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Synthesis by DNA Polymerase I on Bleomycin-Treated Deoxyribonucleic Acid: A Requirement for Exonuclease III†

Osami Niwa and Robb E. Moses*

ABSTRACT: ΦX174 RFI DNA treated with bleomycin (BLM) under conditions permitting nicking does not serve as a template-primer for *Escherichia coli* DNA polymerase I. Purified exonuclease III from *E. coli* and extracts from wild-type *E. coli* strains are able to convert the BLM-treated DNA to suitable template-primer, but extracts from exonuclease III deficient strains are not. Brief digestion by exonuclease III is enough to create the template-primer, suggesting that the exonuclease III is converting the BLM-treated DNA by a modification of 3′ termini. The exonucleolytic rather than the phosphatase activity of exonuclease III appears to be involved in the conversion. Comparative studies with micrococcal nuclease indicate that BLM-created nicks do not have a simple 3′-P structure. Bacterial alkaline phosphatase does not convert BLM-treated DNA to template-primer. The endonuclease VI activity associated with exonuclease III does not incise DNA treated with BLM under conditions not allowing nicking, in contrast to DNA with apurinic sites made by acid treatment, arguing that conversion does not require the endonuclease VI action on uncleaved sites.

Bleomycin (BLM),[1] a glycopeptide antibiotic produced by *Streptomyces verticillus* (Umezawa et al., 1966), is used therapeutically on certain types of tumors. The main target of the drug is DNA (Muller & Zahn, 1977). BLM can produce loss of bases from DNA, alkali-sensitive sites, and under appropriate conditions strand scissions (Haidle et al., 1972; Muller & Zahn, 1977; Ross & Moses, 1978; Sausville et al., 1978a). Analyses of the products (oligonucleotides) of BLM-treated DNA have shown that BLM may create heterogeneous breakdown products in respect to the terminal structures (Sausville et al., 1978; D'Andrea & Haseltine, 1978). Lack of 3′-P at the nicked sites has been suggested since inorganic phosphate is not released by exonuclease III (Kuo & Haidle, 1973; Kappen & Goldberg, 1978).

Apparently due to the combination of strand scissions and sites of base loss, BLM causes inhibition of DNA synthesis both in vivo and in vitro (Yamaki et al., 1971; Kuo & Haidle, 1973; Muller & Zahn, 1977; Kappen & Goldberg, 1978). However, little is known about the reactivity of BLM-produced lesions in the DNA molecule or repair reactions of cells exposed to this drug. On the basis of the above data, it appears reasonable that the cellular response to BLM involves steps of the excision repair process, differing perhaps in the early events, depending on whether the damaged nucleotides must be excised or not. We have studied the response to BLM in permeable *Escherichia coli* (Ross & Moses, 1977) and found that the agent stimulates nonconservative repair synthesis, but does not inhibit semiconservative synthesis, in contrast to ultraviolet (UV) irradiation. Thus, BLM appears to be a useful probe of cellular DNA repair processes with some features differing from UV irradiation.

To understand the cellular repair synthesis in response to BLM and to analyze the requirements for such repair synthesis, we have investigated the ability of DNA polymerase

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[1] Abbreviations used: BLM, bleomycin; RFI, covalently closed circular replicative form I DNA; dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, disodium ethylenediaminetetraacetate.

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I to utilize the sites caused by BLM in ΦX174 RFI DNA for synthesis. We find that DNA polymerase I from *E. coli* will not use the BLM-cleaved DNA as a primer for synthesis. However, treatment of such DNA with exonuclease III converts the DNA to adequate primer. Cell extracts also can convert the DNA to primer, but extracts from cells deficient in exonuclease III activity can not. Our findings thus represent a basis for a coupled assay for exonuclease III activity, using DNA polymerase I synthesis as an index of terminus modification at sites of BLM cleavage. Alkali-labile sites created by BLM under conditions not allowing nicking (Ross & Moses, 1978) are not susceptible to cleavage by the apurinic endonuclease VI activity (associated with exonuclease III) (Weiss, 1976; Linn, 1978), in contrast to depurinated sites produced by acid treatment, arguing against endonucleolytic cleavage playing a role in conversion.

Experimental Procedures

Enzymes. DNA polymerase I was purified from *E. coli* W3110 according to Moses & Richardson (1970a). *E. coli* exonuclease III prepared according to Richardson & Kornberg (1964) was kindly provided by Dr. M. Fuke, Baylor College of Medicine. Bacterial alkaline phosphatase from *E. coli* and micrococcal nuclease were purchased from Worthington. All of these enzymes, except for micrococcal nuclease, were endonuclease free, as determined by filter retention assay with ΦX174 RFI DNA as a substrate (Kuhnlein et al., 1976).

DNA Preparation. The procedure used for the preparation of ΦX174 RFI [^{3}H]DNA was according to Ross & Moses (1978) except the polyethylene glycol phase extraction was omitted. *E. coli* (su$^-$) infected with ΦX174Eam3 (defective in lysis, from Dr. D. Denhardt, McGill University) was labeled with [^{3}H]thymidine in the presence of chloramphenicol. Labeled ΦX174 RFI DNA was purified through an ethidium bromide–cesium chloride density gradient. The RFI fractions were pooled and precipitated by isopropyl alcohol–sodium acetate, and the DNA was resuspended and stored in 50 mM EDTA. Analysis of supercoiled DNA was performed on 5–20% alkaline sucrose isokinetic gradients as described by Ross & Moses (1978).

