Effect of Alkylating Agents on Initiation and Elongation of the *lac* UV5 Promoter

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ABSTRACT: DNA containing the *lac* UV5 promoter was alkylated using bifunctional sulfur and nitrogen mustards and a monofunctional sulfur mustard. The alkylation sites were mapped using *Taq* polymerase, and the effect of alkylation on the formation of the DNA–RNA polymerase complex was determined using gel retardation. Alkylation was observed at all G residues in the template strand. Exposure of the alkylated DNA to *Escherichia coli* RNA polymerase resulted in the formation of a DNA–enzyme complex that was more stable, prior to initiation, than the complex formed with nonalkylated DNA. The DNA–RNA polymerase complex formed with the alkylated DNA also demonstrated decreased ability to progress along the full length of the DNA template. These observations show that, in addition to inducing transcriptional blockages, mustards also influence the interaction between RNA polymerase and its promoter. The ability to interfere with protein–DNA interactions may contribute significantly to the effects of these compounds in eukaryotic systems with their complex array of transcription factors.

Nitrogen and sulfur mustards and a number of their derivatives are potent alkylating agents (Brookes, 1990), and several of the nitrogen mustards are valuable therapeutic agents for the treatment of cancer (Koeller & Murphy, 1989). Sulfur mustard has been used several times this century as a chemical warfare agent (Robinson, 1971), the most notable recent occasion being during the conflict between Iran and Iraq (Willems, 1989). There is a substantial body of evidence which implicates DNA in the mechanism of action of these compounds. The first visible signs of damage appear in the nucleus (Papirmiester *et al.*, 1984; Petrali *et al.*, 1990). Cells which are in the process of growth and division are more sensitive than differentiated cells and are most sensitive during the DNA synthetic phase of the cell cycle. DNA synthesis is one of the first cellular processes to be affected, and DNA repair enzymes influence the toxicity of these compounds (Carr & Fox, 1982; Murnane *et al.*, 1980; Roberts & Kotsake-Kovatsi, 1986; Tan *et al.*, 1987). It is well established that the mustards alkylate nucleic acids. Alkylation occurs in both DNA and RNA at adenine and guanine residues, the preferred sites being guanine N7 and adenine N1 and N3 (Brookes & Lawley, 1960; Shooter *et al.*, 1971; Hemminki & Kallama, 1986; Mattes *et al.*, 1986; Kohn *et al.*, 1987; Papirmeister *et al.*, 1991).

The studies cited above clearly demonstrate an effect of the mustards at the DNA level. DNA synthesis is inhibited by these compounds, and metabolic changes resulting from DNA damage, such as depletion of NAD^+, are observed *in vivo* (Papirmeister *et al.*, 1991). However, a detailed understanding of the consequences of the alkylation of DNA by mustards is incomplete. In particular, the effects on transcription have been only partially defined. Studies using *in vitro* transcription systems have demonstrated that alkylation of DNA by nitrogen mustards results in the termination of transcription (Pieper *et al.*, 1989; Pieper & Erickson, 1990; Gray *et al.*, 1991). These studies concentrated on the process that occurs after binding of the RNA polymerase to the promoter and formation of the initiated promoter–RNA polymerase complex. The

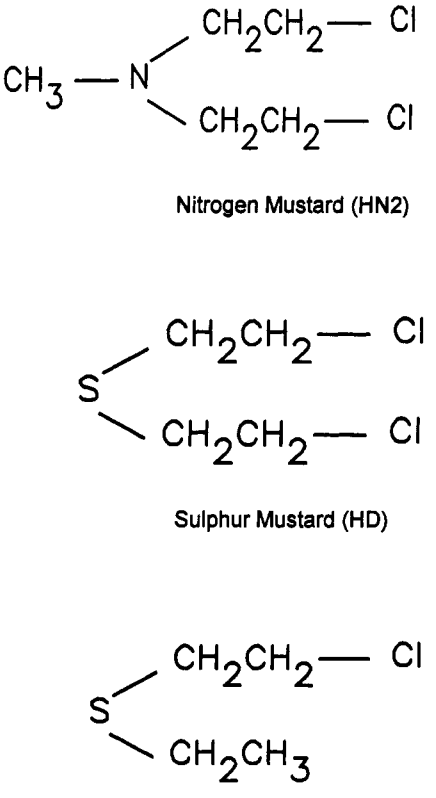

FIGURE 1: Structures of the three alkylating agents used in this work.

lac UV5 promoter incorporates a number of G-containing sequences that are good candidates for the production of interstrand and intrastrand cross-links, as well as a number of isolated G residues on the template strand, and both types of sites occur in the contact region of RNA polymerase within the promoter region (Siebenlist *et al.*, 1980). RNA polymerase has been shown to make contact with, and protect from methylation, several guanine and adenine residues in this

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region, and methylation within the promoter has been shown to decrease the binding of the RNA polymerase to DNA (Siebenlist *et al.*, 1980). It is reasonable, therefore, to suspect that alkylation by the mustards within the promoter region may influence the interaction between the DNA and RNA polymerase. The chance of such interference occurring is enhanced by the bifunctional nature of these chemicals, which allows the possibility of cross-linking between the DNA and the RNA polymerase.

