7), representing 65% and 25% of the bound platinum, respectively. Several results also suggest that the antitumor activity of *cis*-DDP is related to the intrastrand adducts [reviewed by Eastman (1987), Lippard (1987), and Reedijk (1987)]. These findings have stimulated a number of research studies, with the aims of deciphering whether platinated DNA may be biochemically active and of gaining a better understanding of the mechanism of action of the drug at the level of gene expression. For example, the

† This work was supported in part by the Fondation pour la Recherche Médicale and by the Association pour la Recherche sur le Cancer (Grant 6403).

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[1] Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); bp, base pair(s); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane. An asterisk denotes a deoxyribonucleotide modified by *cis*-diamminedichloroplatinum(II). d(GG/CC) is a double-stranded oligodeoxyribonucleotide of 20 bp, containing 18 pyrimidine bases and 2 central dG bases on one strand and 18 purine bases and 2 central dC bases on the complementary strand. d(G*G*/CC) is the corresponding double-stranded sequence in which the dG-dG bases are modified by *cis*-DDP. $d(GG/CC)_m$ and $d(G*G*/CC)_m$ are multimers of the ligated d(GG/CC) and d(G*G*/CC) motifs, respectively. d(AG/TC), d-(A*G*/TC), $d(AG/TC)_m$, and $d(A*G*/TC)_m$ correspond to d(GG/CC), d(G*G*/CC), $d(GG/CC)_m$, and $d(G*G*/CC)_m$ in which one of the central dG·dC bp is replaced by a dA·dT bp, respectively. Dinucleoside monophosphate primers and trinucleoside diphosphate products are referred to as dinucleotides and trinucleotides, respectively.

0006-2960/91/0430-222$02.50/0

effect of the reaction of *cis*-DDP with DNA has been investigated by Bernges and Holler (1988) with regard to DNA synthesis and 3′–5′ (proofreading) and 5′–3′ exonuclease (repair) activities of *Escherichia coli* DNA polymerase I. These studies showed that *cis*-DDP inhibits DNA synthetic activity of the polymerase through an increase in the K_m values and a decrease in V_m values for platinated DNA, but not for the nucleoside 5′-triphosphates as the varied substrates. Other such examples are provided by studies of the hydrolytic activity of several nucleases for cutting DNA treated with *cis*-DDP [for general reviews, see Eastman (1987), Lippard (1987), and references cited therein] as the cytotoxicity of *cis*-DDP is thought to occur through alterations in adduct removal by DNA repair enzymes [for a review on DNA repair, see Van Houten (1990)].

Although inhibition of DNA synthesis provides a plausible model to account for drug action, it is well established that important control of gene expression may also occur at the level of DNA transcription, at least in part, through alterations in DNA structure and conformation [for reviews, see von Hippel et al. (1984), Jovin et al. (1987), and Sawadogo and Sentenac (1990)]. Thus, a consideration of the possible roles attributable to platinated DNA in eucaryotic nuclei and in procaryotic cells requires the evaluation of its effect on the transcription process. The purpose of this work was therefore to investigate whether DNA modified by *cis*-DDP at a d(GG) site or a d(AG) site can be used as template by wheat germ RNA polymerase II or *E. coli* RNA polymerase. We studied the template properties of two platinated double-stranded polymers consisting of repeating motifs of 20 bp. These double-helical motifs contain either a d(G*G*) adduct or a d(A*G*) adduct on one strand. In these duplexes, all the base pairs but one are the same (the 5′ dG·dC bp adjacent to the central dG·dC bp is replaced by a dA·dT bp). These two polymers have been characterized from structural studies, and the results established that d(G*G*) and d(A*G*) adducts bend DNA similarly, but at the nucleotide level they distort the double helix differently (Marrot & Leng, 1989; Schwartz et al., 1989).

Purified wheat germ RNA polymerase II (Dietrich et al., 1985) catalyzes a template-directed single-step condensation of a ribonucleotide to a dinucleotide primer, leading to trinucleotide product formation (abortive elongation reaction), as described by Oen and Wu (1978) for *E. coli* RNA polymerase [reviewed in McClure (1985) and von Hippel et al. (1984)]. With the plant enzyme, it has been shown that trinucleotide synthesis can be a catalytic process (the trinucleotide is released and accumulates) and is much reduced in conditions favoring chain elongation, that is, in the presence of the full set of substrates dictated by the template sequence. These reactions permitted kinetic analysis of the first phosphodiester bond formation, and also of the parameters that have importance to condition the switch from abortive to productive elongation (Job et al., 1987; Job, D., et al., 1988).

Herein, we show that both wheat germ RNA polymerase II and *E. coli* RNA polymerase can catalyze dinucleotide-primed reactions on platinated DNA. Under conditions allowing the synthesis of trinucleotide products, the enzymes behaved very differently on the platinated or the complementary strand of the double-helical template polymers. Thus, single-step addition reactions were inhibited on the platinated strand but were enhanced on the complementary unplatinated strand. Furthermore, in the presence of the full set of triphosphate substrates and for both transcriptases, the two following characteristic features were observed: (i) transcription elongation was completely blocked on the strand carrying the metal complex and (ii) transcription elongation was not blocked on the complementary unplatinated template strand. However, on this latter strand and with the platinated polymers, productive synthesis of long-length RNA species was slightly inhibited. The observed effects may represent an important biological role played by platinated DNA sequences.

Materials and Methods

Reagents. Ultrapure ribonucleoside triphosphates and the dinucleoside monophosphates were purchased from Pharmacia and Boehringer Mannheim. α-^{32}P-Labeled ribonucleoside triphosphates (410 Ci mmol^{-1}) were from Amersham.

Oligodeoxyribonucleotide Synthesis and Ligations. The oligodeoxyribonucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified by ion-exchange FPLC. The reaction between *cis*-DDP and single-stranded oligodeoxyribonucleotides were performed as previously described (Marrot & Leng, 1989). The platinated oligodeoxyribonucleotides were purified by ion-exchange FPLC. The sites of platination were verified by reverse-phase HPLC analysis of the digests obtained after incubation of the platinated oligodeoxyribonucleotides with P1 nuclease (Boehringer Mannheim) (Fichtinger et al., 1985), and then with alkaline phosphatase (Bethesda Research Laboratories) (Eastman, 1986). The sequences of the repeating units of the two duplexes used in this work are

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d(GG/CC) :  5' C T T C T C T T C T G G T C T T C T C T
               A A G A G A A G A C C A G A A G A G A G  5'

d(AG/TC) :  5' C T T C T C T T C T A G T C T T C T C T
               A A G A G A A G A T C A G A A G A G A G  5'
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After reaction with the single-stranded oligodeoxyribonucleotides (upper strand), *cis*-DDP was bound either to the adjacent dG residues or to the dA residue adjacent to the dG residue. The double-stranded oligodeoxyribonucleotides were obtained by mixing the platinated or the unplatinated 20-mer with the complementary strands (lower strands), adjusted so that the resulting duplexes have cohesive ends (19 bp were formed). Care was taken to hybridize the oligodeoxyribonucleotides so as to avoid contamination of the duplexes by residual amounts of the free single-stranded oligomers. As before, we shall name the 20-mer duplexes by their two central d(GG/CC) or d(AG/TC) base pairs (Schwartz et al., 1989). Wheat germ RNA polymerase II requires a stretch of about 40 bp in order to bind to DNA (Chandler & Gralla, 1980) and to catalyze dinucleotide-primed trinucleotide product formation (de Mercoyrol et al., 1990). Therefore, multimers of the above 20-mer duplexes were prepared with T4 DNA ligase (Koo et al., 1986). This yielded double-helical polymers containing on their upper strand dG-dG or dA-dG dinucleotides regularly spaced by 18 pyrimidine bases. It was verified by gel electrophoresis that the size of the major part of the multimers was greater than 100 bp. We shall refer to these multimers as d(GG/CC)$_m$ and d(AG/TC)$_m$ (unplatinated DNA) and as d(G*G*/CC)$_m$ and d(A*G*/TC)$_m$ (platinated DNA).