Preparations of Cell Extract. E. coli strains W3110 (wild type), P3478 (*polA*$^-$) (DeLucia & Cairns, 1969), and BW9091 (*xthA*$^-$, obtained from B. Weiss) were used. Cells were grown at 37 °C in L broth containing the following (per liter of water): bactotryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; thymine, 5 mg. They were harvested at the concentration of 8×10^8 cells/mL and suspended in 50 mM phosphate buffer (pH 7.4) to give 2×10^{10} cells/mL. After 10 min of toluene treatment (Moses & Richardson, 1970b), toluene was washed out, and cells were resuspended in one-seventh volume of the same buffer. EDTA and lysozyme were added at final concentrations of 1 mM and 160 μg/mL, respectively, and incubated at 37 °C for 15 min. The cell lysate was centrifuged in a Beckman 50 Ti rotor at 40 000 rpm for 30 min, and the supernatant was used as cell extract. All extraction procedures were done at 0–4 °C, unless indicated otherwise.

Chemicals. BLM mixture was from Bristol Laboratories. Deoxynucleoside triphosphates (dNTP's) were purchased from PL Biochemicals. [α-^{32}P]dATP and [^{3}H]thymidine were purchased from Amersham.

Filter Retention Assay. The fraction of nicked circular DNA after treatment was determined according to Kuhnlein et al. (1976). A 0.6-mL aliquot of 0.01% $NaDodSO_4$ and 0.25 mM EDTA (pH 7.5 was added to the reaction mixture (0.1 mL) to stop the reaction. Then, 0.2 mL of 0.3 M phosphate buffer (pH 12.3) was added, followed by a 4-min incubation at room temperature. To neutralize the solution, 0.1 mL of 1 M phosphate buffer (pH 4.0) was added; subsequently, 0.2 mL of 5 M NaCl and 5 mL of 1.0 M NaCl and 50 mM Tris-HCl (pH 8.1) were added. These conditions correspond to "neutral" conditions and irreversibly denature only nicked ΦX174 RFI DNA. Alkali-sensitive sites from acid depurination were not hydrolyzed by this treatment. The sample was held at pH 12.3 for 1 h at room temperature before neutralizing to hydrolyze alkali-sensitive sites. The solution was slowly (5–10 mL/min) passed through a nitrocellulose filter (Schleicher and Schuell BA85, pore size 0.45 μm). The filter was washed twice with 3 mL of 0.3 M NaCl and 0.03 M sodium citrate, dried, and counted. DNA remaining in supercoiled form passes through the filter while nicked molecules are denatured and retained. This assay affords rapid quantitation of strand scissions in RFI DNA.

Acid Depurination of DNA. ΦX174 RFI DNA was depurinated by incubation with 3 volumes of 50 mM sodium citrate (pH 3.5) at 60 °C for 15 min. Immediately after the incubation, the mixture was chilled and dialyzed against 50 mM Tris-HCl (pH 7.4) for 5 h. This treatment gave approximately two alkali-sensitive sites per DNA molecule, as determined by the filter retention assay described above.

BLM Treatment of DNA. The reaction mixture (90 μL), unless otherwise noted, contained 11 mM Tris-HCl (pH 7.4), 11 mM $MgCl_2$, 4.5–6.0 nmol of ΦX174 RFI DNA, 0.2 μg of BLM, and, where indicated, 2 mM dithiothreitol (DTT). The mixture was incubated at 0 °C for 10 min and subsequently treated with other enzymes as indicated below. This treatment of BLM produced 1–2 nicks per DNA molecule, which was calculated from the data shown in Figure 1. In some experiments, DTT was replaced by cell extract.

Micrococcal Nuclease Treatment of DNA. ΦX174 RFI DNA was incubated with micrococcal nuclease to give 1.5 nicks per DNA duplex at 25 °C for 5 min. After incubation, protein was removed by phenol extraction, and the aqueous phase was dialyzed against 10 mM Tris-HCl (pH 7.4) and 1 mM sodium citrate for 24 h at 4 °C with three changes of dialysis buffer.

Exonuclease III of Alkaline Phosphatase Treatment of DNA. BLM-nicked DNA and acid-depurinated DNA were treated with exonuclease III or alkaline phosphatase for some experiments. The reaction mixture (100 μL), unless otherwise noted, contained 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 4.5–6.0 nmol of DNA, and 0.5 unit of exonuclease III (0.3 or 0.6 unit of alkaline phosphatase). This represents an addition of the enzyme directly to the BLM reaction mixture. Incubation with exonuclease III was performed at 37 °C for 2 min unless otherwise noted, and the incubation with alkaline phosphatase was at 65 °C (Weiss et al., 1968) for 20 min (half of the enzyme was added at 0 min, and the other half was added at 10 min). After the incubation, exonuclease III was inactivated by 5-min incubation at 60 °C. This step was omitted in some experiments as noted in the figure legends. To study the effect of Mg^{2+} ion on the exonuclease III activity, exonuclease III treatment was performed either in 10 mM $MgCl_2$ or in 5 mM sodium citrate (pH 7.4). After the heat inactivation of exonuclease III, the reaction mixtures were adjusted to 10 mM $MgCl_2$ and 5 mM sodium citrate (pH 7.4) by adding $MgCl_2$ or sodium citrate.