The aims of this study were to map the alkylation of the *lac* UV5 promoter by nitrogen and sulfur mustards (Figure 1) and to examine the effect of these compounds on the subsequent formation of the RNA polymerase–promoter complex and the formation and elongation of the stable initiated complex.

MATERIALS AND METHODS

Materials. Nitrogen mustard [bis(2-chloroethyl)methylamine, HN2[1]] and 2-chloroethyl ethyl sulfide (CEES) were purchased from Aldrich Chemical Company, Inc. Sulfur mustard (bis(2-chloroethyl) sulfide, HD) was synthesized at Materials Research Laboratory, DSTO. The two sulfur mustards were greater than 98% pure as assessed by ^{1}H NMR. *Escherichia coli* RNA polymerase, ribonuclease inhibitor (human placenta), ultrapure ribonucleotides, 3′-*O*-methylnucleoside triphosphates, and BSA (RNase/DNase free) were obtained from Pharmacia. [α-^{32}P]dATP, [γ-^{32}P]ATP, and X-ray film (Hyperfilm-β max) were obtained from Amersham. XAR-5 X-ray film was obtained from Kodak. Heparin and guaninyl(3′–5′)adenosine (GpA) were purchased from Sigma. Urea, bisacrylamide, acrylamide, ammonium persulfate, TEMED, and dithiothreitol were obtained from Bio-Rad as electrophoresis purity reagents. Restriction endonucleases were obtained from either New England Biolabs or Boehringer-Mannheim, and NA45 DEAE membrane filters were obtained from Schleicher and Schuell. Deoxyribonucleotides and Klenow fragment were obtained from Boehringer-Mannheim. The *fmol* DNA sequencing system was obtained from Promega. NENSORB 20 columns and NENSORB PREP cartridges were obtained from DuPont. All other chemicals were of analytical grade, and all solutions were prepared using distilled, deionized, and filtered water from a Milli-Q 4-stage water purification system (Millipore).

Promoter–RNA Polymerase Complex Formation. A 188-bp *Pvu*II/*Eco*RI restriction fragment of a modified pBR322 vector (containing the *lac* UV5 promoter) was used as the template for *E. coli* RNA polymerase. The preparation of the plasmid and the 188-bp fragment was essentially as described by White and Phillips (1988). DNA concentrations were determined using Hoechst 33258 fluorescent dye (Bresatec). The 3′-end of the fragment was labeled by end-filling the 4-bp overhang generated by *Eco*RI. Briefly, approximately 5.4 μg was incubated with 0.125 mg/mL BSA (fraction V, Sigma), 1.25 mM DTT, 200 μCi of [α-^{32}P]dATP, and 10 units of Klenow fragment in 50 mM Tris/10 mM $MgCl_2$ (pH 7.5, total volume 80 μL) at room temperature for 30 min. A mixture of all four deoxyribonucleotides (final concentration 1.25 mM each) was added, and incubation continued for an additional 30 min. The labeled DNA was purified using a NENSORB 20 column, dried, and then dissolved in the appropriate volume of TE buffer.

Equal volumes of alkylating agent in TE buffer (pH 8.0) and labeled 188-bp fragment were mixed together at 37 °C for 60 min. The two sulfur mustards were dissolved initially in ethanol and then subdiluted to the required concentration in TE. The final ethanol concentration in the alkylation reaction was 0.5% (v/v). The reaction volume was 16 μL and contained approximately 75 nM DNA fragment.

Following the alkylation reaction, the DNA–RNA polymerase complex was formed by adding 2 μL of the alkylated DNA to 4 μL of a solution containing *E. coli* RNA polymerase. The reaction buffer contained 40 mM Tris (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM DTT, and 125 μg/mL BSA. After incubation at 37 °C for 15 min, heparin was added to a final concentration of 400 μg/mL, and the mixture was incubated at 37 °C for 5 min. For studies of initiation and elongation the stable ternary complex was then formed by the addition of initiation nucleotides (Phillips & Crothers, 1986; White & Phillips, 1988). The initiation nucleotide mix contained GpA, ATP, GTP, and UTP. Final concentrations were 200 μM for GpA and 5 μM for the other three nucleotides. The transcript was elongated by the addition of a mixture of UTP, ATP, GTP, and CTP (2 mM final concentration) and KCl (400 μM final concentration). The nucleotide mixes were replaced by reaction buffer to allow isolation of the preinitiated and initiated complexes. The incubation times were the same for all solutions.

