Molecular basis of nitrogen mustard effects on transcription processes: role of depurination

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Received July 5, 1994; Revised and Accepted August 23, 1994

ABSTRACT

DNA was alkylated with nitrogen mustard (HN2) and the rate of release of the alkylpurines was quantitated by HPLC. The half life of depurination of the major product (7-alkylguanine) was 9.1 h at 37°C. End-labelled DNA was used to show that depurination occurred dominantly at 5'-GA, 5'-GG and 5'-GT sequences. Although extensive alkylation was observed at all 5'-GNC and 5'-GNT sequences, no depurination was observed at these sites during a depurination time of 20 h at 37°C. Since these sites are potential interstrand crosslinking sequences (G-adduct-G and G-adduct-A, both spanning an intervening base pair), this suggests that these regions have a greatly enhanced stability or that simultaneous depurination of both ends of the crosslink is necessary before these lesions are removed (with a predicted half-life of approximately 80 h at 37°C). Depurination at the *lac* UV5 promoter impaired the association of *Escherichia coli* RNA polymerase with that promoter, while in the elongation phase two distinctly different sequence-specific processes were apparent. At 5'-GNC and 5'-GNT sequences transcriptional blockages were maintained with increasing elongation time, whereas at monoadduct sites, the blockage decreased with elongation time (predominantly at 5'-GG and 5'-GC sequences), with an average half-life of approximately 10.7 h. Collectively, these results suggest that the observed read-through past monoadduct sites is due to depurination of the DNA at those sites. *E.coli* RNA polymerase is therefore able to transcribe efficiently past apurinic sites and presumably does so by incorporating an incorrect base into the nascent RNA.

INTRODUCTION

Nitrogen mustard [bis(2-chloroethyl)methylamine, HN2] was the first effective anticancer agent in clinical use (1). Derivatives such as phenylalanine mustard (melphalan), chlorambucil and cyclophosphamide are used in many chemotherapeutic treatment

regimens (2), despite their non-specific reaction mechanism. In an aqueous environment, these compounds spontaneously form reactive arizidinium ions (3) that may bind to nucleophilic sites within proteins, nucleic acids and other biomolecules.

There is a substantial body of evidence which suggests the involvement of DNA in the mechanism of action of this group of compounds (4–7). Nitrogen mustards alkylate DNA preferentially at the N-7 position of guanine and N-3 of adenine, resulting in the formation of monoadducts, as well as intra- and interstrand crosslinks (8–12). Although the role of these lesions in the cytotoxicity exerted by nitrogen mustards is unclear, there is evidence that the inhibition of DNA replication caused by DNA interstrand crosslinks is of particular importance (13).

Exposure of DNA *in vitro* and *in vivo* to alkylating agents such as nitrogen mustards leads to the formation alkyl-purine derivatives (14–17). A characteristic of this alkylation is that the purine-sugar glycosidic linkage becomes labile, thus increasing the rate of depurination of the DNA (14–17). Failure to repair apurinic sites leads to toxic lesions that have been suggested to play an important role in mutagenesis and carcinogenesis (17–19). The biological consequences of apurinic sites would therefore be expected to have an effect on DNA and RNA synthesis. A number of studies has been carried out with DNA polymerases using templates containing abasic sites, both *in vitro* and *in vivo*, and showed that these lesions act as strong blocks to the progression of DNA polymerases (20–24). There is, however, no information available on the effects of apurinic sites on transcriptional processes, although it is known that alkylation by mustards results in termination of transcription at specific sites corresponding to alkylation sites on the DNA template (25–27).

In the present study we have confirmed that 7-alkylguanine is the major depurinated product resulting from nitrogen mustard alkylation, and shown that the depurination process occurs in a sequence selective manner. If depurination occurs in the promoter region, this impairs the ability of *E.coli* RNA polymerase to associate with the *lac* UV5 promoter. Our data suggest that if an apurinic site is confronted during the elongation process, this does not result in the formation of a blocked transcript and RNA polymerase is able to transcribe efficiently past the apurinic site.