DNA Synthesis. The reaction mixture (130 μL) contained 7.7 mM Tris-HCl (pH 7.4), 7.7 mM $MgCl_2$, 23 mM phosphate buffer (pH 7.4), 0.019 mM of each deoxynucleoside triphosphate (dATP was labeled with α-^{32}P, specific activity about 150 cpm/pmol), 4.5–6.0 nmol of ΦX174 DNA, and

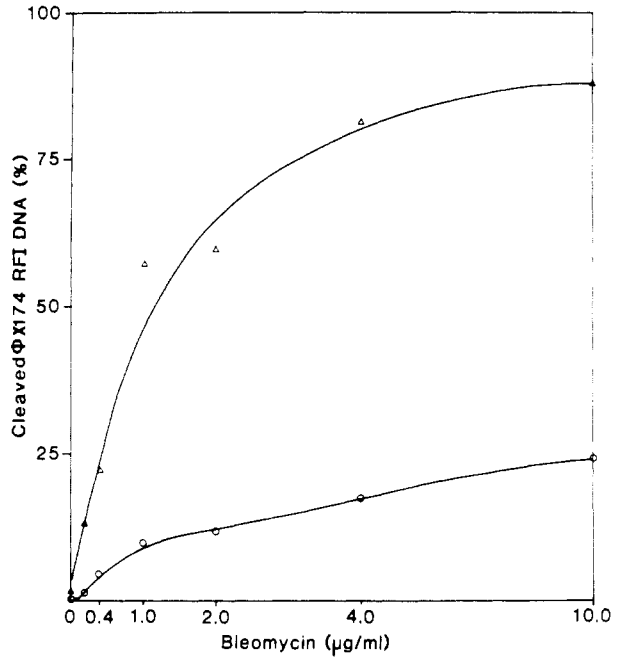

FIGURE 1: BLM-nicking activity. ΦX174 RFI DNA was treated with BLM at 0 °C for 10 min as described under Experimental Procedures. Nicked DNA was determined by the filter retention assay. (○) −DTT; (△) +DTT.

0.15 unit of *E. coli* DNA polymerase I. In the experiments with crude cell extracts, the complete reaction mixture (100 μL) contained 30 mM phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 1 μg of BLM, 2 mM DTT, cell extract (protein concentration was 30 μg/mL), 0.025 mM of each deoxynucleoside triphosphate (dATP was labeled with α-^{32}P, specific activity about 80 cpm/pmol), 4.5–6.0 nmol of ΦX174 RFI DNA, and 0.15 unit of DNA polymerase I. Incubation was at 37 °C for 30 min and was stopped by chilling in ice by adding 100 μL of 1 mg/mL salmon sperm DNA and 3 mL of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate. Acid-insoluble material was collected on a glass filter (Whatman GF/C, 24 mm), washed twice with 3 mL of 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate and once with 10 mL of 0.01 N HCl, dried, and counted.

Results

Template-Primer Activity of BLM-Treated DNA. BLM alone causes very little strand scission in DNA, and BLM combined with a sulfhydryl reagent (DTT) gives efficient strand scission (Figure 1), consistent with previously reported results (Ross & Moses, 1978; Sausville et al., 1978). As shown in Table I, ΦX174 RFI DNA nicked with BLM plus DDT did not serve as a template-primer for DNA polymerase I, suggesting that the nicks created specifically with BLM do not bear simple 3′-OH termini. Addition of *E.coli* exonuclease III to the reaction mixture gave modification of the nicked DNA so that DNA synthesis could take place on the BLM-treated DNA whereas DNA treated with BLM without DTT, as well as nontreated DNA, was not affected by this enzyme. This indicates that the conversion by exonuclease III is specific for the nicks created with BLM plus DTT. Since it is known that BLM produces double-stranded scissions, as well as single-stranded nicks, we minimized the production of double-stranded scissions by using low concentrations of BLM (Lloyd et al., 1978; J. D. Love and R. E. Moses, unpublished experiments). Following treatment with 2 μg/mL BLM, which creates 1–2 nicks per DNA molecule, but leaves most molecules in circular form, we observed similar conversion of

Table I: Template-Primer Activity of BLM-Treated DNA[a]

treatment	pmol of dAMP incorporated
none	1.3
BLM	1.4
BLM + DTT	1.7
exonuclease III	1.5
BLM + exonuclease III	2.4
BLM + DTT + exonuclease III	12.3
BLM (2 μg/mL) + DTT + exonuclease III	10.5

[a] φX174 RFI DNA was treated as indicated. DNA was treated with BLM at 10 μg/mL unless otherwise indicated. DNA polymerase I was added to the processed DNA solution without removal of BLM or heat inactivation of exonuclease III, and incubation was at 37 °C for 30 min with 19 μM of each dNTP. Other conditions and enzyme concentrations were as described under Experimental Procedures.

