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ARTICLE *in* BIOCHIMIE · FEBRUARY 2000

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DNA synthesis by CHO cell extracts on fork-like DNA templates containing the major cisplatin adduct requires a ligation step

Nicolas Tanguy Le Gac[a], Jean-Sébastien Hoffmann[a], Robert Hindges[b], Giuseppe Villani[a]*

[a]*Institut de Pharmacologie et de Biologie Structurale, CNRS, 205, route de Narbonne, 31077 Toulouse cedex, France*
[b]*Molecular Neurobiology Laboratory, MNL-O, The Salk Institute, 10010 N. Torrey Pines Rd., La Jolla, CA 92037, USA*

(Received 22 July 1999; accepted 30 September 1999)

Abstract — In this work we have examined the role of DNA ligation in the in vitro replication catalyzed by CHO crude extracts on fork-like oligonucleotide substrates containing a unique d(GpG) intrastrand cross-link produced by the antitumor drug cisplatin. We show here that this reaction involves a ligation step, which necessitates excision of the flap strand of the forked substrate. By constructing substrates in which the unannealed tail could not be degraded by a 5' exonuclease, we obtained evidence suggesting that this type of activity participates in the removal of the flap strand. Furthermore, we found that the ligation event played a predominant role in the synthesis of fully replicated products from both intact and platinated templates. Finally, we investigated whether translesion synthesis of the cisplatin lesion could occur concomitantly to ligation by monitoring the incorporation of labeled precursors downstream of the adduct. Our results are compatible with the possibility that some translesion syntheses of the Pt-d(GpG) adduct by the extracts also contributed to the generation of full length molecules. © 2000 Société française de biochimie et biologie moléculaire/Éditions scientifiques et médicales Elsevier SAS

fork-like substrates / cell extracts / ligation / translesion synthesis / cisplatin adduct

1. Introduction

The antitumor drug cis-diamminedichloroplatinum (II) or cisplatin is believed to exert its cytotoxic effects by interacting with DNA where it inhibits both replication and transcription and induces programmed cell death [1]. However, data have been obtained in recent years indicating that mutagenic DNA replication can occur past cisplatin lesions both in vivo and in vitro [2-9]. Experiments with purified enzymes have demonstrated that calf thymus DNA polymerase β, human immunodeficiency virus type I reverse transcriptase and *E. coli* DNA polymerase I can perform in vitro translesion synthesis of the Pt-d(GpG) adduct, the major lesion produced by the cisplatin on DNA [5-7]. Nevertheless, in vitro replication of DNA templates by cellular crude extracts is expected to reproduce cellular DNA replication more faithfully than purified DNA polymerases alone. An SV40 based replication system has been used to examine the effect of cisplatin lesions on DNA replication by cytosolic extracts prepared from human cell lines [10]. Inhibition of DNA synthesis was observed under these conditions, although residual replication (estimated 20% of the control, unplatinated DNA) occurred in the presence of 2.6 adducts per plasmid molecule. In a recent work, we have used oligonucleotides containing a unique cisplatin d(GpG) adduct as template for DNA synthesis by Chinese hamster ovary (CHO) and HeLa cells extracts [11]. Two types of substrates were compared: the first was a 90-mer single-stranded oligonucleotide primed with a 17-mer, while the second was constructed by annealing to the 5' end of the 90-mer template a partially complementary oligonucleotide to form a fork-like structure. Appearance of full-length products in a reaction catalyzed by cell extracts was observed only with damaged fork-like substrate, whereas inhibition of DNA synthesis occurred on damaged single-stranded substrate. We interpreted these results as indicating the participation of additional factors enabling DNA polymerases to bypass the Pt-d(GpG) adduct when present in fork-like oligonucleotide templates. However, fractionation experiments undertaken in our laboratory to identify these putative factors, led to partially purified fractions containing DNA ligase activity and capable of converting a primed platinated fork-like substrate to full length product in a manner largely dependent on the addition of exogenous ATP (results to be published). These findings prompted us to evaluate the role of DNA ligation in the in vitro DNA replication by CHO crude extracts of fork-like templates containing a unique Pt-d(GpG) adduct.

* Correspondence and reprints
Abbreviations: CHO, Chinese hamster ovary; DTT, dithiothreitol; FEN-1, flap endonuclease-1; HMG1, high-mobility group protein 1; ss/ds, single-stranded/double-stranded.

2. Materials and methods

2.1. Enzymes and chemicals

*Hin*dIII and *Ehe*I DNA restriction endonucleases were purchased from New England Biolabs and MBI Fermentas respectively. *E. coli* DNA polymerase I Klenow fragment exonuclease free was from Amersham Life Science.T4 polynucleotide kinase was from United States Biochemical. [γ-^{32}P]ATP and [α-^{32}P]-dCTP and [α-^{32}P]-dATP were from Du Pont/NEN. Unlabeled deoxyribonucleosides triphosphates were from Amersham Life Science. Streptavidin was from Sigma. Proteinase K and phenol/chloroform/isoamyl alcohol solution were from Amresco.