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MATERIALS AND METHODS

Nitrogen mustard [bis(2-chloroethyl)methylamine, HN2], heparin, guaninyl (3'-5') adenosine (GpA), adenosine and guanosine were purchased from Sigma Aldrich Chemical Company Inc., Australia. Calf thymus DNA was from Worthington Biochemical Corp., NJ. *Escherichia coli* RNA polymerase, Ribonuclease Inhibitor (Human Placenta), ultrapure ribonucleotides triphosphates, 3'-O-methyl nucleoside triphosphates and BSA (RNase and DNase free) were obtained from Pharmacia Biotech, WI, USA [α - 32 P] ATP and [α - 32 P] dATP were purchased from Amersham, UK. Urea, bisacrylamide, acrylamide, ammonium persulfate, TEMED and DTT were obtained from Bio-Rad, CA, USA as electrophoresis grade reagents. Restriction enzymes were obtained from either Boehringer Mannheim, Germany or New England Biolabs, CT, USA. The NA45 DEAE membrane was purchased from Schleicher & Schuell, Germany and Nensorb 20 columns were from Dupont, Australia. All other chemicals were of analytical grade and all solutions were prepared from distilled, deionized and filtered water from a Milli-Q 4 stage water purification system (Millipore, MA).

HPLC analysis of depurinated products

The measurement and identification of depurinated HN2 alkylation adducts were carried out according to published procedure (28). Briefly, 10 mg/ml calf thymus DNA was reacted with 1 mM HN2 in TE buffer (pH 8.0) for 60 min at 37°C. Non-covalently bound drug was removed by ethanol precipitation of the DNA. The modified DNA pellet was then resuspended in 10 mM phosphate buffer (pH 7.0) containing 2 M NaCl. Alkylated DNA was depurinated by neutral hydrolysis at 70 or 37°C and aliquots removed at desired time intervals and the DNA was then precipitated with ethanol. The supernatant containing the depurinated products was dried under vacuum and dissolved in 50 mM ammonium formate (pH 5.1). HPLC analysis was performed using a Waters 600E System Controller and Waters 990 Photodiode Array Detector with a reverse phase C-18 4.6 mm $\times$ 25 cm μ Bondapak column. The sample volume was 200 μ l, the eluent was 50 mM ammonium formate (pH 5.1) and the flow rate was 1.0 ml/min. The amount of depurinated product was quantitated using peak area at 254 nm.

The HPLC reference compounds 7-alkylguanine and 3-alkyladenine were prepared by reacting guanosine and adenosine, respectively, with HN2 overnight at 37°C in trifluoroethanol (29), heated at 100°C for 10 min, dried under vacuum and the residue dissolved in methanol. Insoluble materials were removed by centrifugation and the supernatant dried and resuspended in 50 mM ammonium formate (pH 5.1).

Labelling of DNA fragment

A 188 bp *PvuII/EcoRI* restriction fragment of a modified pBR322 vector was used and contained the *lac* UV5 promoter. The preparation of the plasmid and the 188 bp fragment was as described by White and Phillips (30). The 3' overhang generated by *EcoRI* was filled using Klenow DNA Polymerase. Briefly, approximately 10 μ g of the 188 bp DNA fragment was incubated with 0.125 mg/ml BSA, 1.25 mM DTT, 200 μ Ci [α - 32 P] dATP and 15 units of Klenow fragment in 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ (final volume of 100 μ l) at room temperature for 30 min. A mixture of all four deoxynucleotides (at a final concentration of 1.25 mM each) was added and the reaction was

continued for a further 30 min. The labelled DNA fragment was purified using a Nensorb 20 chromatography column, dried and resuspended in an appropriate volume of TE buffer containing sonicated calf thymus DNA to a final concentration of 150 μ M bp.