Table II: Cell Extract Activity on BLM-Treated DNA[a]

reaction conditions	pmol of dAMP incorporated
P3478 (exoIII$^+$) extract	
complete	13.4
−extract	2.5
−BLM-DTT	4.8
−DTT	8.6
−polymerase	1.5
−φX174 DNA	1.9
BW9091 (exoIII$^-$) extract	
complete	2.6
−BLM-DTT	3.0
−DTT	3.7
−polymerase	1.2

[a] The complete reaction mixture contained φX174 RFI DNA, BLM (10 μg/mL), DTT, DNA polymerase I, four dNTP's, and cell extract in phosphate buffer (pH 7.4). Reaction was at 37 °C for 30 min. Protein concentration of the extract in the reaction was 30 μg/mL.

BLM-treated DNA by exonuclease III (Table I). Thus, it appears that BLM-treated DNA identifies a requirement for terminus modification to allow synthesis.

The data in Table I also suggest that the modifying activity of exonuclease III is not replaced by the 3′-5′-exonuclease activity of DNA polymerase I. This was confirmed by preincubating the BLM-treated DNA with various amounts of DNA polymerase I prior to the polymerization reaction. We observed no detectable increase in DNA synthesis by preincubation of BLM-treated DNA with up to 0.3 unit of DNA polymerase I (data not shown).

Cell Extract Modification of BLM-Treated DNA. Modifying activity similar to exonuclease III could be shown by using a crude extract from an exonuclease III$^+$ *E. coli* strain P3478 (Table II). Another wild-type strain, W3110, had a similar modifying activity (data not shown). On the other hand, extract from the exonuclease III deficient strain (BW9091) had much reduced modifying activity. This result, agreeing with the conclusion described above, suggests that exonuclease III is a component in the cell modifying the BLM nicked DNA. This is also supported by heat inactivation of the modification activity in the extract. When extract from exonuclease III$^+$ cells is treated at 70 °C, the modifying activity disappears as rapidly as purified exonuclease III (Figure 2).

As shown in Table II, the DNA synthesis stimulated by the cell extract is specific for BLM, but in contrast to the results shown in Table I, the synthesis is much less dependent on the presence of DTT. This suggests that the cell extract contains an activity stimulating BLM nicking. We, therefore, directly

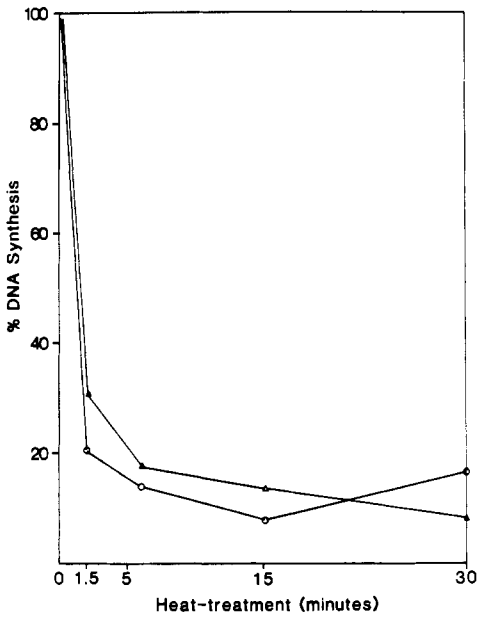

FIGURE 2: Heat inactivation of the DNA-modification activity. Purified exonuclease III or *E. coli* (P3478) cell extract was heat treated at 70 °C for the period indicated. These were then added to the polymerization mixture, which contained BLM (10 μg/mL) nicked DNA, DNA polymerase I, and dNTP's. Reaction was at 37 °C for 30 min. Incorporated dAMP is shown as percent of the amount with non-heat-treated enzyme. 100% for purified exonuclease III, and the cell extracts are 5.0 and 12.2 pmol of dAMP, respectively. (○) Purified exonuclease III; (△) cell extract.

Table III: Cell Extract Stimulation of BLM Nicking[a]

treatment	heat-treated extract temp (°C)	reaction temp (°C)	retained DNA (%)
BLM		0	23.6
extract		0	15.5
		37	29.9
	60	37	9.5
extract + BLM		0	78.2
		37	81.4
	70	37	76.0
	100	37	57.4

[a] φX174 RFI DNA was treated as indicated. BLM, when present, was at 4 μg/mL. Extract was from W3110 (65 μg/mL, final protein concentration). Heat-treatment of the cell extract was done at the indicated temperature for 20 min at 650 μg/mL protein in 20 mM Tris-HCl (pH 7.4). Nicking of the DNA was determined by the filter retention assay as described under Experimental Procedures.

measured nicking by filter retention assay; BLM-specific nicking is enhanced by addition of the extract (Table III). Such a stimulation could be due to an enzymatic activity, since *E. coli* contains multiple apurinic endonuclease activities which might attack the sites of base loss caused by BLM. Alternatively, the extract might contain sufficient reducing compounds to mimic the action of DTT. Since the extracts were prepared from permeable cells, low molecular weight substances should be quite diluted.