DNA-Dependent RNA Polymerases. RNA polymerase II was purified from wheat germ, much as described by Jendrisak and Burgess (1975), with the following modifications: Fraction 3 of Jendrisak and Burgess was purified by successive column chromatography on Fractogel TSK DEAE-650 (S) (Merck), Heparin-Ultrogel A4R (IBF), Fractogel TSK HW-55 (S) (Merck), Phospho-Ultrogel A6R (IBF), and single-stranded DNA Ultrogel A4R (IBF). The enzyme preparations migrated as a single band ($M_r \approx 550000$) on electrophoresis in native 7.5% polyacrylamide gels, and in native gels containing

a continuous gradient from 8 to 25% polyacrylamide. The specific activity of the enzyme preparations was typically of the order of 2000 units mg^{-1} on poly[d(A-T)] as template and with 20 μM ATP, 5 μM UTP, and 1.5 mM $MnCl_2$, 1 unit being equivalent to the incorporation of 10 pmol of UMP into poly[r(A-U)] in 15 min at 35 °C. *E. coli* RNA polymerase holoenzyme was a generous gift from Henri Buc (Institut Pasteur, Paris). It was used as a stock 8.45 μM solution. The specific activity of the procaryotic enzyme was of the order of 65000 units mg^{-1}, with transcription assays as described above.

Reaction Assays. Unless otherwise noted in the figure legends, the reaction mixtures contained 20 nM wheat germ RNA polymerase II or 2.4 nM *E. coli* RNA polymerase, 5 $\mu g\ mL^{-1}$ DNA, 1.5 mM $MnCl_2$, 0.2 mM dinucleotide primer, 1 μM ^{32}P-labeled ribonucleoside triphosphate, and appropriate amounts of unlabeled nucleoside triphosphates. In all assays, other components were 40 mM Tris-HCl, pH 7.8 buffer, 7.9% (v/v) glycerol, 7.9 mM 2-mercaptoethanol, 3.2 mM α-thioglycerol, 0.7 mM DTT, 0.03 mM EDTA, 0.03% Triton X-100, and 1.5 mM NaF. Final volumes were 20 μL. Assays were incubated for 1 h (wheat germ enzyme) or 30 min (*E. coli* enzyme), at 35 °C. Reactions were stopped by mixing the 20-μL reaction mixtures with 20 μL of stop solution containing 1 mM EDTA, 80% formamide, and 0.1% xylene cyanol.

Activity Measurements. The reaction mixtures were processed as follows (Job et al., 1987). (i) Abortive syntheses of trinucleotides were detected after ascending TLC on poly(ethylenimine)–cellulose sheets of 4 μL (reaction mixtures + stop solution) (Randerath & Randerath, 1967). Solvent systems were prepared so as to provide a clear separation between the unused triphosphate substrate (which generally remained bound at the origin) and the trinucleotide products. This was achieved by employing the following solvent systems: LiCl (0.1 M)/HCOOH (1 M), LiCl (0.2 M)/HCOOH (1 M), and LiCl (0.8 M)/HCOOH (1 M), for analysis of GpUpC, ApGpU, and GpGpU syntheses, respectively. Following radioautography, all spots containing radioactivity were cut out and counted in a liquid scintillation counter. (ii) The distribution of RNA chain lengths was analyzed by electrophoresis on 20% polyacrylamide gels (0.03 cm × 30 cm × 40 cm), with an 8 M urea and 50 mM Tris–borate, pH 8.0, buffer (Maniatis et al., 1982). Gels were run at a constant 2000 V, until the xylene cyanol dye marker had migrated 10 cm. The gels were then covered with Saran wrap and exposed to Fuji RX films at −80 °C with a Cronex Li-Plus intensifying screen from Du Pont. In these gels, trinucleotides migrated more slowly than expected, as observed by Levin et al. (1987) and de Mercoyrol et al. (1990) for very short transcripts with free 5′-hydroxyl groups.

Results

The sequences of the repeating units of the double-helical $d(GG/CC)_m$, $d(G^*G^*/CC)_m$, $d(AG/TC)_m$, and $d(A^*G^*/TC)_m$ polymers used in the present study contain two central dG or dA and dG bases on the upper strands and two central dC or dT and dC bases on the complementary lower strands. These polymers should permit, therefore, the direction of the enzymic synthesis of several types of trinucleotide products: each of these should correspond to a unique array of three bases within the repeats and result from single-step addition reaction of a single triphosphate substrate to a dinucleotide primer. For both *E. coli* (Sylvester & Cashel, 1980) and wheat germ RNA polymerase II (Dietrich et al., 1985), it has been shown that trinucleotides terminated by a pyrimidine base are formed in much higher amounts than the purine-terminated triplets. For this reason, the analysis of the abortive elongation reaction was restricted in this study to the use of CTP or UTP as the single substrate. Owing to the nucleotide sequences of the polymers, primers and substrates were selected so as to direct transcription from the dG-dG- or the dA-dG-containing strands or from the complementary strands of the duplexes (referred to as the "upper" or the "lower" strands, respectively).

Dinucleotide-Primed Reactions Directed by the Lower Strands of the Templates with Wheat Germ RNA Polymerase II. In a typical reaction assay, the synthesis of GpGpU occurred after incubation for 1 h of reaction mixtures containing wheat germ RNA polymerase II, GpG as primer, [α-^{32}P]UTP as substrate, and $d(GG/CC)_m$ as template. When analyzed by TLC on poly(ethylenimine)–cellulose sheets, GpGpU migrated with a R_f value of 0.3, whereas unreacted UTP remained bound at the origin (Figure 1A, lane 1). As shown in Figure 1A (lane 2), the formation of this transcription product was strongly inhibited by α-amanitin, a specific inhibitor of class II RNA polymerases (Sawadogo & Sentenac, 1990). Indicative of elongation of the triplet, the reaction was abolished upon addition of CTP (lane 3) and of CTP and GTP together (lanes 4–6): in the former case, formation of a 20-mer transcript is expected from the nucleotide sequence of the lower strand of the template because further elongation requires that two GMP residues must be incorporated at positions 21 and 22, numbering by 1 the first G residue at the 5′ end of the primer; in the latter case, chains longer than a 20-mer are expected because full copying of the lower strand of $d(GG/CC)_m$ only necessitates UTP, CTP, and GTP as substrates. Elongation of GpGpU was strictly dependent on the presence of CTP (the nucleotide required at position 4 in the transcript), as the level of triplet formation was not affected when transcription assays leading to GpGpU synthesis were carried out in the additional presence of GTP alone (Figure 1A, lane 7). These results demonstrate that the GpG-primed reaction exhibited all the features expected from initiation of transcription of the lower strand of the $d(GG/CC)_m$ template, at the level of the two central dC bases within the repeating 20-mer unit, and testify to the absence of any detectable cross contamination of the ribonucleotide substrates.