The RNA polymerase concentration (prior to quenching with heparin) was approximately 615 nM. This estimated concentration is based on the assumption of 100% purity of the enzyme and a molecular weight of 460 000. The resulting enzyme/DNA ratio (25:1) was chosen by serial dilution experiments to provide a measurable level of enzyme–DNA complex under the gel electrophoresis conditions used to separate free and bound DNA fragments.

Primer Extension. Unlabeled 188-bp DNA fragments were alkylated with 200 μM mustard at 37 °C for 60 min. The DNA concentration used was the same as that used for formation of the DNA–RNA polymerase complex. After alkylation, the samples were frozen in liquid nitrogen and then stored at −20 °C until required. Alkylation sites were mapped with *Taq* polymerase using a Promega Corporation *fmol* DNA sequencing system. The sequencing reactions were carried out according to the Promega protocol. Control and alkylation site mapping reactions were carried out using nucleotide mixes containing a 3:1 ratio of 7′-deaza-dGTP/dGTP. A 20-mer primer was synthesized using an Applied Biosystems Model 381A DNA synthesizer, purified using a NENSORB PREP cartridge, and 5′-labeled by incubating oligonucleotide with [γ-^{32}P]ATP and T4 polynucleotide kinase in accordance with the Promega protocol. The primer was complementary to the −118 to −99 region of the promoter. The labeled primer was stored at −20 °C and used without further purification. All reactions were carried out also using a Perkin-Elmer/Cetus thermal cycler. Approximately 8.5 fmol of the 188-bp fragment and 2 pmol of the labeled 20-mer primer were used for each cycling reaction (volume of 5 μL). The cycling conditions involved a 5-min incubation at 94 °C followed by 30 cycles at 94 °C for 30 s and at 70 °C for 30 s, followed by a 5-min extension at 70 °C. At the completion of cycling, the samples were cooled to 4 °C and 3 μL of stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added. The samples were then heated to 90 °C for 5 min, cooled rapidly in ice, centrifuged, and loaded onto a denaturing polyacrylamide gel as described below.

[1] Abbreviations: HN2, nitrogen mustard, bis(2-chloroethyl)methylamine; HD, sulfur mustard, bis(2-chloroethyl) sulfide; CEES, 2-chloroethyl ethyl sulfide; TE, Tris–EDTA; DTT, dithiothreitol; TBE, Tris–borate–EDTA; bp, base pair.

