In Vitro Transcription Analysis of DNA Alkylation by Nitrogen Mustard†

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ABSTRACT: A synchronized in vitro transcription assay has been used to probe the sequence specificity of alkylation of DNA by nitrogen mustard. Transcriptional blockages were detected with use of a 497-base-pair *Pvu*II/*Sal*I restriction fragment of a modified pBR322 vector when initiation of transcription was commenced after the DNA had been alkylated but not if the initiated transcription complex was subjected to alkylation before the elongation phase. The intensity of transcriptional blockages increased with alkylation time and was maximal after 1.5 h at a mustard concentration of 200 μM. There was also evidence of alkylation of the promoter region with increasing mustard concentration. The transcriptional blockage pattern changed at some sites as elongation time was increased and three types of blockages were observed—partial transcription (one or two nucleotides) past an initial blockage site, delayed but normal transcription past some sites, and complete termination at most sites. Eight of the nine blockage sites detected were at G or GG sequences on the template strand, with an apparent specificity for 5′-CTGT sequences of the template strand. Seven of the nine sites were capable of inter- or intrastrand cross-links, including three possible G-G interstrand cross-links spanning an intervening base-pair. In the 103-bp segment probed by this procedure, transcriptional blockages were detected (with one exception) only at sites corresponding to G on the template strand where inter- or intrastrand cross-linking was possible but not for similar sequences on the non-template strand.

Nitrogen mustard, bis(2-chloroethyl)methylamine, and its derivatives are among the most useful clinical agents used in the management of lymphoma (Koeller & Murphy, 1989). The exact mechanism by which the nitrogen mustards exert their effects is yet to be established. However, ultrastructural studies of cells treated with sulfur mustard indicate that the first signs of damage are visible in the nucleus (Papirmiester et al., 1984; Petrali et al., 1990). Further evidence for a central role for DNA in the toxicity of these compounds is provided by the observation that resistant cells show increased activity of topoisomerase II, an enzyme thought to play a role in DNA repair (Tan et al., 1987). Furthermore, the enhancement of the lethality of nitrogen mustard by caffeine (Murnane et al., 1980; Roberts & Kotsake-Kovatsi, 1986) is thought to be due to the synthesis of DNA on a damaged template (Carr & Fox, 1982).

Nitrogen mustards alkylate DNA and RNA at guanine and adenine residues (Brookes & Lawley, 1960; Hemminki & Kallama, 1986; Shooter et al., 1971). The detailed consequences of alkylation of DNA are unknown; however, transcription of DNA alkylated by nitrogen mustard has been observed to produce RNA of reduced chain length (Kann & Kohn, 1972). The sequence specificity of the alkylation of DNA by these compounds has been investigated by use of piperidine-induced cleavage at N7 guanine alkylation sites (Mattes et al., 1986; Kohn et al., 1987). This technique has proved to be very useful in identifying alkylation of N7 guanine sites but is limited in that adducts at all other nucleotide sites are not observed (Somani & Babu, 1989). A technique that does not suffer from this limitation is the recently established in vitro transcription footprinting procedure (Phillips & Crothers, 1986; White & Phillips, 1988), which has yielded detailed sequence specificity of a range of DNA-binding drugs (Phillips et al., 1990). This procedure should therefore detect additional forms of DNA alkylation. In addition, elucidation of the effects of alkylation on RNA polymerase provides a more biologically relevant insight into the mechanism of action of these compounds since inhibition and termination of transcription can be detected at individual blockage sites under conditions of active transcription of the DNA. Pieper et al. (1989) and Pieper and Erickson (1990) have demonstrated, using a similar system, termination of transcription by several nitrogen mustard derivatives and mapped the dominant termination sites. However, their studies have raised a number of important questions about the precise relationship between the location of the lesion and the point at which transcription is terminated.

The aims of this study were to determine, with in vitro transcription, the transcription termination sites generated by nitrogen mustard on DNA, to establish the optimal conditions for identification of these sites, their sequence specificity, and the nature of the termination process, and to define the "rules" for interpreting alkylation-induced transcriptional blockages.

Materials and Methods

Materials. Nitrogen mustard [bis(2-chloroethyl)methylamine], HN2,[1] was purchased from Aldrich Chemical Co., Inc., *Escherichia coli* RNA polymerase, ribonuclease inhibitor (Human Placenta), ultrapure ribonucleotides, 3′-*O*-methylnucleoside triphosphates, guaninyl(3′–5′)adenosine (GpA), and BSA (RNase/DNase free) were obtained from Pharmacia. [α-^{32}P]ATP and X-ray film (Hyperfilm-β max) were obtained from Amersham. Heparin was purchased from Sigma. Urea, bisacrylamide, acrylamide, ammonium persulfate, TEMED, and dithiothreitol were obtained from Bio-Rad as electro-

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[1] Abbreviations: HN2, nitrogen mustard; TE, Tris–EDTA; DTT, dithiothreitol; TBE, Tris–borate–EDTA; bp, base-pair.