2.2. Construction of forked DNA substrates

The intact and cisplatin-modified 90-mer and the 17-mer primer were prepared and purified as described [11]. The 17-mer was 5' labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP following standard procedures. PAGE purified oligonucleotides, spanning from 53 to 65-mer in length and partially complementary to the 90-mer, were provided by Genosys and hybridized to 90-mer to construct forked DNA substrates. The 59-mer containing phosphorothioate bonds and the 5'-biotinylated 59-mer were also purchased from Genosys; when required streptavidin was coupled to biotin by incubating the biotinylated forked substrates at 37 °C for 5 to 30 min with a 50 molar excess of streptavidin in the same reaction buffer used to monitor DNA synthesis by crude extracts (see below). Forked substrate ^{32}P labeled at the 3' end was prepared as follows: 17 nM of 90/53 hybrid substrate *(figure 1B)* was incubated in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 5 µM [α-^{32}P]dCTP, 25 µM dATP and 0.5 unit of exonuclease free Klenow fragment for 10 min at 30 °C. The reaction product was then heated at 70 °C for 10 min, slowly cooled and ethanol-precipitated.

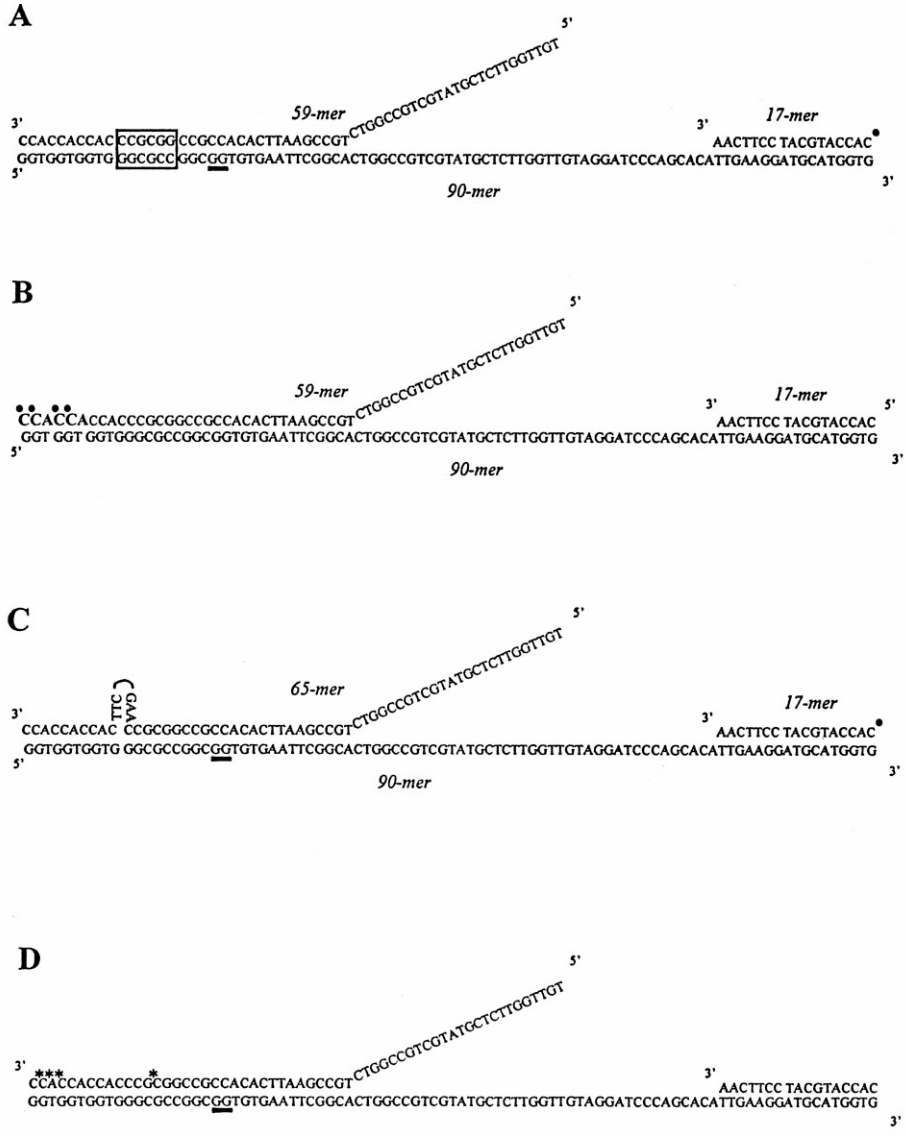


Figure 1. DNA substrates used in this study. Underlined GG residues show the positions of the guanines involved in the cisplatin adduct. •, the ^{32}P labeled nucleotides present in the substrates. Box in substrate A indicates the position of a *Ehe*I restriction site. Bold-faced type nucleotides in substrate B represent the residues incorporated *by E. coli* polymerase I Klenow fragment. Asterisk in substrate D indicates the presence of phosphorothioate bonds.