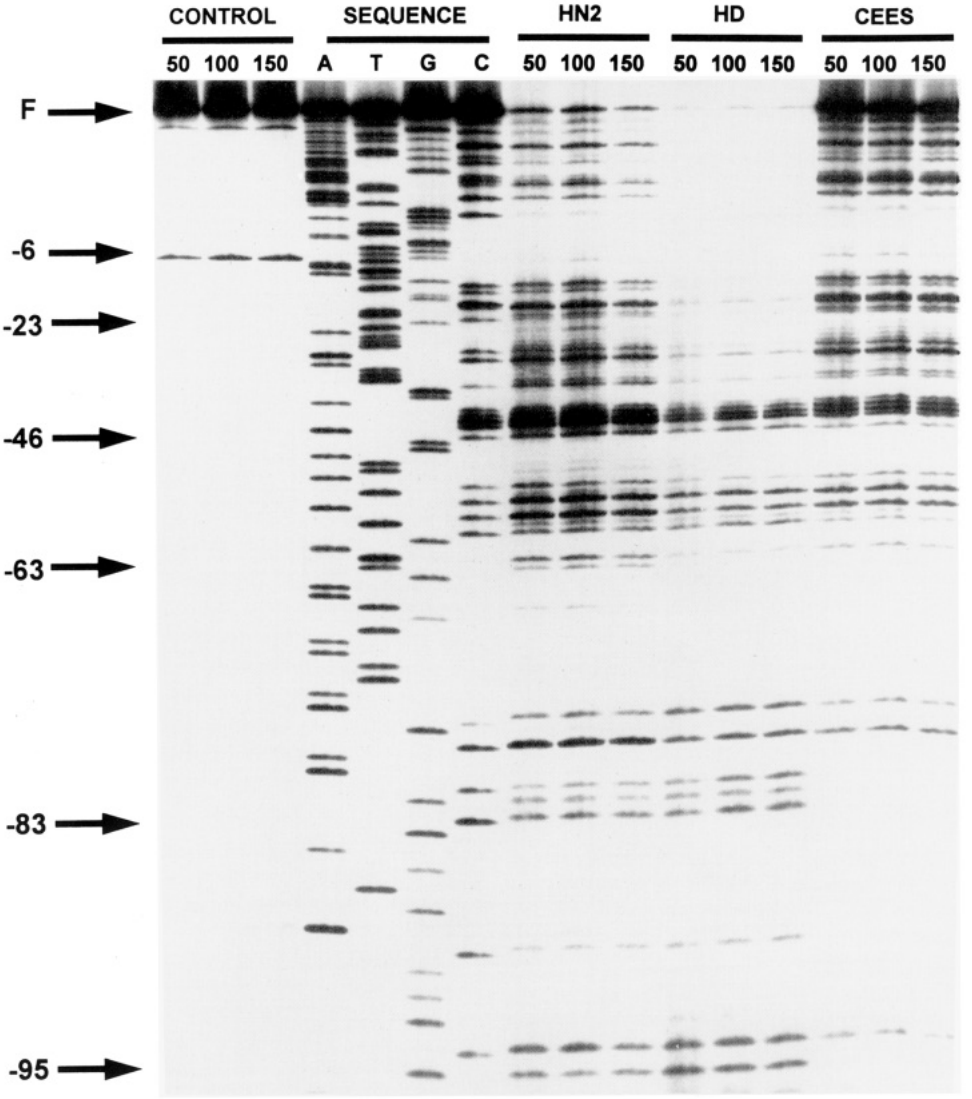

FIGURE 2: (a, top) Effect of alkylation on the processivity of *Taq* polymerase. DNA was alkylated at 37 °C for 60 min with 200 μM nitrogen and sulfur mustards and then subjected to 30 cycles of denaturation, primer annealing, and extension. The lanes labeled A, T, G, and C are the sequencing lanes. The length of the newly synthesized DNA is numbered with respect to +1 (the start of RNA synthesis) of the promoter region. F denotes the full-length extension from the primer. (b, bottom) Mustard alkylation sites. The alkylation sites on the template (bold type) strand are indicated by ▲. The primer sequence is underlined. The regions of contact by *E. coli* RNA polymerase in the closed promoter state are known to reside between –50 and +20 (von Hippel *et al.*, 1984). The position of insertion of the labeled nucleotides is indicated by *.

Electrophoresis and Quantitation. After treatment of the DNA with alkylating agent, initiation, and elongation by RNA polymerase, 1.5 μL of 60% sucrose (containing 0.25% xylene cyanol and 0.25% bromophenol blue) was added to the reaction (total volume 15 μL). Samples (4 μL) were loaded onto a 5% [19:1 acrylamide/bisacrylamide] nondenaturing submarine gel in TBE buffer. The gels were prepared using a Bio-Rad subcell PAGE caster and contained 3 mM $MgCl_2$. Electrophoresis was carried out at 100 V for 3.5 h. The electrophoresis buffer was TBE and also contained 3 mM $MgCl_2$.