DNA alkylation and depurination sites

Determination of alkylation and depurination sites was carried out according to published procedures (11,31). Briefly, a 188 bp DNA fragment was end-labelled as described above. The labelled DNA (20 000 c.p.m.) was then incubated with various concentrations of HN2 for 60 min at 37°C, and DNA then precipitated with ethanol. For simultaneous determination of both alkylation and depurination sites, the modified DNA was resuspended in 100 μ l of 1 M piperidine. In contrast, for the determination of depurination sites, the DNA was resuspended in TE buffer, incubated at 37°C for 10 and 20 h, dried under vacuum and then resuspended in 100 μ l of 10 mM NaOH. Both sets of samples were then heated at 90°C for 10 min. The piperidine-treated samples were dried under vacuum and the NaOH samples were neutralized with HCl, ethanol precipitated and dried. All samples were dissolved in a loading buffer containing 95% (v/v) formamide, 1 mM EDTA and 0.1% (w/v) of both xylene cyanol and bromophenol blue. Samples were denatured at 90°C for 5 min prior to electrophoresis using a 12% denaturing polyacrylamide gel.

RNA polymerase-promoter binding

HN2 was initially dissolved in ethanol and then diluted to the required concentration in TE buffer such that the final concentration of ethanol was less than 0.4% (v/v). The final volume of reaction was 70 μ l and contained approximately 1 μ g of the labelled 188 bp DNA fragment. Following alkylation at 37°C for 60 min, unreacted drug was removed by ethanol precipitation of the DNA. The modified DNA pellet was washed, dried and resuspended in TE buffer. Depurination of the DNA was performed by further incubating the alkylated and non-alkylated DNA solution at 37°C and removing 10 μ l aliquots at appropriate times up to 50 h. These aliquots were stored at -20°C until required.

The RNA polymerase-promoter complex was formed as described previously (32). Briefly, 100 nM of depurinated DNA was added to 600 nM *E. coli* RNA polymerase in a transcription buffer containing 40 mM Tris-HCl (pH 8.0), 100 mM KCl, 0.1 mM EDTA, 10 mM DTT and 125 μ g/ml BSA. After incubation at 37°C for 15 min heparin was added to a final concentration of 400 μ g/ml to remove non-specifically bound RNA polymerase, and the mixture was incubated at 37°C for a further 5 min. A stable ternary complex was formed by the addition of initiation nucleotides consisting of GpA (200 μ M final concentration), ATP, UTP and GTP (5 μ M final concentration of each) and further incubating at 37°C for 5 min. An equal volume of loading buffer [containing 60% sucrose, 0.2% (w/v) each of xylene cyanol and bromophenol blue] was added to the initiated complex and samples analysed on a 5% (19:1 acrylamide/bis-acrylamide) non-denaturing submarine gel in TBE buffer. Electrophoresis was carried out at 100 V for 3 h. The gel was fixed in 10% acetic acid and dried using a Bio-Rad Gel Drier.

In vitro transcription

A 497 bp *PvuII/SalI* restriction fragment of a modified pBR322 vector containing the *lac* UV5 promoter was used for *in vitro*

transcription with *E. coli* RNA polymerase, as described previously (25). Briefly, 100 mM RNA polymerase in transcription buffer was added to 100 nM alkylated DNA fragment to form a stable binary complex. After 15 min incubation at 37°C heparin was added to a final concentration of 400 μ g/ml to remove any non-specifically bound RNA polymerase. A stable ternary complex was then formed as described above, and elongated by addition of a mixture of UTP, ATP, GTP and CTP (2 mM final concentration of each) and KCl (100 mM final concentration). The elongation reaction was stopped at various time points with the addition of an equal volume of loading/termination buffer comprising of 10 mM urea, 10% (w/v) sucrose, 40 mM EDTA, 0.1% (w/v) xylene cyanol and bromophenol blue in 2 $\times$ TBE buffer. Electrophoresis was carried out as described previously (30).

Gel analysis and quantitation

Gel analysis was performed using a Molecular Dynamics Model 400B PhosphorImager with quantitation carried out using the Molecular Dynamics ImageQuant software.

RESULTS

Depurination products

Calf thymus DNA (10 mg/ml) was reacted with 1 mM HN2 for 60 min and the alkylated DNA was then separated from unreacted HN2 by ethanol precipitation. The alkylated DNA was resuspended in 10 mM phosphate buffer (pH 7.0), subjected to hydrolysis at 37°C or 70°C and the depurinated products then resolved by HPLC.