2.3. DNA synthesis by crude extracts

Crude extracts from Chinese hamster ovary (CHO) cells were prepared as described [11]. DNA synthesis reactions were performed at 37 °C in 45 mM Hepes-KOH, pH 7.8, 7 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 2 mM ATP, 500 μM each dATP, dCTP, dGTP and dTTP (unless otherwise indicated), 3.5% glycerol, 65 mM potassium glutamate and 500 μg/mL of BSA. Incubation times, amounts of DNA substrates and of cell extracts are indicated in the figure legends. At the end of the reaction, 5–10 μL of stopping buffer (90% formamide/0.1% xylene cyanol/0.1% bromophenol blue/1 mM EDTA) were added. Samples were denatured for 5 min at 80 °C and analyzed on a 15% polyacrylamide/7M urea/30% formamide gel followed by autoradiography. When necessary, gels were scanned using a PhosphorImager and bands intensity quantitated using the ImageQuant software.

2.4. EheI restriction endonuclease digestion of DNA synthesis products

After completion of the reaction, EDTA and sodium dodecyl sulphate were added to a final concentration of 40 mM and 0.25% respectively. Subsequently, proteinase K was added to a concentration of 1 mg/mL and incubation continued for an additional 30 to 60 min. The volume of the samples was brought to 200 μL with water and the DNA extracted with phenol/chloroform/isoamyl alcohol followed by ethanol precipitation. Half of the resuspended material was then digested with 0.5 to 2 units of *Ehe*I in 33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate and 0.1 mg/mL BSA. The reaction mixture was incubated for 60 min at 37 °C.

3. Results

3.1. Replication of a fork-like DNA template by CHO crude extracts involves a ligation step

Generation of full length product (90-mer) by in vitro replication of intact or platinated substrate A depicted in *figure 1* by CHO crude extracts can be achieved at least in two ways: a) continuous DNA synthesis to the 5' end of the 90-mer template, which requires both the displacement of the 59-mer strand and, when the lesion is present, the capacity to perform translesion synthesis (see model A of *figure 2*); or b) DNA synthesis until the single-double strand junction, followed by nuclease digestion of the flap strand to create a nick that can be sealed by a DNA ligase (see model B of *figure 2*). Replication via model B implies that the portion of the 59-mer which is fully hybridized to the 90-mer should be present in the final replication product. To test this possibility we constructed substrate B of *figure 1* by extending the 3'-OH of a 53-mer partially annealed to the 90-mer template with *E. coli* DNA polymerase I Klenow fragment in presence of dATP and [α-^{32}P] dCTP to obtain a 90/59-mer duplex in which the 59-mer is 3' end labeled. Inspection of the reaction products shows a major band at the 59-mer position when loaded on a denaturing gel (see lane 4 of *figure 3*). Substrate B was then primed with unlabeled 17-mer and subjected to DNA replication by cell extracts. As shown in *figure 3*, radioactive, full length 90-mer product was obtained, indicating that ligation took place during the reaction, even if some material remained unligated after a prolonged incubation time (see lane 3, *figure 3*). To allow ligation on substrate B, the flap part of the partially double stranded substrate should be excised or degraded. As shown in the model B of *figure 2*, DNA synthesis to one nucleotide preceding the double strand junction of the fork template will create a structure which could be efficiently cleaved by a nuclease activity similar to the one of the cellular 5' to 3' exo-endonuclease FEN-1 [12]. FEN-1 must enter at the 5' end of the unannealed tail and then slide to the region of hybridization where the cleavage occurs. It has been shown that biotinylation of a nucleotide at the 5' end does not inhibit FEN-1 action, while

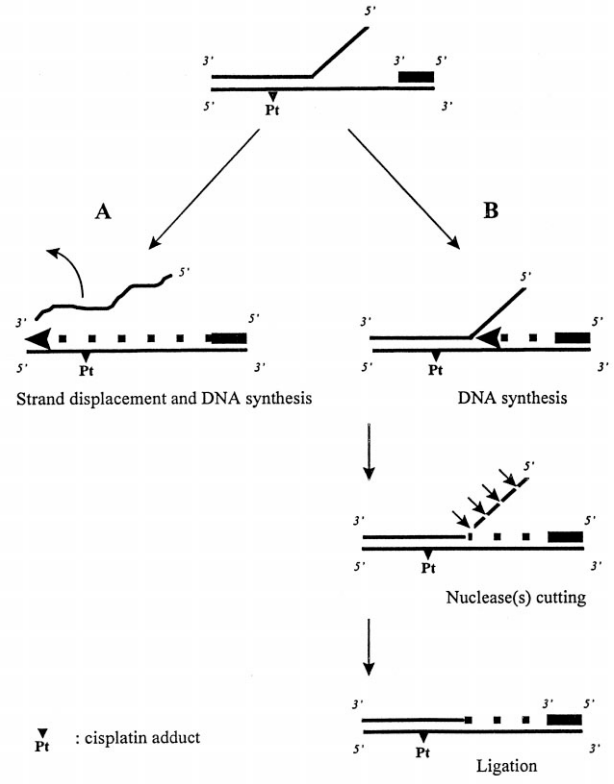


Figure 2. Possible pathways for in vitro generation of full-length products from fork-like DNA substrates with cell extracts.