Extract from BW9091 (exonuclease III deficient) contains about the same level of nicking activity as the wild-type extract (data not shown) whereas the conversion activity for DNA synthesis after nicking is deficient. This indicates that extract-stimulated BLM nicks must be modified so that DNA polymerase can start DNA synthesis at the nicks and that an exonuclease III associated activity does not appear to be responsible for nicking.

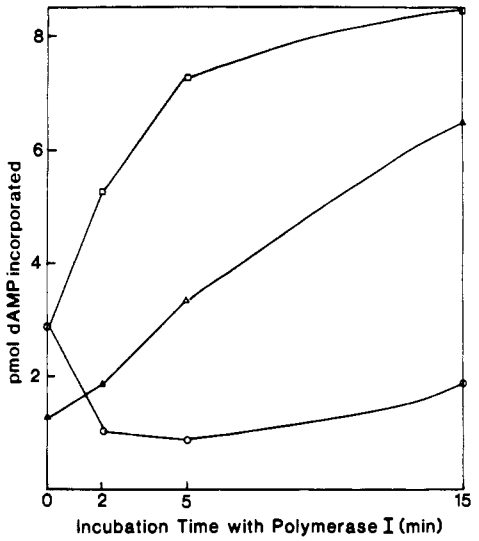

FIGURE 3: Time course of exonuclease III action on BLM-treated DNA. ΦX174 RFI DNA was treated with BLM (10 μg/mL), and DTT was treated with exonuclease III at 37 °C for the period indicated: (□) 10 min; (△) 1 min; (○) minus exonuclease III. After the digestion, the enzyme was inactivated by a 5-min heat treatment at 60 °C. The template-primer activity of the DNA was then assayed by adding DNA polymerase I and dNTP's and incubating the mixture for the period indicated.

Action of Exonuclease III on BLM-Treated DNA. To study the role of exonuclease III in the activation of BLM-treated DNA more carefully, we determined the extent of reaction required for conversion. Figure 3 shows the influence of incubation time with exonuclease III on the template-primer activity of BLM-treated DNA. In this experiment, 3′–5′-exonucleolytic digestion by exonuclease III was stopped by 5 min of heat treatment, which was adequate for a complete inactivation of the enzyme, followed by the addition of DNA polymerase I and deoxynucleoside triphosphates. Even a short exposure to exonuclease III was enough to convert the DNA to good template-primer. This indicates that very limited digestion is enough to modify the BLM-treated nicked DNA, suggesting that the role of exonuclease III in activation of the nicked DNA is to modify termini rather than to create large single-stranded gaps. For example, upon 2-min digestion of nicked DNA (2–3 nicks/molecule) by exonuclease III, about 30 nucleotides (0.3% of total DNA) were released (data not shown), and the DNA polymerase I incorporated 100–200 nucleotides on this DNA.

Bacterial Alkaline Phosphatase Activity on BLM-Treated DNA. As described in the preceding sections, the inability of DNA polymerase I to use BLM-nicked DNA could be due to lack of 3′-OH termini at the nicked sites. If the terminal structure is a 3′-P, alkaline phosphatase should be able to remove inorganic phosphates and to create 3′-OH termini. Since micrococcal nuclease produces single-stranded nicks which carry 3′-P termini, we used DNA treated with micrococcal nuclease as a control. As shown in Table IV, alkaline phosphatase was able to modify the micrococcal nuclease-treated DNA as well as exonuclease III to a good template-primer for DNA polymerase I. Under the experimental conditions, micrococcal nuclease-treated DNA was activated more efficiently by alkaline phosphatase (0.6 unit) than by exonuclease III. This is the result expected for a 3′-P terminus. In contrast to this, BLM-treated DNA was poorly activated by alkaline phosphatase, suggesting that at least a part of the termini of BLM-treated nicked DNA is neither a simple 3′-OH nor 3′-P. It also suggests that the exonuclease activity rather than the phosphatase activity of exonuclease III might be

Table IV: Modification of BLM-Treated DNA and Micrococcal Nuclease-Treated DNA[a]

nicking agent	treatment prior to polymerase I	pmol of dAMP incorporated
none	none	1.7
	alkaline phosphatase (0.3 unit)	3.0
	alkaline phosphatase (0.6 unit)	3.8
	exonuclease III	2.5
micrococcal nuclease	none	2.7
	alkaline phosphatase (0.3 unit)	7.2
	alkaline phosphatase (0.6 unit)	10.2
	exonuclease III	7.2
BLM + DTT	none	2.9
	alkaline phosphatase (0.3 unit)	4.7
	alkaline phosphatase (0.6 unit)	6.3
	exonuclease III	15.0

[a] Nicked φX174 RFI DNA was treated either with bacterial alkaline phosphatase or with exonuclease III (0.5 unit) prior to synthesis by DNA polymerase I. All other conditions and enzyme concentrations are described under Experimental Procedures. All DNA was extracted with phenol and extensively dialyzed before assay.