GpGpU synthesis was also evidenced in the presence of platinated $d(G^*G^*/CC)_m$ as the template, exhibiting the similar characteristic features observed by using the corresponding unplatinated $d(GG/CC)_m$ sequence (Figure 1A, lanes 8–14). It is noticeable, however, that the level of trinucleotide formation was higher with $d(G^*G^*/CC)_m$ as opposed to that with $d(GG/CC)_m$ as the template. Thus, under the standard conditions given under Materials and Methods, the concentration of substrate UTP converted into GpGpU was 181 and 110 nM with $d(G^*G^*/CC)_m$ and $d(GG/CC)_m$ as the template, respectively (Figure 1A).

Figure 1B shows the same reaction mixture as in panel A, but analyzed by high-resolution gel electrophoresis, with results that are consistent with those obtained by TLC. First GpGpU migrated as a single species in the gel (Figure 1B, lanes 1 and 8). Second, the gels resolved the various RNA chains resulting from the different conditions used for elongation of the triplet to longer RNA chains. Thus, in the sole presence of elongating CTP, GpGpU synthesis was markedly diminished, whereas a discrete-length product of lower mobility than the trinucleotide was detected, corresponding most probably to the synthesis of the 20-mer transcript expected from the template sequence (Figure 1B, lanes 3 and 10). In the presence of both CTP and GTP, full transcription of the repeating units occurred, as much longer RNA chains than the 20-mer transcript

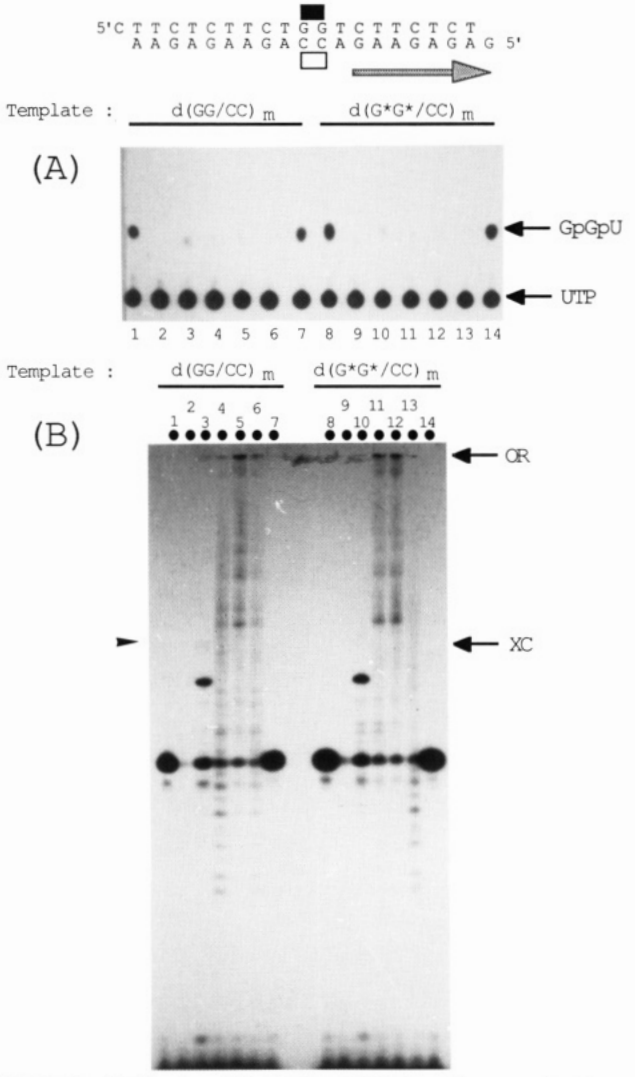

FIGURE 1: GpG-primed GpGpU and RNA syntheses on the lower strand of $d(GG/CC)_m$ and $d(G^*G^*/CC)_m$ templates with wheat germ RNA polymerase II. The dinucleotide primer was GpG. Aliquots of 4 μL (A) or 10 μL (B) (reaction mixture + stop solution) were analyzed. The upper part depicts the sequence of the double-helical repeat motif of the unplatinated $d(GG/CC)_m$ and the platinated $d(G^*G^*/CC)_m$ polymers. The positions of the platinated bases and of the dinucleotide primer are shown by the black and white rectangles, respectively. The direction of transcription on the lower strand is indicated by an arrow. (A) Radioautographic analysis by TLC. The template was unplatinated $d(GG/CC)_m$ (lanes 1–7) or platinated $d(G^*G^*/CC)_m$ (lanes 8–14): (lanes 1 and 8) complete reaction mixtures leading to GpGpU synthesis with 1 μM [α-^{32}P]UTP as substrate; (lanes 2 and 9) +10 μg mL^{-1} α-amanitin; (lanes 3 and 10) +20 μM CTP; (lanes 4, 5, and 6 and 11, 12, and 13) +20 μM CTP and 0.5, 2, or 10 μM GTP, respectively; (lanes 7 and 14) +2 μM GTP. The concentration of UTP substrate converted into GpGpU was calculated to be 110, 106, 181, and 171 nM for lanes 1, 7, 8, and 14, respectively. (B) Analysis by polyacrylamide–urea gel electrophoresis: (lanes 1–14) see conditions for lanes 1–14 in (A). The arrowhead shows the longest oligonucleotide synthesized under conditions allowing the formation of a 20-mer transcript (lanes 3 and 10). OR, origin; XC, xylene cyanol.

were synthesized from the $d(GG/CC)_m$ and the $d(G^*G^*/CC)_m$ templates (Figure 1B, lanes 4–6 and 11–13). However, such productive elongation reactions carried out with the platinated DNA were inhibited only slightly by comparison with those with the unplatinated templates (compare lanes 6 and 13 of Figure 1B).

To extend these results to other possible dinucleotide-primed reactions on the lower strand of the platinated templates, transcription experiments were carried out with either GpU or ApG as the primer. These primers allowed direction of transcription close to the central dC-dC [$d(GG/CC)_m$ and $d(G^*G^*/CC)_m$ polymers] or the central dT-dC nucleotides [$d(AG/TC)_m$ and $d(A^*G^*/TC)_m$ polymers] within the repeating units of the lower strands of the multimers, complementary to one or two bases of the dG-dG or dA-dG sequences forming the platination sites on the upper strands of the multimers. The results are presented in Figure 2A–C for the TLC analysis and in Figure 2D,E for the gel electrophoresis analysis. In all cases, similar results to those obtained in Figure 1 were observed; that is, on the lower strands, trinucleotide product formation was enhanced, whereas productive elongation was only slightly inhibited. The results show, therefore, that the RNA polymerase was capable of using dinucleotide primers to initiate RNA synthesis at sequences fully or partly complementary to the $d(G^*G^*)$ or $d(A^*G^*)$ adducts. Furthermore, from a comparison of the electrophoretic mobilities of the products formed in the presence of an incomplete set of triphosphate substrates [for example, the conditions yielding a 19-mer transcript (lanes 1, 3, 5, and 7 of Figure 2D), or a 20-mer transcript (lanes 1 and 3 in Figure 2E)] with those formed with the full set of substrates (lanes 2, 4, 6, and 8 in Figure 2D and lanes 2 and 4 in Figure 2E), the results demonstrate that the enzyme was able to translocate past the next site(s) complementary to the platinated nucleotides.