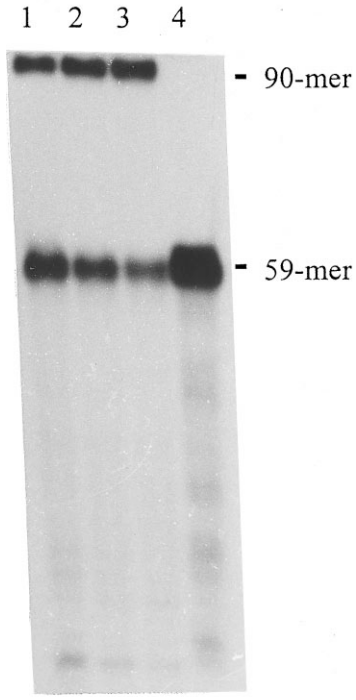


Figure 3. Replication by cell extracts of substrate B. Autoradiogram of a 15% polyacrylamide/7 M urea/30% formamide gel of the reaction products. 2 ng of substrate B, primed with an unlabeled 17-mer, was incubated at 37 °C with 2 μg of cell extracts in a 15-μL reaction for the indicated times. Lane 1, 30 min of incubation; lane 2, 60 min of incubation; lane 3, 90 min of incubation; lane 4, 90 min of incubation, no cell extract. Positions of 90-mer (full size product) and 59-mer (labeled strand) are indicated.

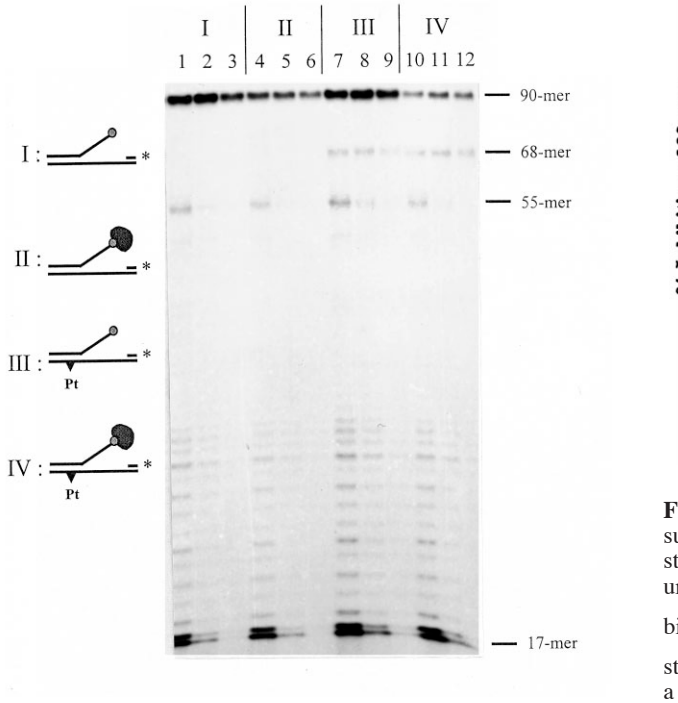
additional binding of streptavidin completely abolishes it [13]. To investigate the possible contribution of FEN-1 in creating a substrate suitable for ligation, intact or platinated substrate A (see *figure 1*), bearing either biotin alone or biotin plus streptavidin at the 5' end of the 59-mer, were constructed and subjected to replication by cellular extracts. We observed 20 to 40% reduction in the appearance of full length products *(figure 4A, B)* when streptavidin was present on intact or platinated substrates, therefore suggesting that a FEN-1 like activity plays a role in the digestion of the flap strand.

3.2. Attempts to evaluate the contribution of ligation to the replication of intact and platinated fork-like DNA substrates