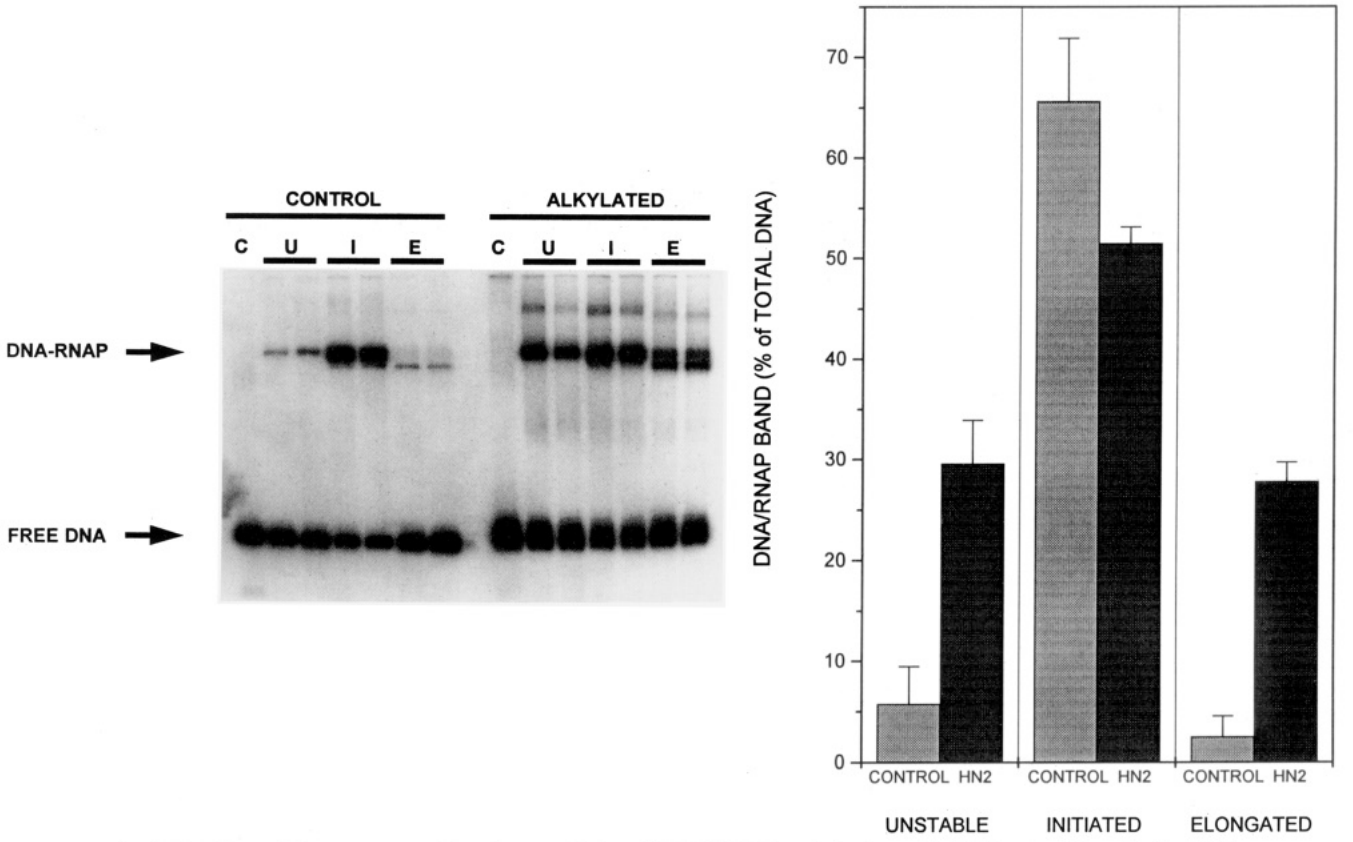

FIGURE 3: (a, left) Effect of nitrogen mustard on the association of *E. coli* RNA polymerase with DNA containing the *lac* UV5 promoter. Reactions were carried out in duplicate. U denotes the complex formed before the addition of the first nucleotide, I denotes the stable complex formed by the addition of the first 10 nucleotides, and E denotes those reactions in which sufficient nucleotides were available to allow complete transcription. (b, right) Quantitation of the DNA–RNA polymerase bands of a. The results shown are the mean and standard deviation of duplicate measurements from a minimum of three independent alkylation and retardation experiments.

Primer extension samples were loaded onto a 38 × 50 × 0.4 cm 12% denaturing gel [19:1 acrylamide/bisacrylamide] containing 7.5 M urea. Electrophoresis was then continued at 2000 V until the xylene cyanol had moved 25 cm down the gel. The electrophoresis buffer was TBE. After electrophoresis, the gels were dried and exposed to either XAR-5 X-ray film or a Molecular Dynamics phosphor storage screen. Sequencing gels were washed for 20 min in an aqueous solution of 10% methanol and 10% acetic acid prior to drying. Densitometric analysis was performed using a Molecular Dynamics Model 400B PhosphorImager.

RESULTS

The experimental approach used to investigate the effects of the mustards on the interaction between the *lac* UV5 promoter and *E. coli* RNA polymerase entailed two stages. The first stage was the determination of the location of the alkylation sites within the promoter using primer extension analysis, and the second stage was the quantitation of the interaction between the promoter and enzyme using gel retardation.

Alkylation of the Promoter Region. The DNA fragment containing the *lac* UV5 promoter was alkylated for 60 min at 37 °C using 200 μM nitrogen mustard, sulfur mustard, or CEES. Figure 2a shows the effect of the alkylation on the progress of the *Taq* DNA polymerase. The assay allows excellent resolution of a region of 130 bp encompassing the promoter region. Primer extension was carried out using nucleotide concentrations of 50, 100, and 150 μM. This procedure was followed as a control measure to examine whether or not high levels of nucleotides would allow the polymerase to bypass alkylation sites. There was no significant difference in the amount of full-length product in either the control reactions or the alkylation reactions subjected to variations in nucleotide concentration. In the control lanes, a small reproducible band occurred at position –6. The intensity of this band was 5% of the full-length band. Significant differences were observed in both the amount of full-length product and the total bands observed for each alkylating agent. The relative amounts of full-length product were CEES > HN2 > HD in the ratio 115:5:1, and for the total bands the ratio was 28:18:1. The intensity of the bands resulting from interruption of the passage of the *Taq* polymerase varied between positions on the DNA, with only very weak bands observed at –23 and at position 10. HN2 alkylated the DNA fragment at every G in the template strand (i.e., corresponding to C in the sequencing lanes). The behavior of CEES was similar to that of HN2, with the exception that no pausing was observed in the region –83 to –95. For HD, the conditions employed allowed resolution of the sequence only up to position –23. However, within that region the alkylation pattern was identical to that obtained for nitrogen mustard. In addition, for all three compounds, the progress of the polymerase was terminated at two A residues at –62 and –63. Figure 2b shows the location of alkylation sites in the RNA polymerase template strand as revealed by primer extension analysis. Control experiments showed no effect of either the small amount of ethanol present in the sulfur mustard samples, incubation at 37 °C, or freezing the DNA followed by storage at –20 °C and a thawing step prior to analysis.