Dinucleotide-Primed Reactions Directed by the Lower Strands of the Templates with E. coli RNA Polymerase. In additional experiments, $d(AG/TC)_m$ and $d(A^*G^*/TC)_m$ were used as templates in the presence of *E. coli* RNA polymerase, under experimental conditions allowing transcription of the lower strands of these polymers. As wheat germ RNA polymerase II requires Mn^{2+} to catalyze the single-step addition reactions [trinucleotide formation cannot be detected in the presence of Mg^{2+} (Dietrich et al., 1986)], this comparative study with the procaryotic RNA polymerase was carried out in the presence of $MnCl_2$ as the metal cofactor. Figure 3 shows the results obtained by using ApG as a primer. As in the case of the eucaryotic RNA polymerase, abortive elongation was favored on platinated DNA (compare lanes 1 and 5); however, productive elongation was reduced (compare lanes 3 and 7), although chains longer than a 20-mer transcript were evident in transcription of the lower strand of the platinated $d(A^*G^*/TC)_m$ (compare lanes 6 and 7 of Figure 3). Similar results to those presented in Figure 3 were obtained with the $d(GG/CC)_m$ and $d(G^*G^*/CC)_m$ polymers, and with GpG as primer (not shown). Therefore, we conclude from this comparative study that the eucaryotic and procaryotic enzymes exhibited the same behavior in transcription of the lower strand of the double-helical platinated polymers.

Dinucleotide-Primed Reactions Directed by the Upper Strands of the Templates with Wheat Germ RNA Polymerase II. Primers and pyrimidine substrates were selected so as to direct transcription from the strands of the double-helical polymers containing the dG-dG or dA-dG dinucleotides. The sequences of the repeating units of the upper strands of these polymers mean that there are only two possible combinations per polymer: GpA + CTP or ApC + CTP for the polymers containing dG-dG, and GpA + CTP or ApC + UTP for the polymers containing dA-dG.

In contrast to the results obtained for trinucleotide formation on the lower strands, we observed in the case of the upper strands that the triplets were formed in too low amounts to allow quantification by TLC analysis. As this was found with both the platinated and the unplatinated templates, it appears that the enzyme behavior was somehow dependent on the