The experiment depicted in *figure 3* shows that ligation contributed to the synthesis of full length product by crude extracts but does not rule out the possibility that some continuous DNA synthesis to the end of the 90-mer also took place, since no labeled primer or labeled deoxyribonucleoside triphosphates were present in the reaction. To investigate this possibility we constructed substrate C of *figure 1*, in which the 65-mer strand, annealed to the 90-mer, possesses a loop of six extra bases placed in the hybridized part of the duplex. We reasoned that DNA synthesis plus ligation by cell extracts will yield a 96-mer product while synthesis without intervention of ligation will result in a 90-mer product. The outcome of such experiments is shown in *figure 5A*, where one can distinctly see both 96- and 90-mer products. It should be mentioned that, taking advantage of the fact that the six base loop sequence contains a *Hin*dIII restriction site, we found that the 96-mer product, following hybridization with a 40-mer oligonucleotide complementary to the restriction site, was sensitive to cutting by this endonuclease (data not shown). This demonstrated unambiguously that the sequence was ligated into the final product. A quantification of the gel depicted in *figure 5A* showed that, for the higher quantity of extract used, the amount of 90-mer produced reached 70% and 50% of the amount of the 96-mer for intact and platinated substrate respectively *(figure 5B)*. Nevertheless, in addition to continuous DNA synthesis, at least two other events catalyzed by cell extracts could generate labeled 90-mer. The first one is the excision of the non-paired six bases, thus converting the 96- to 90-mer. An activity in human cell extracts has been described that repairs DNA with loops of five or more unpaired bases [14]; however, circular heteroduplexes are much better substrates for mismatch repair proteins than linear molecules such as those used in this study [15]. The second one implies the existence of some excision/resynthesis events at the end of the substrate downstream of the lesion. This second possibility will be addressed experimentally further on.

3.3. Incorporation of labeled precursor downstream of the platinum adduct on the fork-like DNA substrate

Translesion synthesis on platinated substrate A could be monitored by detecting incorporation of a radioactive deoxyribonucleoside monophosphate downstream of the Pt-d(GpG) adduct instead of using a radioactive primer. As shown in *figure 1*, substrate A contains an *Ehe*I restriction site six nucleotides downstream of the platinum lesion; cutting by the restriction endonuclease will yield two fragments of 77 and 13 nucleotides in length respectively. Translesion synthesis will lead to detection of radioactivity within the 13-mer resulting from digestion of the platinated substrate. To test this prediction we performed replication by cell extracts of intact and platinated substrate A using [α-^{32}P]-dCTP as radioactive precursor. After incubation, reaction products were treated with proteinase K, phenol extracted and ethanol precipitated. Resuspended material was split in two, and one sample



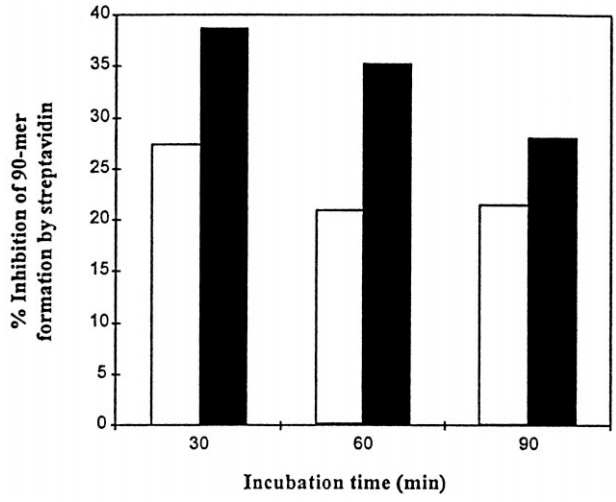


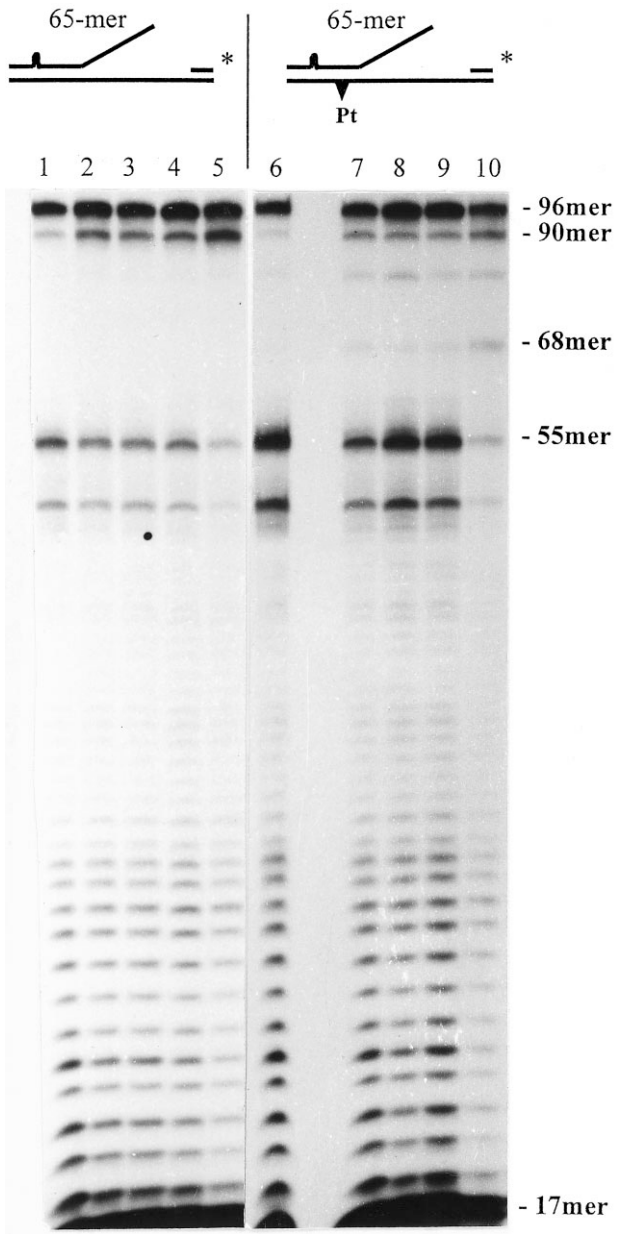
Figure 4. Replication by cell extracts of intact and platinated substrate A either complexed with biotin or with biotin plus streptavidin. **A.** Autoradiogram of a 15% polyacrylamide/7M urea/30% formamide gel of the reaction products. 2 ng of biotinylated substrate A, complexed ( ) or not ( ) with streptavidin, were incubated at 37 °C with 2 μg of cell extracts in a 15-μL reaction for the indicated times. Lanes 1 to 6, intact substrate; lanes 7 to 12, platinated substrate; lanes 1,4,7 and 10, 30 min of incubation. Lanes 2, 5, 8 and 11, 60 min of incubation; lanes 3, 6, 9 and 12, 90 min of incubation. Positions of the 90-mer (full size product), 68-mer (product of synthesis to the base preceeding the lesion), 55-mer (ss/ds junction), and 17-mer (primer) are indicated. I and II, intact substrates; III and IV, platinated substrates. **B.** Quantification of the effect of streptavidin on 90-mer formation catalyzed by cell extract. Bands at the 90-mer position of the autoradiogram depicted in **A** were quantified as described in *Materials and methods*. The amount of radioactivity detected in reactions with biotinylated substrates reacted with streptavidin were compared to those obtained with biotinylated, unreacted substrates for each time point. Open bars represent intact substrate and filled bars platinated substrate.