The major depurinated product had an elution time of 11.5 min, identical to that of the reference compound, 7-alkylguanine while 3-alkyladenine was a minor product with an elution time of 23 min. Free guanine eluted at 8 min. After 10 h hydrolysis at 70°C, the amount of 7-alkylguanine was approximately 13 times that of 3-alkyladenine (data not shown). Figure 1 shows the time course of release of 7-alkylguanine at 70°C and 37°C and free guanine at 70°C. The amount of 7-alkylguanine released at 70°C is approximately six times that released at 37°C after 10 h depurination time, and approximately 60 times that of free guanine at 70°C. The half-life of release of 7-alkylguanine was

Figure 1. Release of depurination products. Calf thymus DNA was reacted with HN2 and then subjected to hydrolysis at neutral pH. The depurinated products shown are 7-alkylguanine released at 70°C (■) and 37°C (●) and free guanine released at 70°C (▲), and were resolved by HPLC.

Figure 2. Alkylation and depurination sites. (a) Autoradiogram of a 12% denaturing polyacrylamide gel showing DNA strand breaks. Labelled 188 bp DNA fragment was reacted with 50, 100 and 200 μ M HN2 and subjected to piperidine treatment (lanes 2–4 respectively). For lanes 5 and 6, DNA was reacted with 200 μ M HN2 and depurinated at 37°C for 10 and 20 h respectively and then subjected to NaOH treatment. Lane 1 is a control (unmodified DNA). (b) Quantitation of alkylation and depurination sites. The relative intensity of DNA strand breaks and the sequence of the 3'-end labelled DNA strand are shown. The intensity of the alkylation sites (from lane 4; a) appears above the sequence of the labelled DNA strand and relative intensity of the depurination (from lane 6; a) appears below the sequence of the labelled DNA strand. The numbering is with respect to +1 for the start of the transcribed message.

1.8 h at 70°C and 9.1 h at 37°C (calculated from a first-order kinetic analysis).

Sequence specificity of alkylation and depurination sites

End-labelled 188 bp DNA was reacted with HN2 for 60 min and the alkylated DNA was separated from unreacted HN2 by ethanol precipitation. The alkylated DNA was then subjected to piperidine hydrolysis (yielding the sum of both alkylated and depurinated sites) or alkaline hydrolysis (revealing just depurinated sites) (31). Because the DNA was reacted with mustard for only 1 h, there is essentially no depurination occurring within this time (see Figure 1). Although piperidine treatment is known to reveal both alkylation and depurination sites (11,31), under the present experimental conditions these sites are therefore almost exclusively just alkylation sites. As shown in Figure 2a, the level of alkylation increased with increasing HN2 concentration, with all guanine residues being alkylated at 200 μ M HN2 (lanes 2–4).

Figure 3. Effect of mustard-induced apurinic sites on promoter binding. (a) Autoradiogram of a 5% polyacrylamide gel mobility shift assay of the association of *E. coli* RNA polymerase with the *lac* UV5 promoter. Alkylated and unmodified DNA (labelled 188 bp) were incubated at 37°C for 0–50 h prior to the formation of the initiated transcription complex. The lane (–) indicates uncomplexed DNA in the absence of RNA polymerase. (b) Quantitation of the RNA polymerase–DNA complex. The percentage of the DNA in the form of the DNA–RNA polymerase complex is shown as a function of depurination time for HN2 alkylated/depurinated DNA (■) and unmodified/depurinated DNA (●).

Lanes 5 and 6 show sites where HN2 alkylation resulted in depurination after 10 and 20 h, respectively, at 37°C. Of the 22 alkylation sites observed, seven resulted in a detectable level of depurination. The intensity of depurinated sites increased with increasing depurination time and some sites were only detectable after 20 h of hydrolysis time. The sequence specificity and relative intensity of alkylation and depurination sites are shown in Figure 2b. Depurination was detected at the following sequences: 5'-G-A (positions +30 and –1), 5'-GT (positions +5, –4, –6 and –13) and 5'-GG (position –2). Sites that were alkylated but did not depurinate occur at 5'-GNT (positions +47, +36, +12, –17, –24 and –38), 5'-GNC (positions +41, –18, –39 and –47). Other less well defined sites which may also be in this category are 5'-CGG (positions +29 and +11), 5'-AGG (position –48) and 5'-GC (position +9). The 5'-GNT and 5'-GNC sequences are possible GA and GG interstrand crosslinking sites spanning over an intervening base pair.