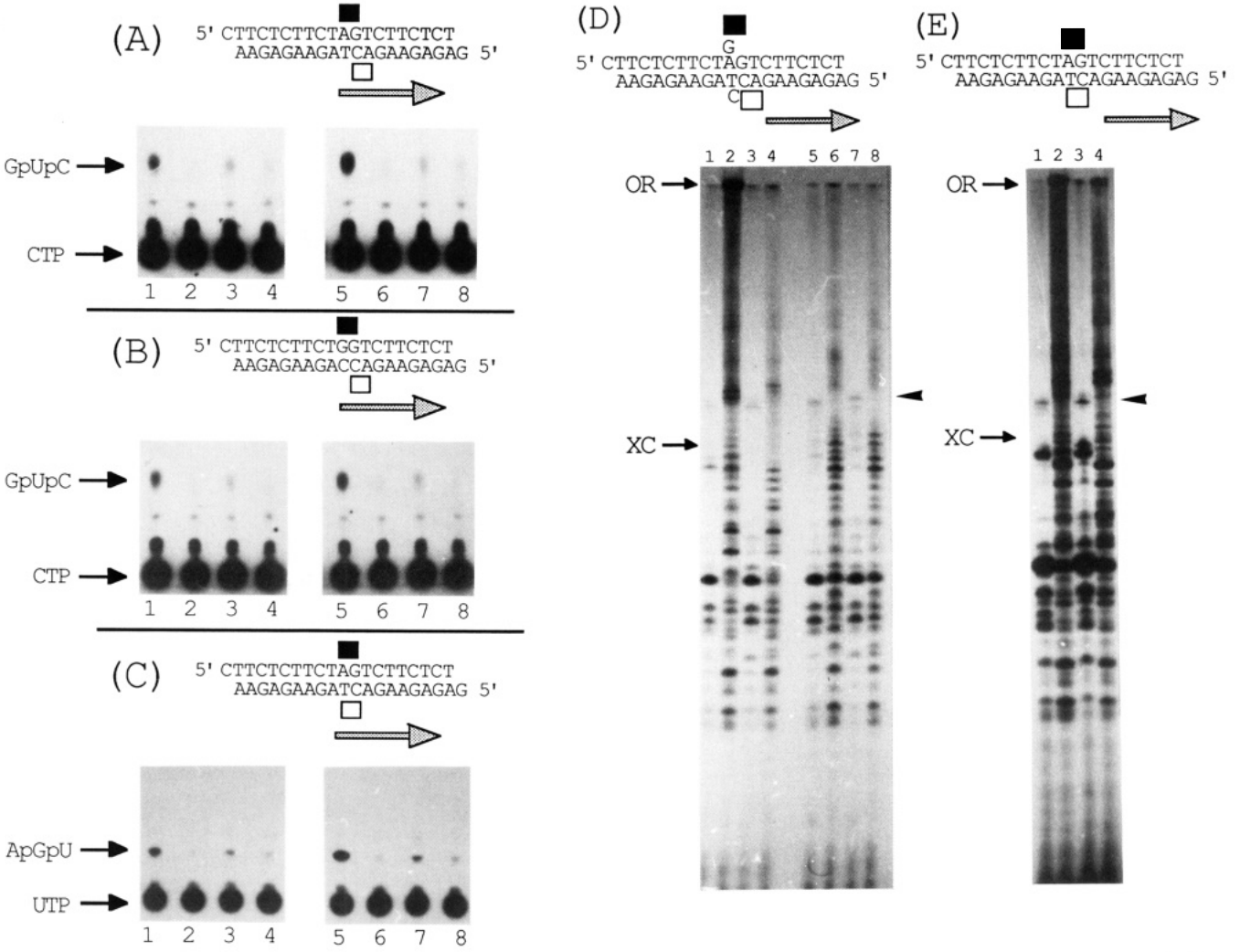

FIGURE 2: Dinucleotide-primed reactions of trinucleotide and RNA syntheses on the lower strand of $d(GG/CC)_m$, $d(G^*G^*/CC)_m$, $d(AG/TC)_m$, and $d(A^*G^*/TC)_m$ templates with wheat germ RNA polymerase II. The dinucleotide primer was GpU or ApG. Aliquots of 4 μL (A–C) or 10 μL (D and E) (reaction mixture + stop solution) were analyzed. In the upper parts, the positions of the platinated bases and of the dinucleotide primer are shown by the black and white rectangles, respectively. The direction of transcription on the lower strands is indicated by an arrow. (A–C) Radioautographic analysis by TLC. (A) The template was unplatinated $d(AG/TC)_m$ (lanes 1–4) or platinated $d(A^*G^*/TC)_m$ (lanes 5–8): (lanes 1 and 5) complete reaction mixtures leading to GpU-primed GpUpC synthesis with 1 μM [α-^{32}P]CTP as substrate; the concentration of CTP substrate converted into GpUpC was 7 and 32 nM for lanes 1 and 5, respectively; (lanes 2 and 6) +10 μg mL^{-1} α-amanitin; (lanes 3 and 7) +20 μM UTP; (lanes 4 and 8) +20 μM UTP, 2 μM ATP, and 2 μM GTP. (B) The template was unplatinated $d(GG/CC)_m$ (lanes 1–4) or platinated $d(G^*G^*/CC)_m$ (lanes 5–8): (lanes 1 and 5) complete reaction mixtures leading to GpU-primed GpUpC synthesis with 1 μM [α-^{32}P]CTP as substrate; the concentration of CTP substrate converted into GpUpC was 9 and 27 nM for lanes 1 and 5, respectively; (lanes 2 and 6) +10 μg mL^{-1} α-amanitin; (lanes 3 and 7) +20 μM UTP; (lanes 4 and 8) +20 μM UTP and 2 μM GTP. (C) The template was unplatinated $d(AG/TC)_m$ (lanes 1–4) or platinated $d(A^*G^*/TC)_m$ (lanes 5–8): (lanes 1 and 5) complete reaction mixtures leading to ApG-primed ApGpU synthesis with 1 μM [α-^{32}P]UTP as substrate; the concentration of UTP substrate converted into ApGpU was 35 and 86 nM for lanes 1 and 5, respectively; (lanes 2 and 6) +10 μg mL^{-1} α-amanitin; (lanes 3 and 7) +20 μM CTP; (lanes 4 and 8) +20 μM CTP, 2 μM GTP, and 2 μM ATP. (D and E) Analysis by polyacrylamide–urea gel electrophoresis: (D, lanes 1 and 2) $d(AG/TC)_m$ as template, GpU as primer, and 1 μM [α-^{32}P]CTP, 20 μM UTP or 1 μM [α-^{32}P]CTP, 20 μM UTP, 2 μM ATP, and 2 μM GTP as substrates, respectively; (D, lanes 3 and 4) as for lanes 1 and 2 in (D) but with $d(A^*G^*/TC)_m$ as template; (D, lanes 5 and 6) $d(GG/CC)_m$ as template, GpU as primer, and 1 μM [α-^{32}P]CTP, 20 μM UTP or 1 μM [α-^{32}P]CTP, 20 μM UTP, and 2 μM GTP as substrates, respectively; (D, lanes 7 and 8) as for lanes 5 and 6 in (D) but with $d(G^*G^*/CC)_m$ as template. The arrowhead shows the longest oligonucleotide synthesized under conditions allowing the formation of a 19-mer transcript (lanes 1, 3, 5, and 7). (E, lanes 1 and 2) $d(AG/TC)_m$ as template, ApG as primer, and 1 μM [α-^{32}P]UTP, 20 μM CTP or 1 μM [α-^{32}P]UTP, 20 μM CTP, 2 μM ATP, and 2 μM GTP as substrates, respectively; (E, lanes 3 and 4) as for lanes 1 and 2 in (E) but with $d(A^*G^*/TC)_m$ as template. The arrowhead shows the longest oligonucleotide synthesized under conditions allowing the formation of a 20-mer transcript (lanes 1 and 3). OR, origin; XC, xylene cyanol.

nature and base composition (pyrimidine versus purine rich) of the transcribed strand. Nevertheless, enzymic RNA synthesis with wheat germ RNA polymerase II could easily be detected, by using the above primer–triphosphate substrate combinations, the unplatinated templates, and all of the nucleotides required for transcription elongation on their upper strands. These RNA products were analyzed by gel electrophoresis (Figure 4). Figure 4 (lane 1) shows the GpA-primed RNA synthesis directed by the upper strand of the $d(GG/CC)_m$ unplatinated polymer, with the CTP, ATP, and GTP triphosphate substrates. Long RNA chains were also synthesized from transcription of the upper strand of $d(AG/TC)_m$ (GpA as primer; lane 3), of $d(GG/CC)_m$ (ApC as primer; lane 5), and of $d(AG/TC)_m$ (ApC as primer; lane 8) (Figure 4).

Under the same experimental conditions, but with the platinated polymers as templates, only short-length species were being synthesized from transcription of the upper strands. The results are shown in lanes 2 [$d(G^*G^*/CC)_m$ as template; GpA as primer], 4 [$d(A^*G^*/TC)_m$ as template; GpA as primer], 6 [$d(G^*G^*/CC)_m$ as template; ApC as primer], and

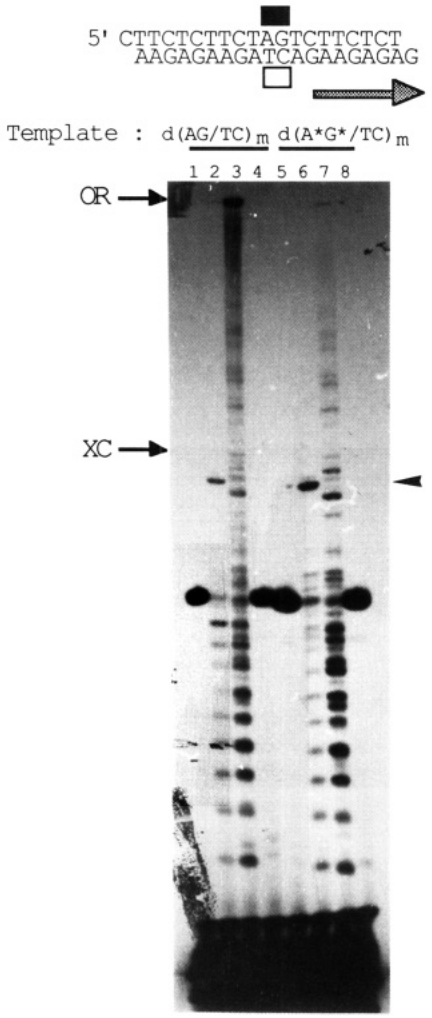

FIGURE 3: ApG-primed reactions of trinucleotide and RNA syntheses on the lower strand of $d(AG/TC)_m$ and $d(A^*G^*/TC)_m$ templates with *E. coli* RNA polymerase. The dinucleotide primer was ApG. Aliquots of 10 μL (reaction mixture + stop solution) were analyzed by polyacrylamide–urea gel electrophoresis. In the upper part, the positions of the platinated bases and of the dinucleotide primer are shown by the black and white rectangles, respectively. The direction of transcription on the lower strand is indicated by an arrow. The template was unplatinated $d(AG/TC)_m$ (lanes 1–4) or platinated $d(A^*G^*/TC)_m$ (lanes 5–8): (lanes 1 and 5) complete reaction mixtures leading to ApGpU synthesis with 1 μM [α-^{32}P]UTP as substrate; (lanes 2 and 6) +20 μM CTP; (lanes 3 and 7) +20 μM CTP, 2 μM GTP, and 2 μM ATP; (lanes 4 and 8) +2 μM GTP and 2 μM ATP. The arrowhead shows the longest oligonucleotide synthesized under conditions allowing the formation of a 20-mer transcript (lanes 2 and 6). OR, origin; XC, xylene cyanol.