Formation of the DNA–RNA Polymerase Complex. Figure 3a shows a typical retardation gel. The experiments were

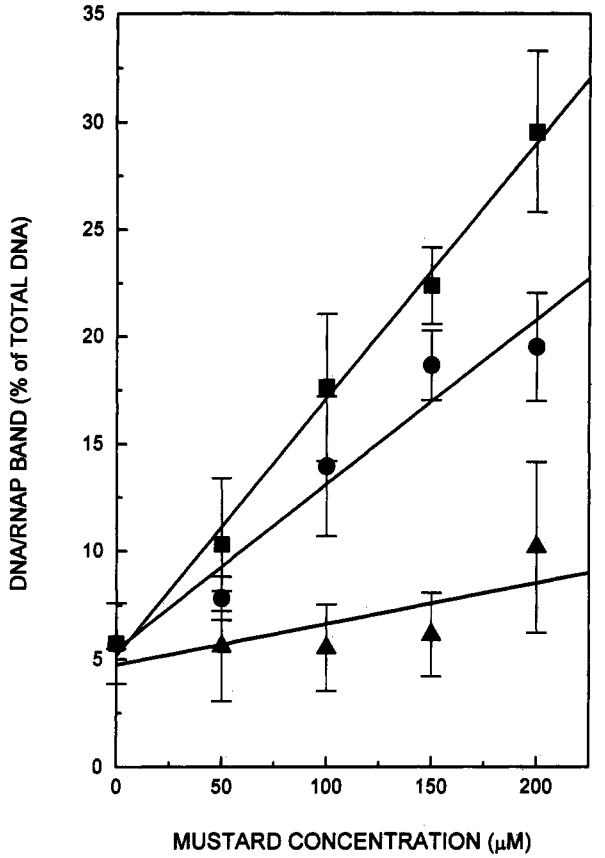

FIGURE 4: Quantitation of the uninitiated (unstable) DNA–RNA polymerase bands. The results are the mean and standard deviation of duplicate measurements from a minimum of three independent alkylation and retardation experiments: ■, HN2; ●, HD; ▲, CEES.

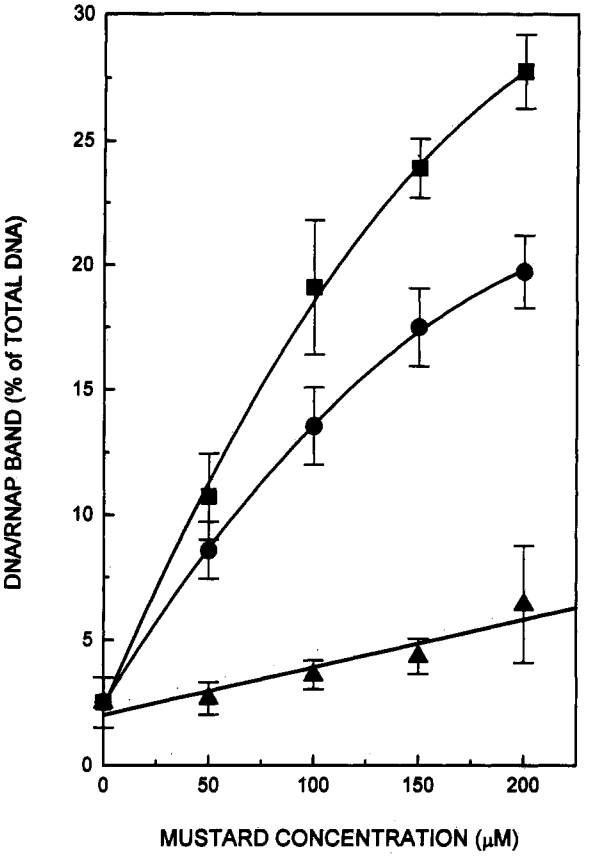

FIGURE 5: Quantitation of the elongated DNA–RNA polymerase bands. The results are the mean and standard deviation of duplicate measurements from a minimum of three independent alkylation and retardation experiments: ■, HN2; ●, HD; ▲, CEES.

performed in duplicate and show the effect of 200 μM nitrogen mustard on the formation of the DNA–RNA polymerase complex, as well as the initiation process and elongation of the initiated complex. The unstable complex shown in Figure 3a is the DNA–RNA polymerase complex formed prior to incorporation of the first nucleotide into nascent RNA. Under the experimental conditions used, this complex was unstable and the electrophoretic treatment almost completely separated the DNA and the RNA polymerase. The "initiated" lanes contained the complexes resulting from incorporation of the first 10 nucleotides (i.e., transcription up to the first C at position +10, as shown in Figure 2b) and represent the stable ternary complex. The "elongated" lane contained those samples to which high concentrations of all four nucleotides had been added.