Depurination of *lac* UV5 promoter

Initial studies on the effect of apurinic sites on transcription were performed by reacting the 497 bp DNA fragment with HN2, subjecting the alkylated DNA to hydrolysis at 70°C, and then using this depurinated DNA to quantitate the extent of *in vitro* transcription. Results from preliminary studies showed that with increasing depurination, less transcriptional activity was observed and the amount of the initiated transcription complex decreased with increasing depurination time. Taken together, these results suggest that depurination of the promoter was occurring, and this decreased the extent of binding of the RNA polymerase, hence a decrease in the formation of the initiated complex and subsequent decrease of the extent of transcription. In order to test this hypothesis, the capacity of RNA polymerase to bind a depurinated *lac* UV5 fragment was measured by the gel mobility shift assay. Figure 3a shows the gel mobility shift analysis of the DNA–RNA polymerase initiated complex and the quantitation of these bands is shown in Figure 3b. Since the total amount of DNA loaded in each lane varied by up to $\pm 20\%$, the amount of retarded complex was expressed as a percentage of the total DNA in each lane in order to allow for this differential loading. In the absence of mustard, the DNA–RNA polymerase complex comprised approximately 59% of the total DNA. The amount of this complex decreased linearly with increasing incubation time at 37°C, and after 50 h incubation the amount of the DNA–RNA polymerase complex decreased to 44%. With the mustard-alkylated DNA, the DNA–RNA polymerase complex prior to depurination was 54% of the total DNA and following depurination decreased linearly with only 16% of the DNA–RNA polymerase complex evident after 50 h depurination at 37°C.

Effect of elongation time

The transcription system used employed a synchronized population of the initiated complex with the nascent RNA being only 11 nucleotides long (30,33). The movement of RNA polymerase from the the initiated transcription complex is reflected by the extent of elongation of the initiated transcripts. For alkylated DNA, the processive movement of the RNA polymerase along the DNA template is blocked at sites of alkylation and is revealed as a blocked transcript (25). Figure 4a shows mustard-induced transcriptional blockages which occur at (or 1 bp prior to) the alkylation site on the template strand. Two types of effect are apparent from the transcriptional blockage

sites shown in Figure 4a. At some sites (43/44-mer, 83-mer, 102-mer and 117-mer), the transcriptional blockage decreases with increasing elongation time, whereas at other sites (80-mer, 110-mer and 126/127-mer), the blockage is decreased to a significantly lesser extent or is maintained. The read-through of the RNA polymerase past the 43/44-mer, 83-mer, 102-mer and 117-mer blockages were quantitated and shown to represent a first-order process in each case (Figure 5), displaying half lives

of 4.7, 11.5, 20.5 and 6.3 h respectively. Since with increasing elongation time there is an increase in the amount of depurination, this should result in an increasing amount of full length transcript if the *E. coli* RNA polymerase is able to by-pass apurinic sites. For this reason the amount of full length transcript was quantitated from the digitized image of Figure 4b, and is shown in Figure 6 (panel B). The observed increase in the amount of full length transcript (from 12 to 25% of the total transcriptional activity) with elongation time is not due to increased release of RNA polymerase from the initiated transcription complex since the total transcriptional activity remains essentially constant throughout the duration of the elongation process (Figure 6, panel A).

Accompanying the decay of the transcriptional blockage at position 83 there is a build up of new blockages at somewhat longer transcript lengths (85–93-mer). This has been noted previously with other drug-induced blockages (30,34) and is thought to be due to the formation of a RNA hairpin helix in the blocked transcript immediately upstream of the retarded transcription bubble (34). Since there is an inverted repeat in the DNA sequence at positions 54–69 (Figure 4b), and there is ample time for this 6 bp helix to form before the polymerase is released from the adduct blockage at position 83, the observed 'delayed termination' is probably due to the lagging portion of the polymerase encountering the hairpin after release of the enzyme from the adduct site (34).