required for the modification of BLM-treated DNA. A similar conclusion has been suggested by Kappen & Goldberg (1978). However, it may not be ruled out that a portion of BLM-produced nicks can be modified by the phosphatase activity, since a low but significant amount of DNA synthesis occurred after alkaline phosphatase treatment of BLM-nicked DNA. It should be pointed out that in these experiments BLM-treated DNA as well as micrococcal nuclease-treated DNA was phenol extracted and extensively dialyzed before assay. Since BLM is a glycopeptide and its molecular weight is about 1500 (Muller & Zahn, 1977), it is likely that any substantial amount of BLM which may bind to the nicked sites would have been removed after phenol extraction and dialysis. As shown in Table IV, BLM-treated DNA after this treatment still has low template-primer activity for DNA polymerase I, suggesting that the inability of DNA polymerase I to use BLM-treated DNA is not due to the presence of BLM bound to the nicked sites, blocking the primer activity. It is also worth noting that the effect of exonuclease III demonstrated here shows that this enzyme has access to the nicked site, either with or without phenol extraction.

Exonuclease III Activity in the Absence of Mg^{2+}. *E. coli* exonuclease III has an endonuclease activity (endonuclease VI) attacking apurinic DNA (Weiss, 1976). Figure 4 shows that exonuclease III introduces nicks in apurinic DNA prepared by acid treatment whereas the enzyme has essentially no activity on the DNA treated with BLM (–DTT) in a manner to create alkali-sensitive sites without nicks (Ross & Moses, 1978), as well as the nontreated DNA. This argues that the site of base loss produced by BLM is not susceptible to cleavage by this enzyme although the sites of cleavage are available for modification. This conclusion agrees with the data in Table I, arguing against endonucleolytic cleavage.

It is known that exonuclease III requires Mg^{2+} for its exonucleolytic activity but does not rigidly require the ion for the apurinic endonuclease activity (Ljungquist et al., 1975). The enzyme is able to convert acid-depurinated DNA to suitable template-primer for DNA polymerase I by cleaving apurinic sites (Verley et al., 1974). We tested the effect of

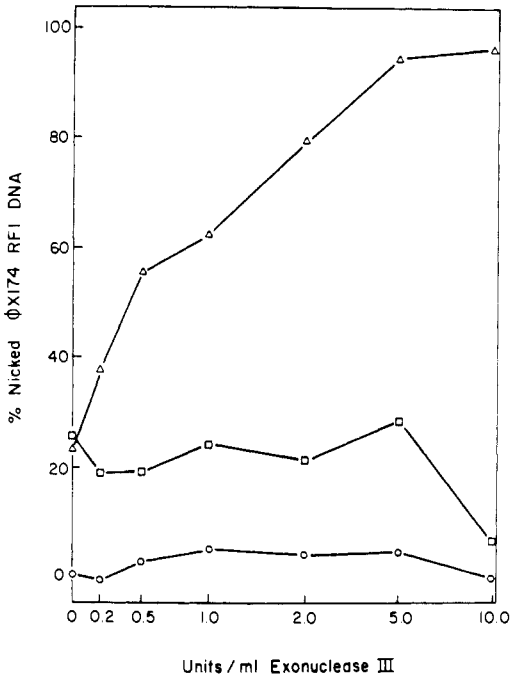

FIGURE 4: Endonuclease activity of exonuclease III. The fraction of nicked DNA was determined by the filter retention assay, as under Experimental Procedures. Incubation was for 5 min at 37 °C. (○) Nontreated DNA; (□) DNA treated with 10 μg/mL BLM (–DTT); (△) acid-depurinated DNA.

Table V: Effect of Mg^{2+} on Exonuclease III Activity to Create Template-Primer for DNA Polymerase I[a]

treatment prior to polymerase I	exonuclease III treatment in	pmol of dAMP incorporated
none		4.3
exonuclease III	$MgCl_2$	4.3
	sodium citrate	4.8
BLM + DTT + exonuclease III	$MgCl_2$	20.9
	sodium citrate	5.1
acid depurination		7.9
acid depurination + exonuclease III	$MgCl_2$	21.6
	sodium citrate	15.0
micrococcal nuclease + exonuclease III	$MgCl_2$	8.4
	sodium citrate	8.3

[a] BLM-treated, acid-depurinated, or micrococcal nuclease-treated DNA was treated with exonuclease III either in the presence of $MgCl_2$ or in the presence of sodium citrate. Exonuclease III was inactivated by heat treatment, and the incubation mixtures were brought to the same ionic condition prior to the reaction of DNA polymerase I, as described under Experimental Procedures.

Mg^{2+} on the ability of exonuclease III to create a template-primer for DNA polymerase I on apurinic DNA and BLM-treated DNA.