digested with *Ehe*I: both undigested and digested samples were then analyzed on a denaturing gel. As can be seen in *figure 6*, two main radioactive digestion products of 13 and 12 nucleotides in length were clearly detected on both intact and platinated substrates. Quantification indicated that they accounted for roughly 20% of the total radioactivity loaded on the gel.

The data presented in *figures 5* and *6* are compatible with the possibility that CHO cell extracts can perform some translesion synthesis in replicating a fork-like substrate containing the Pt-d(GpG) cisplatin adduct. However, other in vitro molecular events, such as terminal incorporation, could account, at least in part, for the appearance of both the 90-mer products shown in *figure 5A* or the radioactive, short digestion products detected in *figure 6*. In the attempt to distinguish between these two possibilities we prepared a substrate identical to A but having the three last nucleotides linked by phosphorothioate bonds to abolish or greatly reduce terminal degradation which can result in resynthesis (see *figure 1*, substrate D). In addition, a phosphorothioate bond was also introduced between the two nucleotides cleaved by the *Ehe*I restriction enzyme, the rationale for that being that incorporation of nucleotides by 'genuine' error free DNA synthesis will recreate a cleavable site while limited terminal excision may not affect the thioate bond. The capacity of *Ehe*I to cleave substrate D, replicated by cell extracts in the presence of either [α-^{32}P]-dCTP or [α-^{32}P]-dATP, and to generate short radioactive products was tested. Although some digestion was still possible at the restriction site containing the thioate bond on intact and platinated substrate D, we detected a reduced amount of digestion products compared to those obtained with substrate A, indicating that terminal resynthesis played a role in their generation (data not shown).

4. Discussion

The antitumor drug cisplatin binds to DNA, forming adducts which inhibit the progression of the replication fork. Nevertheless, this inhibition does not seem to be