DISCUSSION

DNA depurination is a spontaneous process which under physiological conditions is estimated to occur at a rate of approximately 3×10^{-11} nucleotides/s (35). The depurination can also be enhanced by enzymatic processes such as those involving DNA glycosylases, which catalyse the release of damaged purine bases. The process is enhanced by low pH and elevated temperatures and is influenced by the nature and site of attached groups (15–17).

The results from this work showed that 7-alkylguanine is the major depurinated product, about 13 times more than that of 3-alkyladenine. This is in agreement with the relative amounts of these products obtained with sulphur mustards (36). The amount of the 7-alkyl derivative is about 60 times more than that

Figure 4. Effect of transcription elongation time. (a) The autoradiogram shows the transcriptional blockages and read-through sites for elongation time from 1 to 50 h. DNA was reacted with 200 μ M HN2 and then the initiated transcriptional complex formed. Elongation nucleotides were added and 5 μ l aliquots of the transcription reaction were removed and added to an equal volume of loading/termination buffer. Lanes G and C are sequencing lanes using termination of transcription by 3'-O-methyl-GTP and 3'-O-methyl CTP respectively. The lengths of several of the major block and read-through sites are numbered on the right-hand side of the autoradiogram. The full length transcript is denoted as FLT. (b) Location of transcriptional blockage and read-through sites. The transcriptional block sites that are relatively constant are marked with an asterisk (*); the filled rectangle ■ indicates sites of significant read-through. The open rectangle □ on the DNA sequence shows all potential 5'-GNC interstrand crosslinking sites.

Figure 5. Decay of transcriptional blockage. Read-through past the 43/44-mer (■), 83-mer (●), 102-mer (▲) and 117-mer (▼) sites are shown as first-order process for the decay of the mole fraction of each blocked transcript.

Figure 6. Effect of depurination and elongation. The absolute amount of the full length transcript (panel B) was quantitated from Figure 4a (as pixel intensity X number of pixels in each band) for the elongation times of 1–50 h. The total intensity of all transcripts in each lane of Figure 4a is shown in panel A.

of unmodified guanine, and this ratio is similar to that obtained for DNA modified at N-7 by dimethyl sulphate (28).

The pattern for guanine N7 alkylation observed in this study is as reported previously (11). However, the depurination event accompanying this alkylation exhibits an unusual sequence specificity, with depurination observed predominantly at 5'-GA, 5'-GG and 5'-GT sequences. Depurination at the two latter sequences has been reported for other alkylating agents (31). The sites that were alkylated but did not result in depurination were mainly at 5'-GNC and 5'-GNT sequences, both of which are possible sites for GG and GA interstrand DNA crosslinks extending over an intervening base pair. Such an HN2 induced DNA interstrand crosslinks have been reported for the 5'-GNC sequence (37). If GNC and GNT are sites of preferred interstrand crosslinking, and if simultaneous depurination of both ends of the crosslink is necessary before these lesions are removed, then the half-life of such process should be approximately 80 h at 37°C (based on the observed half life of 9.1 h for the release of 7-alkylguanine). Therefore, after a depurination process of 20 h, only approximately 10% or so of the crosslinked site would be detected as depurinated. This would therefore be consistent with the observed depurination at isolated guanine residues, and the low level of depurination at apparent sites of interstrand crosslinking (GNC and GNT). Alternatively, the nature of the local DNA structural changes induced by the crosslink (37) may result in a decreased rate of depurination of either (or both) end of the crosslink.

The first step in the transcription process is the correct binding of RNA polymerase to the promoter. Although this is fundamental to the process of gene expression, effects of covalent modification of the promoter has received little attention. Gray and Phillips (32) showed that alkylation of the *lac* UV5 promoter results in formation of a more stable DNA–RNA polymerase complex, with a decreased ability of the polymerase to escape from the initiated transcription complex. The results from the present work demonstrate a second effect of alkylation of the promoter—apurinic sites induced by HN2 diminish the ability of RNA polymerase to associate with the promoter. Both effects result

in a decrease of transcriptional activity from that promoter. Since alkylation occurs at possible DNA interstrand crosslinking sites in the promoter region (Figure 2b) this may have the additional effect of blocking progression of the closed complex to the open complex, but this has yet to be confirmed.