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phoresis 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. All other chemicals were analytical grade, and all solutions were prepared with the use of distilled, deionized, and filtered water from a Milli-Q four-stage water purification system (Millipore).

Transcription. A 497-base-pair *Pvu*II/*Sal*I restriction fragment of a modified pBR322 vector containing the *lac* UV5 promoter was used as the template for in vitro transcription with *E. coli* RNA polymerase as described by White and Phillips (1988). Nitrogen mustard, prepared immediately prior to use in TE buffer, pH 8.0, was added to an approximately 50 nM solution of the restriction fragment, also in TE buffer, and incubated at 37 °C. For studies of the effect of alkylation time on adduct formation, the reaction was terminated by freezing the reaction mixture with liquid nitrogen. After alkylation, 100 nM *E. coli* RNA polymerase in transcription buffer was added to form a stable binary complex (Phillips & Crothers, 1986; White & Phillips, 1988). The transcription buffer contained 40 mM Tris-HCl, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 mM DTT, 125 μg/mL BSA, and 1.6 units/μL RNase inhibitor. Heparin was added to a final concentration of 400 μg/mL to remove any nonspecifically bound RNA polymerase, and the mixture was incubated at 37 °C for 5 min. A stable ternary complex was then formed by adding a mixture of nucleotides containing GpA (final concentration 200 μM) and UTP, GTP, and ATP (final concentration 5 μM) and [α-^{32}P]ATP in transcription buffer and incubated for 5 min at 37 °C. The resulting initiated complex was elongated by the addition of a mixture of UTP, ATP, GTP, and CTP (2 mM final concentration) and KCl (400 mM final concentration). The high levels of nucleotides during elongation dilute the radioactive nucleotide to such an extent that subsequent incorporation is negligible. Therefore, the intensity of each transcript is a direct measure of the relative concentration of each RNA species present since labeled nucleotides were incorporated only into the initiated complex—all transcripts, irrespective of length, therefore contained the same amount of radioactivity. The elongation reaction was stopped with the addition of an equal volume of loading/termination buffer comprising 10 M urea, 10% sucrose, 40 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue in 2× TBE. Electrophoresis and autoradiography were carried out as described by White and Phillips (1988). Densitometric analysis was performed with use of a Molecular Dynamics 300A computing densitometer.

Results

Preliminary experiments showed that the transcription buffer itself inhibited the alkylation reaction. As a consequence, exposure of the initiated transcription complex to nitrogen mustard produced no transcriptional blockages, and only full-length transcripts were observed. However, exposure of the DNA to nitrogen mustard prior to formation of the initiated transcription complex did produce blockages, and this was the standard sequence adopted for all subsequent studies.

Reaction Time. In order to determine the rate of production of mustard-induced transcription blockages, 200 μM nitrogen mustard was reacted with the DNA before transcription was initiated. Figure 1 shows the effect of this exposure on the transcription of the 497-bp fragment. The control elongation lane shows minimal pausing of transcription compared to the intensity of the band corresponding to the full-length transcript. Exposure of the DNA to nitrogen mustard for increasing periods of time resulted in decreasing amounts of full-length