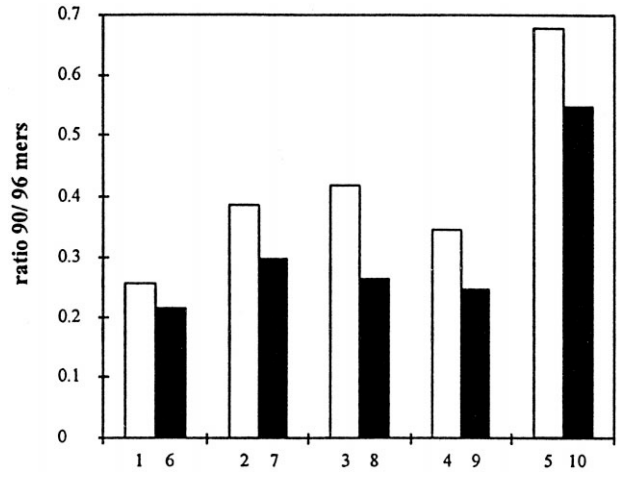


Figure 5. Replication by cell extracts of intact and platinated substrate C. **A.** Autoradiogram of a 15% acrylamide/7M urea/ 30% formamide gel of the reaction products. 3 ng of intact or platinated substrate C was incubated in a 15-μL reaction at 37 °C for 45 min with the indicated amounts of cell extracts. Lanes 1 to 5, intact substrate; lanes 6 to 10, platinated substrate; lanes 1 and 6, 0.2 μg of extracts; lanes 2 and 7, 0.4 μg of extracts; lanes 3 and 8, 0.6 μg of extracts; lanes 4 and 9, 1.2 μg of extracts; lanes 5 and 10, 2 μg of extracts. Position of the 96-mer (full size product plus six extra bases), 90-mer (full size product), 68-mer (product of synthesis to the base preceding the lesion), 55-mer (ss/ds junction), and 17-mer (primer) are indicated. **B.** Quantification of the amount of 96- and 90-mer products shown in **A**. Bands at 96- and 90-mer position of the autoradiogram depicted in **A** were quantified as described in *Materials and methods* and the ratio of 90- over 96-mer calculated. Numbers in abscissa correspond to the lanes of **A**. Open bars represent intact substrate and filled bars platinated substrate.

always complete; in recent years data have accumulated indicating that translesion synthesis of cisplatin lesions can occur either in vivo or in reactions catalyzed in vitro by purified DNA polymerases [2-8]. Recently we have found that cellular crude extracts were able to generate full length products in replicating an oligonucleotide containing a single cisplatin adduct if the substrate was in a fork-like conformation, while inhibition by the adduct occurred on a single stranded template [11]. We proposed that the differential processing of the Pt-d(GpG) lesion by the extracts could be attributed to the fact that protein complexes, eventually leading to strand displacement and translesion synthesis, formed only on the fork-like substrate (see model A of *figure 2*). We also investigated the capacity of the high-mobility group protein 1 (HMG1) to affect processing of the Pt-d(GpG) adduct by CHO cell extracts. The choice of HMG1 was motivated by reports establishing that this protein bound preferentially to DNA containing the Pt-d(GpG) adduct [16] and inhibited its removal by the excinuclease complex [17]. We confirmed

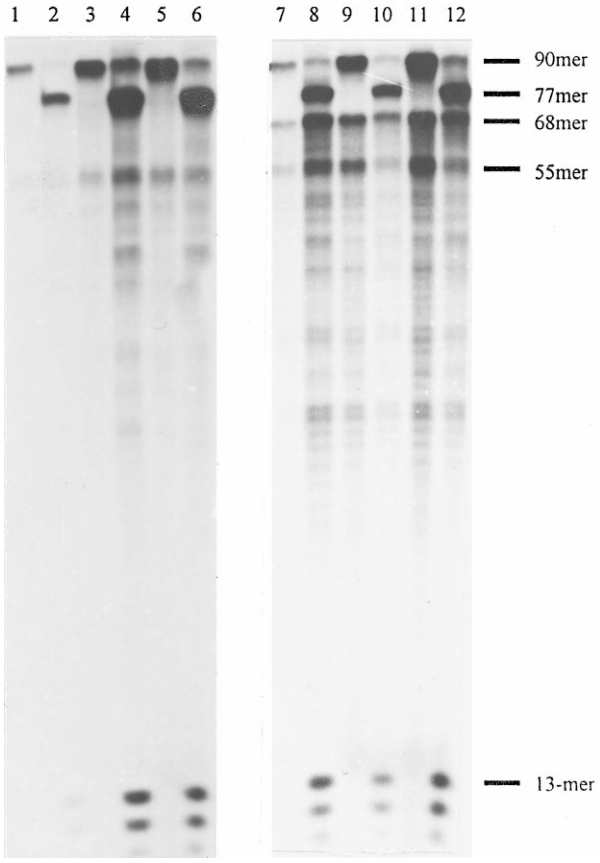


Figure 6. *Ehe*I digestion of the replication products of intact and platinated substrate A by cell extracts. Autoradiogram of a 15% acrylamide/7M urea/30% acrylamide gel of the reaction products. 3 ng of intact or platinated substrate A were incubated in a 15-μL reaction at 37 °C with 3 μg of cell extracts for the indicated times. Concentration of unlabeled dATP, dGTP and dTTP was of 500 μM while [α^{32}P]-dCTP was at 25 μM. Substrates were primed with unlabeled 17-mer. At the end of the reaction, samples were processed as described in *Materials and methods*. Lanes 1 to 6, intact substrate; lanes 7 to 12, platinated substrate. Odd numbers indicate undigested samples and even numbers are *Ehe*I digested samples. Lanes 1-2 and 7-8, 60 min of incubation; lanes 3-4 and 9-10, 90 min of incubation; lanes 5-6 and 11-12, 120 min of incubation. Position of 90-mer (full size product), 77-mer (longer *Ehe*I digestion product), 68-mer (product of DNA synthesis to the base preceding the lesion), 55-mer (ss/ds junction) and 13-mer (shorter *Ehe*I digestion product) are indicated.

the preferential binding of HMG1 to the adduct and found that, at high concentrations, it reduced the amount of full length product synthesized by cell extracts on the platinated forked substrate, suggesting that its binding interfered with the assembly of proteins necessary for translesion synthesis [18]. In this respect, it may be interesting to note that it has recently been shown that HMG1 inhibits the displacement of a cisplatin-damaged oligonucleotide by the calf thymus DNA helicase E [19].