Little information is currently available regarding the response of the transcriptional elongation process at apurinic sites. Analysis of the transcriptional elongation experiment (Figure 4a) showed two sequence specific processes occurring with increasing elongation time. The amount of transcriptional blockage is largely maintained at some sites but disappears at others. Sites where transcriptional blockages decrease sharply with time occur at the 5'-GG (43/44-mer, 102-mer), 5'-GA (83-mer) and 5'-GC (117-mer) sequences, and these are sites of occurrence of monoadducts. Sites where blockages are maintained with time occur at 5'-GTC (80-mer), 5'-GGC (110-mer) and 5'-GGCC (126/127-mer), all of which are possible DNA interstrand crosslinking sites. This is consistent with previous observations (37) that HN2 crosslinks duplex DNA fragments through the distal deoxyguanosine at the sequence 5'-GNC (where N = G or C). This fundamental difference of response between sites of monoadducts and interstrand crosslinks may therefore account for one level of the observed sequence dependence of read-through past some transcriptional blockage sites. With regard to monoadducts there are three possible mechanisms to account for the observed read-through past these sites. First, the covalent adduct itself may be inherently labile and with increasing elongation times, as it dissociates, it allows the RNA polymerase to continue transcription past that site. The second possible mechanism is that the adduct has sufficient flexibility about the site of attachment to permit slow movement of the polymerase past that site. The third possibility is that depurination occurs at the site of alkylation, thus allowing the RNA polymerase to read-through past the apurinic site. Each of these possibilities are now considered.

Although the antitumour antibiotic CC-1065 is known to bind to DNA, this covalent binding has been shown to be reversible at 37°C (38). However, there is no evidence in the literature to suggest that the classic covalent bonds formed between DNA and mustards are reversible and it must therefore be assumed that these bonds are stable under the experimental conditions employed. The second possibility for the decay of transcriptional blockage is that flexibility of the adduct may allow the elongation complex to proceed past the drug site. While this phenomenon would be consistent with the observation that possible DNA interstrand crosslinking sites are more difficult for the RNA polymerase to by-pass, there is no other direct evidence, either in the present work or elsewhere, to support this possibility. The third possible mechanism, involving depurination, does have good support from the present experimental results. The half-life of the major depurinated product is 9.1 h at 37°C as measured by HPLC, and this is almost the same as that measured for the decay of the transcriptional blockages (average of 10.7 h for the four sites quantitated). This therefore suggests that the decrease of transcriptional blockages with time is likely to arise from depurination of alkylated bases. Furthermore, the sequence selectivity of mustard-induced depurination (Figure 2b) coupled with similar sequence dependence of the read-through at the transcription block site (Figure 4a) is further support for the depurination mechanism. The observed increase in the amount of full length transcript, with the total transcriptional activity remaining essentially constant with increasing elongation time,

suggests that RNA polymerase is transcribing efficiently past the nitrogen mustard-induced apurinic sites. This conclusion is also consistent with the recent study by Zhou and Doetsch (39), who demonstrated efficient by-pass by *E.coli* and *SP6* RNA polymerases at abasic sites on the DNA template.

DNA has repair mechanisms which have been shown to repair both DNA adducts and apurinic lesions (16,40–42). Several studies have reported preferential repair of actively transcribing genes compared with non-transcribing genes (42) and mechanisms as to how this process occurs have been proposed (43). The critical (rate-limiting) step in this repair process is the chain elongation process at the apurinic site. The kinetics of the two competing transcription processes of elongation and pausing (to allow DNA repair to take place) is therefore important. If the lesion is an apurinic site and is not repaired, an incorrect ribonucleotide is likely to be inserted into the RNA chain (39). The biological effect of this misincorporation on the translated protein is therefore of great interest and is currently being investigated as a potential mechanism of mutation.

ACKNOWLEDGEMENTS

This work was carried out with the support of the Australian Research Council (D.R.P.) and a scholarship from the Australian International Development Assistance Bureau (A.M.).

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