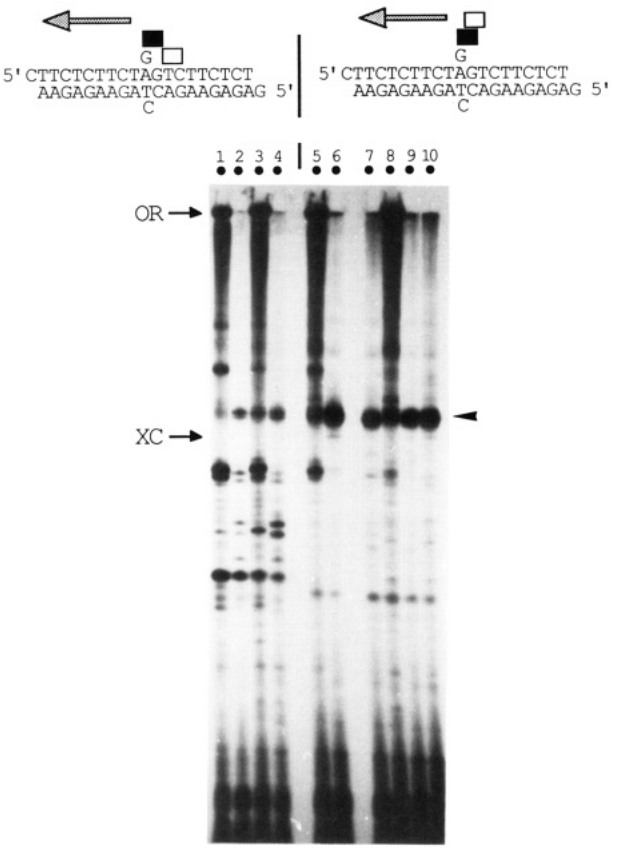

FIGURE 4: Dinucleotide-primed reactions of RNA syntheses on the upper strand of $d(GG/CC)_m$, $d(G^*G^*/CC)_m$, $d(AG/TC)_m$, and $d(A^*G^*/TC)_m$ templates with wheat germ RNA polymerase II. The dinucleotide primer was GpA or ApC. Aliquots of 10 μL (reaction mixture + stop solution) were analyzed by polyacrylamide–urea gel electrophoresis. In the upper parts, the positions of the platinated bases and of the dinucleotide primer are shown by the black and white rectangles, respectively. The direction of transcription on the upper strands is indicated by an arrow. (Lanes 1 and 2) $d(GG/CC)_m$ or $d(G^*G^*/CC)_m$ as template, respectively, GpA as primer, and 1 μM [α-^{32}P]CTP, 2 μM GTP, and 5 μM [α-^{32}P]ATP as substrates; (lanes 3 and 4) $d(AG/TC)_m$ or $d(A^*G^*/TC)_m$ as template, respectively, GpA as primer, and 1 μM [α-^{32}P]CTP, 2 μM GTP, 5 μM [α-^{32}P]ATP, and 1 μM UTP as substrates; (lanes 5 and 6) $d(GG/CC)_m$ or $d(G^*G^*/CC)_m$ as template, respectively, ApC as primer, and 1 μM [α-^{32}P]CTP, 2 μM GTP, and 5 μM [α-^{32}P]ATP as substrates; (lanes 7 and 9) $d(AG/TC)_m$ or $d(A^*G^*/TC)_m$ as template, respectively, ApC as primer, and 1 μM [α-^{32}P]UTP, 2 μM GTP, and 5 μM [α-^{32}P]ATP as substrates (the arrowhead shows the longest oligonucleotide synthesized; the conditions allow the formation of a 21-mer transcript); (lanes 8 and 10) $d(AG/TC)_m$ or $d(A^*G^*/TC)_m$ as template, respectively, ApC as primer, and 1 μM [α-^{32}P]UTP, 2 μM GTP, 5 μM [α-^{32}P]ATP, and 1 μM CTP as substrates. OR, origin; XC, xylene cyanol.

10 [$d(A^*G^*/TC)_m$ as template; ApC as primer] of Figure 4. In the case of transcription of the upper strand of the $d(AG/TC)_m$ polymer, one should expect, from the sequence of this strand, the synthesis of a 21-mer transcript in the presence of the ApC primer, and a limited set of triphosphate substrates composed of UTP, ATP, and GTP (the nucleotide required for incorporation at position 22 is CTP). Figure 4 (lane 7) shows that such a discrete-length product was formed under these experimental conditions. This type of synthesis was also evidenced with same primer and substrates, but with use of the platinated $d(A^*G^*/TC)_m$ polymer (Figure 4, lane 9). It is noteworthy that this oligoribonucleotide comigrated in the polyacrylamide gel with the longest chains that could be synthesized from the upper strands of the $d(G^*G^*/CC)_m$ and $d(A^*G^*/TC)_m$ templates in the presence of the full set of substrates required for a complete copying of the repeating units forming the polymers (compare lanes 2, 4, 6, and 10 and lanes 7 and 9 of Figure 4). From these results, we conclude that, although wheat germ RNA polymerase II was able to use dinucleotide primers to initiate RNA synthesis within the $d(G^*G^*)$ or $d(A^*G^*)$ adduct, transcription elongation was irreversibly blocked on the upper strand of the platinated templates, thereby implying that the eucaryotic enzyme could not translocate past the next platination site.

Dinucleotide-Primed Reactions Directed by the Upper Strands of the Templates with E. coli RNA Polymerase. In the following experiments $d(AG/TC)_m$, $d(A^*G^*/TC)_m$, $d(GG/CC)_m$, and $d(G^*G^*/CC)_m$ were used as templates in the presence of *E. coli* RNA polymerase, under experimental conditions allowing transcription of the upper strands of these polymers. Figure 5 shows the results after gel electrophoresis of the reaction assays. In the case of the procaryotic RNA polymerase, trinucleotide formation from ApC primer and