However, subsequent work undertaken in our laboratory to identify the proteins implicated in the in vitro replication of the platinated forked substrate, revealed that synthesis of full-length products by partially purified cellular fractions involved a ligation step. We therefore decided to perform a new set of experiments, aimed at clarifying the role of ligation in reactions catalyzed by crude extracts. The data we obtained clearly indicate that ligation is involved in the generation of full length products *(figure 3)*. To allow the ligases present in the cell extracts to act on the oligonucleotides employed in our studies, the part not hybridized of the substrates should be excised or degraded. Based on its mode of action, one could expect that a nuclease like FEN-1 would play a role in this nucleolytic process [12, 20]. To test this hypothesis, we performed experiments in which the substrate was modified in a way that it could no longer be processed by FEN-1. The extent of inhibition observed in presence of biotin plus streptavidin suggests that a FEN-1 like activity contributes only partially to the generation of full length products via ligation and consequently other nucleases participate in the digestion of the flap strand. Clearly, more experiments are required to clarify the role of FEN-1 as well as to establish which DNA ligase(s) is implicated in the ligation process.

In an attempt to quantify the contribution of ligation, we performed experiments with substrate C of *figure 1*. Results shown in *figure 5* are compatible with the possibility that some DNA synthesis to the end of the template occurred concomitant with ligation, implying that the crude extracts displayed the capacity to perform translesion synthesis in the case of the platinated DNA. Consequently, we set up to monitor direct incorporation of labeled precursor into intact and platinated substrates by taking advantage of the fact that a restriction site for the endonuclease *Ehe*I exists downstream the Pt-d(GpG) adduct. The fact that two main radioactive digestion products could be generated suggested the existence of DNA synthesis to the end of the template *(figure 6)*.The presence of the 12-mer digestion product might result from incomplete DNA synthesis past the lesion. However, in vitro events other than bona fide DNA synthesis could have led to detection of the 90-mer and of the short digestion products shown in *figures 5A* and *6* respectively. For instance, extracts may induce partial degradation at the 3' OH end of the annealed 59-mer of the substrate, allowing terminal incorporation of the radioactive precursors. In order to test this hypothesis we used substrate D of *figure 1*, in which both the three terminal phosphate bonds of the 59-mer and one of those implicated in the *Ehe*I restriction site were substituted by phosphorothioate residues, that are known to be resistant to the action of a variety of nucleases [21, 22]. Accordingly, we found that

the presence of phosphorotioate bonds at the *Ehe*I site of substrate D rendered it resistant to the action of the enzyme (data not shown). Following incubation with crude extracts, the amount of digestion products detected with substrate D was reduced compared to substrate A, establishing that terminal incorporation was indeed implicated in the formation of labeled 90-mer. Partial resynthesis following terminal degradation of the 59-mer could therefore contribute to the appearance of the 12 mer digestion product. On the other hand, we also detected a small amount of digestion products with platinated substrate D, leaving open the possibility that some translesion synthesis was indeed performed by the cellular extracts.

In summary, we present here evidence that DNA synthesis catalyzed in vitro by CHO cell extracts on intact and platinated forked oligonucleotides templates shown in *figure 1* involves a ligation step which, at variance with the conclusions of a previous article [11], seems to play a major role in the formation of fully replicated products. Previous results showing that addition of HMG1 protein reduced the synthesis of full length products on the forked substrate A of *figure 1* could also be explained, at least in part, by the capacity of this protein to inhibit ligase activity. In this respect, it should be noted that the DNA binding site of the protein, estimated to be 15 nucleotides [23, 24], would allow HMG1 to encompass the single to double strand junction in the forked substrate and therefore affect the ligation process.

We think that, taken together, the data of this work support the conclusion that the route presented in model B of *figure 2* is the prominent one used by crude extracts to replicate the forked substrates employed here. The nature of the proteins participating in the process remain to be determined, although a FEN-1 like nuclease activity seems to play a role in the digestion of the flap strand necessary to permit ligation.

Our data do not rule out the capacity of extracts to perform translesion synthesis but they show that, in any case, this event does not play a major role in the production of fully replicated molecules. In addition, from this study it also appears that, to obtain unambiguous evidence for in vitro translesion synthesis catalyzed by CHO crude extracts at a replication fork containing a Pt-d(GpG) adduct, the construction of more physiological fork-like DNA substrates than those used here will be necessary.

Acknowledgments

We are grateful to Drs. Paul E. Boehmer for critical reading of the manuscript. This work was supported in part by grant 9584 from the Association pour la Recherche sur le Cancer (to G.V.).

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