Typical results are shown in Table V. Exonuclease III (endonuclease VI) retained its ability to convert acid-depurinated DNA to a template-primer for DNA polymerase I even in the absence of Mg^{2+} (plus citrate), at about 50% or more of its activity in the presence of Mg^{2+}. On the DNA treated with BLM plus DTT, the activity of exonuclease III was reduced in the absence of Mg^{2+}. Exonuclease III activity on micrococcal nuclease-treated DNA was essentially unaffected by Mg^{2+}, indicating that the 3′-phosphatase activity of exonuclease III does not require Mg^{2+} ion. These results indicate that for the activity of exonuclease III to create a suitable template-primer for DNA polymerase I Mg^{2+} is required for the BLM-treated DNA. On the other hand, Mg^{2+} is not required for micrococcal nuclease-treated DNA conversion. Thus, these results show an enzymatically defined difference

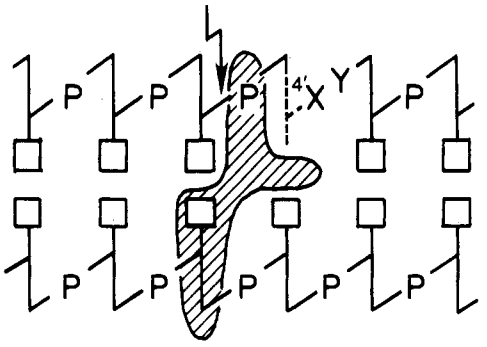

FIGURE 5: Model for the BLM-created nick and recognition by exonuclease III. The enzyme is illustrated as a shaded area. The phosphoester bond pointed to by the arrow is cleaved.

in the structure of BLM-created nicks from micrococcal nuclease-produced nicks.

Discussion

Our present work shows (i) that BLM-treated nicked DNA is unable to be used as a template-primer for DNA polymerase I, (ii) that purified *E. coli* exonuclease III can convert the BLM-treated DNA into a good template-primer, (iii) that cell extracts from exonuclease III$^+$ strains can activate the BLM-treated DNA whereas exonuclease III deficient cell extracts cannot, (iv) that very limited digestion at the nicked sites by exonuclease III is enough for the modification, and (v) that the terminus structure does not seem to be a simple 3′-P but requires exonucleolytic attack.

In order to avoid the complexity caused by nonspecific DNA synthesis, we used ΦX174 RFI DNA and produced a limited number of nicks by BLM to allow synthesis specific for this drug. Our experiments with crude extracts suggest that exonuclease III is a component in the cell which may be involved in the modification of BLM-treated DNA. This suggests an important role of exonuclease III in the repair response of the cell exposed to BLM. However, this does not necessarily exclude the possibility that other enzyme(s) may have similar functions in the cell.

The fact that *E. coli* DNA polymerase I requires the modifying activity of exonuclease III to allow synthesis on the BLM-treated DNA suggests the lack of proper 3′-OH termini at the nicks. Our present work also demonstrates that the nicks have a different structure from micrococcal nuclease-produced nicks, which have 3′-P, 5′-OH. The evidence for this is (i) that the modifying activity of exonuclease III on BLM-treated DNA requires Mg^{2+} ion whereas on the micrococcal nuclease-treated DNA the 3′-P phosphatase activity of exonuclease III is able to work in the absence of Mg^{2+} and (ii) that bacterial alkaline phosphatase is not able to convert the BLM-treated nicked DNA to a template-primer for DNA polymerase I under conditions where the enzyme works well on micrococcal nuclease-treated DNA.

BLM interacts with DNA, resulting in the release of bases and strand nicks with 3′-terminal structure different from normal 3′-P or 3′-OH (Haidle et al., 1972; Takeshita et al., 1978). Cleavage of the 3′–4′ carbon bond of the 3′-terminal deoxyribose moiety has been suggested, giving a 3′ terminus with a two-carbon fragment attached to phosphate. Inorganic phosphate is not released during incubation with BLM (Haidle et al., 1972), arguing against 3′-P termini. Exonuclease III was reported not able to release inorganic phosphates from BLM-treated DNA whereas bacterial alkaline phosphatase can, suggesting 5′-P termini (Kuo & Haidle, 1973; Kappen & Goldberg, 1978). The 5′ termini created with BLM may be variant in structure, that is, 5′-OH or 5′-P, since about 70% of 5′ termini could be labeled with ^{32}P by phosphokinase without phosphatase treatment, but the remaining portion of them required pretreatment of phosphatase to be labeled (Kuo & Haidle, 1973). Thus, the evidence is not in agreement but suggests a heterogeneity of termini configurations.

Weiss (1976) proposed a model which explains the wide variety of activities of exonuclease III: 3′–5′-exonuclease, 3′-phosphatase, apurinic endonuclease, and RNase H. According to this model, exonuclease III has three recognition sites: one site recognizes phosphoester bonds, and this activity cleaves such bonds, leaving a 3′-OH terminus; the second site recognizes a deoxynucleoside moiety sitting in the opposite strand, and the third one recognizes a space created either by the removal of a base or by the displacement of a base located at the strand end.

Figure 5 illustrates a general model of a BLM-created nick which contains the features described above (models similar in principle have been proposed by other workers: Muller & Zahn, 1977; Takeshita & Grollman, 1979), indicating modification of this site by exonuclease III can be plausibly explained by the model of Weiss.