Quantitative analysis of the bands in Figure 3a is shown in Figure 3b. In the absence of mustard treatment, the DNA–RNA polymerase band comprised approximately 5.7% of the total DNA. After stabilization by the addition of initiation nucleotides, this value rose to 66%, an 11.5-fold increase. Following elongation of this initiated complex, the amount of DNA remaining in the DNA–RNA polymerase complex fell to 2.5%. After alkylation by 200 μM nitrogen mustard, the DNA–RNA polymerase complex formed prior to initiation comprised 30% of the total, an increase by a factor of 5 compared to the non-drug-treated control. Upon initiation the amount rose to 52%, which was significantly less than the non-drug-treated control, and on elongation 28% of the DNA remained associated as a DNA–RNA polymerase complex (i.e., 10-fold more than in the comparable non-drug-treated control).

The effect of mustard type and concentration on formation of the preinitiated DNA–RNA polymerase complex is shown in Figure 4. All three alkylating agents caused a linear, concentration-dependent increase in the formation of this complex. The effect of the bifunctional agents was much more profound than that of the monofunctional CEES. For HN2, the amount of this complex increased by a factor of 5.2 as the mustard concentration was increased to 200 μM. For HD, the increase was by a factor of 3.4 and for CEES a factor of 1.9. These compounds also promoted a concentration-dependent increase in the amount of DNA–RNA polymerase complex remaining after the addition of high concentrations of elongation nucleotides (Figure 5). For HN2 and HD (200 μM), the amounts remaining in this form were 11 and 7.8 times the control values, respectively. In contrast, the monofunctional sulfur mustard, CEES, produced only a 2.6-fold-enhanced level compared to the non-drug-treated control value.

DISCUSSION

Promoter Alkylation. The DNA fragment chosen for these studies contains the complete *lac* UV5 promoter sequence and sufficient additional base pairs on either side of the promoter to allow the formation of a stable ternary complex (von Hippel *et al.*, 1984). The primer extension studies demonstrate that all G residues on the template strand of the promoter region are susceptible to alkylation by the mustards. Because there have been reports that prokaryotic DNA polymerases are able to bypass some DNA lesions at high nucleotide concentrations (Taylor & O'Day, 1990), this aspect was investigated with respect to drug-induced blockages detected by *Taq* polymerase. The observation in this study

that the blockages detected in the primer extension assay did not vary over the nucleotide concentration range of 50–150 μM suggests that the *Taq* polymerase is unable to bypass the alkylation sites and that they have been mapped accurately. However, the conclusion that the *Taq* polymerase is unable to bypass the alkylation sites assumes that the pause in the progress of the enzyme is due to the presence of intact alkylation sites. Repeated exposure to the 94 °C denaturation temperature may depurinate the alkylated bases and cause strand cleavage. Under these circumstances, the primer extension pattern would of necessity be independent of nucleotide concentration.

The three mustards showed considerable variation in their effects. The amount of full-length product produced on the template treated with monofunctional CEES was far greater than that produced with either of the bifunctional mustards. There are several possible contributions to this difference. Firstly, the half-life of CEES in aqueous solution containing NaCl is on the order of 3 min (Yang *et al.*, 1988), whereas the half-life of HD is approximately 6 min (Meier & Johnson, 1992). This may be expected to result in less alkylation by CEES. However, the full-length product in the CEES-treated reactions exceeds the product in the HD-treated reactions by a factor of 115, and this large difference is unlikely to be due to a difference in half-life of only a factor of 2. Secondly, the effect may be due to the inability of the monofunctional compound to produce interstrand cross-links. Interstrand cross-links are favored at 5′-GNC sequences (Millard *et al.*, 1990), and such sequences occur at positions −16, −37, −46, and −90. Interstrand cross-links would be expected to prevent completely the progress of the DNA polymerase. This explanation for the difference in the production of full-length product therefore implies that the DNA polymerase may not be halted permanently by the monofunctional adducts. Finally, there are several locations within the region to which the primer binds that could allow the formation of interstrand cross-links. Such cross-linking may prevent strand separation during the denaturing phase of the reaction cycle. The lower overall level of full-length product formation in the HN2- and HD-treated samples may reflect, therefore, interference with the formation of the DNA–primer complex. This explanation is supported by the observation that the total product (truncated plus full-length) is much greater for CEES than for the bifunctional alkylating agents. If the priming reaction was unaffected, similar amounts of total product would be expected.