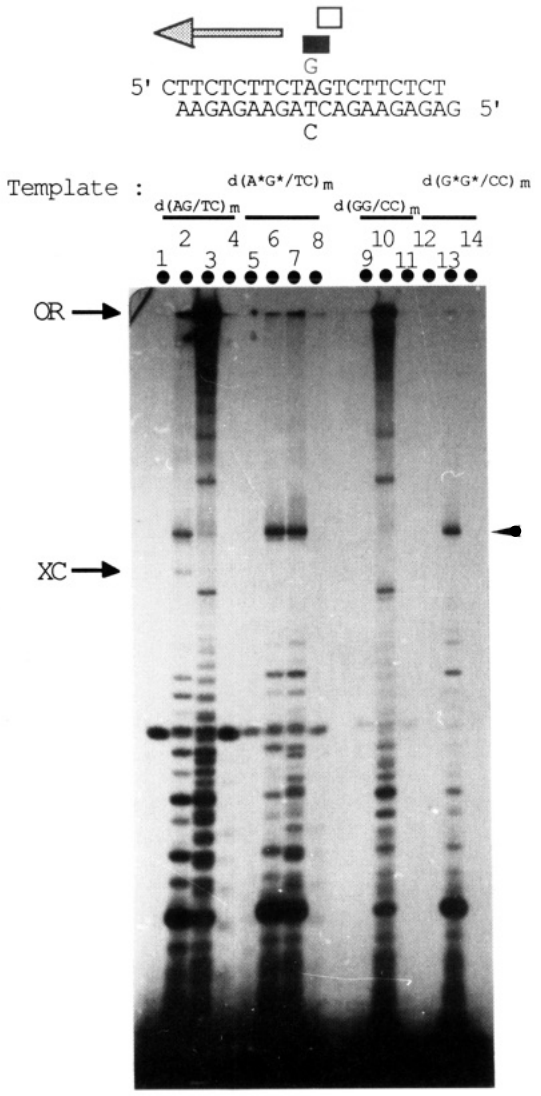

FIGURE 5: ApC-primed reactions of trinucleotide and RNA syntheses on the upper strand of $d(GG/CC)_m$, $d(G^*G^*/CC)_m$, $d(AG/TC)_m$, and $d(A^*G^*/TC)_m$ templates with *E. coli* RNA polymerase. The dinucleotide primer was ApC. Aliquots of 10 μL (reaction mixture + stop solution) were analyzed by polyacrylamide–urea gel electrophoresis. In the upper part, the positions of the platinated bases and of the dinucleotide primer are shown by the black and white rectangles, respectively. The direction of transcription on the upper strands is indicated by an arrow. The template was unplatinated $d(AG/TC)_m$ (lanes 1–4), platinated $d(A^*G^*/TC)_m$ (lanes 5–8), unplatinated $d(GG/CC)_m$ (lanes 9–11), or platinated $d(G^*G^*/CC)_m$ (lanes 12–14): (lanes 1 and 5) complete reaction mixtures leading to ApCpU synthesis with 1 μM [α-^{32}P]UTP as substrate; (lanes 2 and 6) as in lanes 1 and 5, +5 μM [α-^{32}P]ATP and 2 μM GTP; (lanes 3 and 7) as in lanes 1 and 5, +5 μM [α-^{32}P]ATP, 2 μM GTP, and 1 μM CTP; (lanes 4 and 8) as in lanes 1 and 5, +2 μM GTP and 1 μM CTP. The arrowhead shows the longest oligonucleotide synthesized under conditions allowing the formation of a 21-mer transcript (lanes 2 and 6). (Lanes 9 and 12) complete reaction mixtures leading to ApCpC synthesis with 1 μM [α-^{32}P]CTP as substrate; (lanes 10 and 13) as in lanes 9 and 12, +5 μM [α-^{32}P]ATP and 2 μM GTP; (lanes 11 and 14) as in lanes 9 and 12, +2 μM GTP. OR, origin; XC, xylene cyanol.

UTP substrate could be detected with the $d(AG/TC)_m$ and $d(A^*G^*/TC)_m$ polymers (Figure 5, lanes 1 and 5). Furthermore, these data show that the synthesis of ApCpU was depressed on the platinated DNA template, as compared to that with the unplatinated $d(AG/TC)_m$ polymer (compare lanes 1 and 5 of Figure 5), contrary to the observed behavior in transcription of the lower strands. This trinucleotide cannot be elongated in the presence of CTP and GTP (Figure 5, lanes 4 and 8), as expected from the template sequence (ATP is required for incorporation in position 4 of the ApC-primed transcript). With transcription assays leading to ApC-primed ApCpU synthesis carried out in the additional presence of ATP, GTP, and CTP substrates, productive elongation yielding long RNA chains was observed in transcription of the upper strand of the $d(AG/TC)_m$ polymer (Figure 5, lane 3). However, under the same experimental conditions, transcription of the upper strand of platinated $d(A^*G^*/TC)_m$ was strongly impeded and only yielded short RNA species (Figure 5, lane 7). The longest of these oligonucleotides comigrated with a 21-mer transcript that can be obtained from transcription of the upper strand of either $d(AG/TC)_m$ or $d(A^*G^*/TC)_m$ in the presence of ApC primer and an incomplete mixture of triphosphate substrates (composed of UTP, ATP, and GTP; incorporation at position 22 in the ApC-primed transcript requires CTP as substrate; Figure 5, lanes 2 and 6). The same behavior was observed in transcription of the $d(GG/CC)_m$ and $d(G^*G^*/CC)_m$ polymers (Figure 5, lanes 9–14). Therefore, we conclude that both the eucaryotic and procaryotic enzymes behave similarly in transcription of the upper strand of the platinated polymers.

Discussion

The present study clearly demonstrates that wheat germ RNA polymerase II can use dinucleotide primers to initiate RNA synthesis on DNA modified at a d(GG) or a d(AG) site by *cis*-DDP. The reaction catalyzed by the eucaryotic RNA polymerase exhibited two remarkable features, depending on whether the primer and substrate combinations allowed transcription of the strand carrying the platinated nucleotides or its complementary strand within the multimerized duplexes. First, abortive elongation leading to dinucleotide-primed trinucleotide formation was *enhanced* on the template strand complementary to that carrying the d(G*G*) or d(A*G*) adducts. Productive elongation of these trinucleotides to long RNA chains was detected from transcription of this strand with the platinated templates, yielding polymeric products that were longer than 20 nucleotides (i.e., the synthesized chains contained a greater number of nucleotides than that comprised between two adjacent adducts on the multimers), findings that suggest that the eucaryotic enzyme was able to translocate past to the platination sites. Second, on the platinated nucleotide containing strands, transcription elongation was irreversibly *blocked*, as dinucleotide-primed RNA synthesis only yielded transcripts of lengths of the order of 20 nucleotides. Therefore, on this strand, the eucaryotic enzyme could not translocate past a platination site. Similar results were obtained with *E. coli* RNA polymerase, indicating that the observed effect of DNA platination is associated with modifications of the basic catalytic properties of the transcriptases.

The in vitro eucaryotic transcription system used in the present study is presumably highly simplified compared with that involved in the synthesis of precursors of messenger RNA in vivo. Thus, it is well established that there are a large number of genetic elements and protein factors needed in conjunction with purified class II RNA polymerases for specific initiation of transcription [for a recent review, see Sawadogo and Sentenac (1990)]. Within these limitations, the simplified transcription assay containing only purified components may be used, however, as a model system to provide information on the ability of the transcriptase to bind to the platinated template, to incorporate the first nucleotide in the nascent RNA chain, and to shift to the polymerization mode. In this context, it is worth mentioning that the kinetic behavior of purified wheat germ RNA polymerase II in the first steps of nucleotide incorporation exhibits similarities with that of

a HeLa cellular extract, containing the RNA polymerase II and the transcription factors, particularly with regard to the use of dinucleotide primers to initiate RNA synthesis (Luse et al., 1987; Luse & Jacob, 1987; Job, C., et al., 1987, 1988). The present results indicate that the priming activity of the RNA polymerase was not abolished on either the platinated template strand or its complementary strand.