Important features of this model include a space created by the removal of a base or by the fragmentation of the deoxyribose moiety. Exonuclease III action would result in the production of a simple 3-OH, which can be used as a primer by DNA polymerase I. It also explains the inability of bacterial alkaline phosphatase to convert the BLM-treated DNA to a template-primer for DNA polymerase I. This model also raises the possibility that DNA polymerase I will not bind to a 3′ structure which lacks a base residue. Such a finding could explain a lack of synthesis even if some 3′ termini (X) were OH groups. The requirement for limited exonucleolytic activity would be consistent, whether X were OH or P.

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Cleavage of Deoxyribonucleic Acid by the 1,10-Phenanthroline–Cuprous Complex. Hydrogen Peroxide Requirement and Primary and Secondary Structure Specificity†

Laura E. Marshall, Daniel R. Graham, Karl A. Reich, and David S. Sigman*

ABSTRACT: 1,10-Phenanthroline (OP) coordinated to copper ion cleaves DNA in a reaction requiring hydrogen peroxide and thiol. The alternating copolymer poly([^{3}H]dA-[^{3}H]T) (10 μg/mL) is made 95% acid soluble in less than 30 s with 20 μM OP, 2 μM copper ion, 7 mM 3-mercaptopropionic acid, and 7 mM hydrogen peroxide under both aerobic and anaerobic conditions. In the absence of added hydrogen peroxide, oxygen is required for the thiol-mediated scission of DNA by 1,10-phenanthroline and copper ion because it serves as a precursor for hydrogen peroxide formed as a result of the in situ oxidation of thiol catalyzed by the 2:1 phenanthroline–cupric complex. In the absence of 1,10-phenanthroline and copper ion, hydrogen peroxide does not measurably degrade poly(dA-dT). In the presence of the ligand and metal ion, the reaction is much faster when both hydrogen peroxide and thiol are present than when either is present alone. The involvement of the cuprous complex is inferred from the requirement for reducing agent and from the inhibition of the reaction by cuprous specific ligands. Intercalation of 1,10-phenanthroline into the DNA during the course of the reaction is suggested by (1) inhibition of DNA scission by intercalating agents such as ethidium bromide and (2) a strong, if not absolute, preference for double-stranded [poly(dA)·poly(T)] as compared to single-stranded [poly(dA) or poly(T)] DNA as a substrate for the cleavage. Electrophoresis of a fragment of *E. coli lac* operon reacted with the coordination complex revealed scission of nearly equal intensity at every base, suggesting little, if any, primary sequence specificity in the reaction. Because of this lack of specificity, this chemistry may be useful in studying the primary sequence specificity of other agents in their interactions with DNA.

1,10-Phenanthroline inhibits the poly(dA-T)-directed polymerization catalyzed by *E. coli* DNA polymerase I via a mechanism dependent on cupric ion and thiol (D'Aurora et al., 1977, 1978). Recently, we have reported that the 2:1 1,10-phenanthroline–cuprous complex [$(OP)_2Cu^+$][1] formed under these conditions cleaves DNA in an oxygen-dependent reaction and that the products of this reaction are effective inhibitors of Pol I (Sigman et al., 1979). The structure(s) of the inhibitory product(s) are not yet known.

Direct physical evidence for the scission reaction has been provided by electrophoretic analysis of poly(dA-T) and SV40 supercoiled DNAs which had been incubated with OP, Cu^{2+}, and thiol under aerobic conditions (Sigman et al., 1979). Downey et al. (1980), working independently, have confirmed that $(OP)_2Cu^+$ reacts with DNA in an oxygen-dependent reaction by showing that the coordination complex alters the sedimentation properties of φX174-RF DNA and releases acid-soluble counts from ^{3}H-labeled poly(dA-T). Since the production of acid-soluble counts from radioactively labeled poly(dA-T) is a more rapid assay than electrophoresis and better reflects the cleavage chemistry than the production of inhibitors of *E. coli* Pol I, we had been using it to study the role of molecular oxygen in the cleavage chemistry and to examine the effectiveness of free-radical traps and intercalating agents as inhibitors of this novel scission reaction.

Previously, we reported that hydrogen peroxide must be involved in the cleavage because catalase blocked the reaction between the coordination complex and poly(dA-T). In the present paper, we demonstrate that the exclusive role of molecular oxygen is to serve as a precursor for hydrogen peroxide in the $(OP)_2Cu^{2+}$-catalyzed oxidation of thiol (Graham et al.,

† From the Department of Biological Chemistry, School of Medicine, and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, California 90024. *Received May 19, 1980.* This work was supported by U.S. Public Health Service Grant GM 21199; L.E.M. was a Trainee of Cell and Molecular Biology Program, U.S. Public Health Service Grant GM 07185-05; D.R.G. was supported by Grant 3T32C-A9030 awarded by the National Cancer Institute.

[1] Abbreviations used: OP, 1,10-phenanthroline; $(OP)_2Cu^+$, 2:1 1,10-phenanthroline–cuprous complex; $(OP)_2Cu^{2+}$, 2:1 1,10-phenanthroline–cupric complex; 4′,6-diamidino-2-phenylindole bis(hydrochloride); SOD, superoxide dismutase; Pol I, *E. coli* DNA polymerase I.

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