Promoter–RNA Polymerase Interaction. Under the experimental conditions used in this study, it is possible to separate the RNA polymerase from the template DNA unless this complex is first stabilized by the addition of the first few nucleotides. The gel retardation studies demonstrate that alkylation of DNA by both sulfur and nitrogen mustards increases the formation of a complex between DNA and RNA polymerase prior to initiation. Prior to addition of the first 8–12 nucleotides, the DNA–RNA polymerase complex is unstable (Gill *et al.*, 1990). The growth of the nascent RNA to a length of 10 or 11 nucleotides results in the production of a stable ternary complex (Straney & Crothers, 1985), and this is also shown by the control data in Figure 2b. Under the experimental conditions used, only a small amount of complex is preserved prior to the incorporation of 10 nucleotides into the nascent RNA. However, if the DNA is alkylated prior to exposure to the RNA polymerase, a much more stable complex results. There are several possible explanations for this increased ability to form a binary complex. In the first instance, the RNA polymerase may be covalently bound to the DNA by the bifunctional mustards. This hypothesis is supported by the data in Figures 3 and 4, which show a much greater effect of the bifunctional mustards compared with the monofunctional CEES. If this explanation is correct, the RNA polymerase may or may not be bound to the promoter region. There is a body of evidence which suggests that RNA polymerase may undergo one-dimensional diffusion along DNA in search of the promoter (von Hippel *et al.*, 1984; Singer & Wu, 1988). RNA polymerase may therefore contact and become covalently linked to a non-promoter site on the 188-bp fragment. Alternatively, the alkylation may distort the DNA structure and create an altered conformation which traps the polymerase. As an extension of this possibility, alkylation may result in increased stability of the open complex prior to incorporation of the first few nucleotides. Again, this event may not occur within the promoter. The RNA polymerase may move some distance along the DNA before it is stopped by a conformational barrier. This barrier may take the form of a cross-linked site which prevents strand separation. Whatever the reason for the formation of this complex, it is very stable and does not lapse either in the electrical field or in the presence of high concentrations of nucleotides. The close similarity between the amount of complex formed prior to initiation on the alkylated template and the amount remaining after elongation (Figure 3b) suggests that this complex is not transcriptionally functional.

The results reported here have interesting implications for our current understanding of the mechanism of action of compounds containing the chloroethyl moiety. Previous studies have shown that adducts produced by the alkylation of DNA by mustard are capable of terminating transcription at the site of alkylation. The observation made in this study that the complex formed between alkylated DNA and RNA polymerase is very stable suggests that the transcription termination observed *in vitro* may take place without separation of the enzyme and DNA. Therefore, alkylation, in addition to causing elongation of the transcript to cease, may act to sequester RNA polymerase molecules. The demonstration that alkylation by these compounds is also able to interfere with the interaction between DNA and the protein RNA polymerase suggests that the mustards may also interfere with the interaction between DNA and other DNA-binding proteins. The occurrence of such interference is supported by the work of Bonfanti *et al.* (1991), who demonstrated that replacement of guanine with O^6-methylguanine was able to inhibit the binding of transcription factors. Proteins, such as the transcription factor SP1, that bind to GC-rich regions (Wingender, 1990) may therefore be particularly sensitive to alkylation by mustards. Clearly, alteration of these types of interactions may have profound implications for the cell.

The primer extension analysis showed alkylation at all G residues in the template strand. However, as Pieper *et al.* (1989) demonstrated using SP6 and T7 RNA polymerases, termination does not occur at all alkylated G residues. Gray *et al.* (1991) also did not detect transcription termination at all G residues transcribed by *E. coli* RNA polymerase. It therefore seems possible that RNA polymerase from some organisms might be able to bypass some alkylation sites, particularly in monofunctional adducts. This is important for several reasons. Firstly, even if the RNA polymerase is able to bypass the adduct, it is not known whether the correct base is inserted into the RNA product. Secondly, since transcription termination is used to map the interaction sites of a variety of drugs of clinical importance (Phillips *et al.*,

1990), it is possible that the same process occurs in these circumstances (i.e., the RNA polymerase is able to bypass some adducts and therefore not reveal some adduct sites). Finally, given the possibility that bacterial and viral enzymes may bypass some adducts, the question that should be asked concerns how applicable these termination studies are to eukaryotic systems. Hartley *et al.* (1992) have demonstrated that the patterns of alkylation *in vivo* are qualitatively the same as the patterns observed using *in vitro* systems. However, eukaryotic enzymes may respond to these adducts in quite a different manner from prokaryotic and viral enzymes.

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