Although in this initial study we have not quantified the interaction between the RNA polymerase and the platinated templates, the results of the transcription experiments described in the present report strongly suggest that binary enzyme/template complexes can be formed with platinated DNA and the eucaryotic RNA polymerase. This observation contrasts with the results on replication by *E. coli* DNA polymerase I that led Bernges and Holler (1988) to conclude that inhibition of replication is the consequence of lowered binding affinity between platinated DNA and DNA polymerase. A mechanistic feature, however, that seems to be in common to both eucaroytic and procaryotic DNA polymerases and RNA polymerases is the fact that in vitro DNA synthesis (Pinto & Lippard, 1985; Bernges & Holler, 1988; Villani et al., 1988; Hoffmann et al., 1989) or RNA synthesis (this study) stops before each nucleotide–platinum(II) adduct on the DNA template strand (i.e., the upper strand in this study). Therefore, all enzymes are blocked by *cis*-DDP adducts under their copying–polymerization modes. For the RNA polymerases, further studies are needed to resolve the questions of whether the strong blockage observed in transcription elongation results from an extreme instability of the ternary transcription complexes or from an impediment in enzyme translocation or nucleotide incorporation at the level of the *cis*-DDP adducts.

In the case of transcription of the complementary unplatinated strands, we noted that trinucleotide synthesis was enhanced under conditions directing the single-step addition at a sequence fully or partly complementary to the d(G*G*) or the d(A*G*) adducts, i.e., when the primers are complementary to the central dC or dT bases in the repeating motifs. With *E. coli* RNA polymerase, productive elongation is essentially an escape from the recycling phenomenon during which abortive short products are released (Carpousis & Gralla, 1980). Comparison of the levels of products formed in the abortive and productive pathways under the experimental conditions allowing transcription of the lower strands of the various polymers used in the present study shows that there is an inverse correlation between the rates for the two pathways. Previous data showed that the extent of abortive synthesis, as well as the apparent nonprocessive behavior exhibited by the wheat germ enzyme, is dependent, at least in part, on the stability of the transcription complexes (Durand et al., 1982; Dietrich et al., 1985; Job, C., et al., 1988; Job, D., et al., 1988; de Mercoyrol et al., 1989, 1990). Therefore, we attribute the effect of *cis*-DDP to a decrease in the stability of ternary transcription complexes formed on the lower strand of the platinated template polymers. This will, as a result, enhance the rate of trinucleotide product release in the single-step addition reactions and disfavor the productive elongation process in the presence of the full set of triphosphate substrates. It is well established that *cis*-DDP adducts decrease the thermal stability of the double helix [for general reviews, see Eastman (1987) and Lippard (1987)] and provoke local bending of DNA (Rice et al., 1988; Marrot & Leng, 1989; Schwartz et al., 1989): these properties suggest that the alterations in the enzyme synthetic mode observed in transcription of the lower strands of the platinated polymers are associated with modifications in the stability of the various helical structures involved in the mechanisms of enzymic polymerization [e.g., the DNA duplexes upstream and downstream to the site of catalysis that intervene in formation and propagation of the transcription bubble (Kane, 1988)]. It is noticeable that the central dC or dT bases in the $d(G*G*/CC)_m$ or $d(A*G*/TC)_m$ polymers also show an altered reactivity toward chemical probes such as (1,10-phenanthroline)copper, osmium tetraoxide, chloroacetaldehyde (Schwartz et al., 1989), and sodium cyanide (Schwartz et al., 1990). Nevertheless, it must be stressed that the present data do not allow us to establish whether the observed modifications of the catalytic properties of the RNA polymerases in transcription of the lower strands of the platinated polymers are mediated by the adduct per se or, alternatively, by distortions in DNA structure. For example, synthetic curved DNA sequences have been shown to act as transcriptional activators in *E. coli* (Bracco et al., 1989).

In conclusion, the current results demonstrate that *cis*-DDP adducts markedly affect the properties of eucaryotic and procaryotic RNA polymerases, suggesting that platinum(II) complexes may exert their effect in vivo, at least in part, at the transcriptional level. In this context, it is worth noting the proposal of Sorenson and Eastman (1988a,b) that *cis*-DDP-induced cell death may be a consequence of the inability of the treated cells to adequately recover transcription of genes required for passage to mitosis, as division of murine leukemia cells and Chinese hamster ovary cells appears to be arrested by *cis*-DDP in the G_2 phase. Another relevant aspect related to the present study is the observation by Buchanan and Gralla (1990) that, in SV40 tumor virus, *cis*-DDP preferentially attacks regulatory DNA elements, the GC box elements, which control both viral transcription and replication.

Registry No. *cis*-DDP, 15663-27-1; d(GG), 15180-30-0; d(AG), 4336-87-2; RNA polymerase, 9014-24-8.

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Hematoporphyrin-Promoted Photoinactivation of Mitochondrial Ubiquinol-Cytochrome *c* Reductase: Selective Destruction of the Histidine Ligands of the Iron–Sulfur Cluster and Protective Effect of Ubiquinone†

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Received January 30, 1990; Revised Manuscript Received August 15, 1990

ABSTRACT: Purified ubiquinol-cytochrome *c* reductase of beef heart mitochondria is very stable in aqueous solution; it suffers little damage upon illumination with visible light under aerobic or anaerobic conditions. However, it is rapidly inactivated when the photosensitizer hematoporphyrin is present during illumination. The hematoporphyrin-promoted photoactivation is dependent on sensitizer dose, illumination time, and oxygen. Singlet oxygen is shown to be the destructive agent in this system. The photoinactivation of ubiquinol-cytochrome *c* reductase is prevented by excess exogenous ubiquinone, regardless of its redox state. This protective effect is not due to protein–ubiquinone interactions but to the singlet oxygen scavenger property of ubiquinone. Ubiquinone also protects against hematoporphyrin-promoted photoinactivation of succinate-ubiquinone reductase and cytochrome *c* oxidase. The photoinactivation site in ubiquinol-cytochrome *c* reductase is the iron–sulfur cluster of Rieske's protein. Two histidine residues, presumably serving as two ligands for the iron–sulfur cluster of Rieske's protein, are destroyed. No polypeptide bond cleavage is detected. Photoinactivation has little effect on the spectral properties of cytochromes *b* and c_1 but alters their reduction rates substantially. This photoinactivation also causes the formation of proton-leaking channels in the complex. When the photoinactivated reductase is co-inlaid with intact ubiquinol-cytochrome *c* reductase or cytochrome *c* oxidase in a phospholipid vesicle, no proton ejection can be detected during the oxidation of their corresponding substrates.

Ubiquinol-cytochrome *c* reductase (commonly known as cytochrome b–c_1 complex or complex III) is a segment of the mitochondrial respiratory chain which catalyzes antimycin-sensitive electron transfer from ubiquinol to cytochrome *c* (Rieske, 1986). The redox components in this complex are two *b* cytochromes (*b*-565 and *b*-562), one *c*-type cytochrome (c_1), one-high potential iron–sulfur cluster (2Fe-2S Rieske center), and a ubiquinone. The complex contains 7–11 protein subunits depending on the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)[1] systems used

† This work was supported by a grant from the National Institutes of Health (GM 30721).

[1] Abbreviations: ISP, iron–sulfur protein; Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone; Q_2H_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; QCR, ubiquinol-cytochrome *c* reductase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

0006-2960/91/0430-230$02.50/0