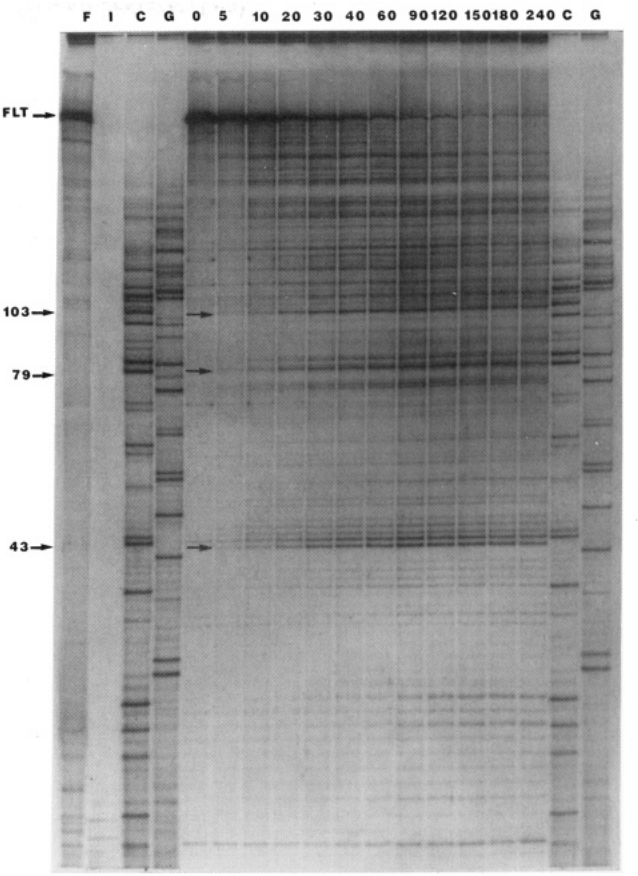

FIGURE 1: Effect of reaction time. The autoradiogram shows the dependence of the appearance of transcriptional blockages on the reaction time at 37 °C of nitrogen mustard with the 497-bp *lac* UV5 containing DNA fragment. Nitrogen mustard (final concentration 200 μM) was added to an approximately 50 nM solution of the DNA fragment (final volume 30 μL) and the mixture was incubated at 37 °C for 0–4 h. At the appropriate time, a 2.0-μL aliquot was removed and frozen instantly in liquid nitrogen. The samples were then thawed, and 6.0 μL of a solution containing RNA polymerase in the transcription buffer was added. Transcription was then carried out as described in the Materials and Methods section. Lane F is the full-length transcript and represents elongation carried out in the absence of HN2. Lane I is the initiated complex in the absence of HN2 and lanes C and G are sequencing lanes using termination by 3′-*O*-methyl-CTP and 3′-*O*-methyl-GTP, respectively. The lengths of several of the major blocked transcripts are shown on the left-hand side of the autoradiogram.

transcript and increasing amounts of shorter length transcripts. This is evident by the appearance of bands corresponding to the shorter length RNA after approximately 5 min and their increase in intensity up to 90 min. The effectiveness of freezing with liquid nitrogen in halting the alkylation reaction is shown by the absence of any significant blockages in the zero time lane and the large amount of full-length transcript at that time.

The kinetics of the decrease in the amount of full-length transcript is shown in Figure 2 together with the increase in the intensity of the five bands in the vicinity of position 43. The full-length transcript shows a continuous decay in intensity over the 4-h alkylation reaction time, reducing to approximately 8% of the zero reaction time value. The intensity of the 43–47-mer region increased continuously for 90 min followed by a small decrease over the next 150 min. For convenience, the alkylation time chosen for further studies was 60 min.

Concentration Dependence. The effect of nitrogen mustard on the transcription system was also concentration dependent (Figure 3). As the mustard concentration was increased from 0 to 500 μM (1-h reaction time), there was a decrease in the

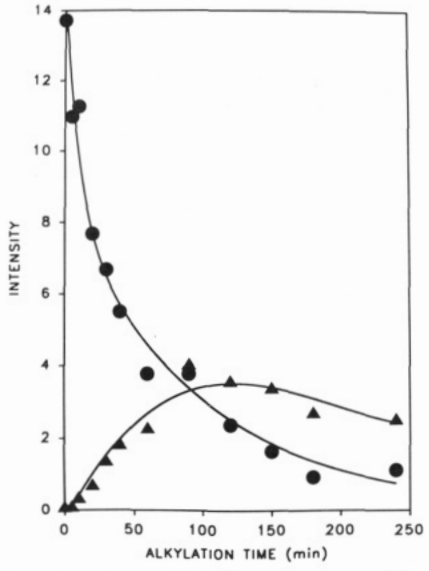

FIGURE 2: Time dependence of blockage formation. The density of the bands corresponding to the full-length transcript (●) and the group of five bands in the vicinity of the 43-mer (▲) are shown as a function of reaction time.

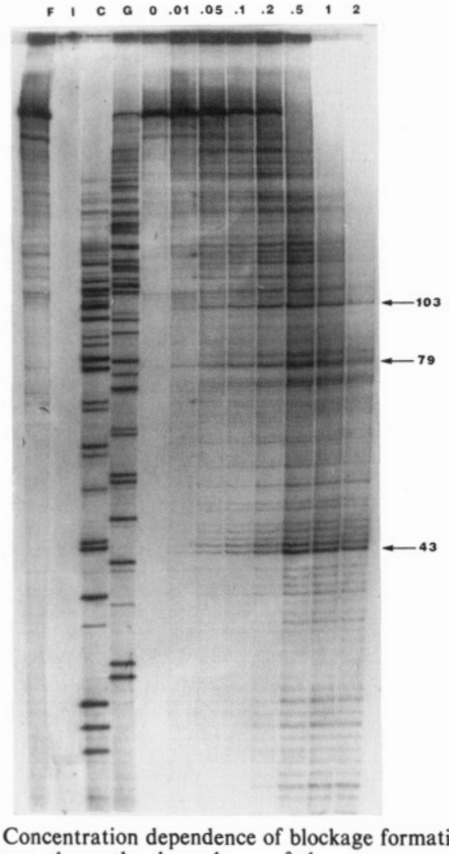

FIGURE 3: Concentration dependence of blockage formation. The autoradiogram shows the dependence of the appearance of transcriptional blockages on the nitrogen mustard concentration. HN2 was added to the DNA fragment (final volume 3 μL) and incubated for 1 h at 37 °C. Initiation and elongation were then carried out as for Figure 1. The nitrogen mustard concentrations ranged from 0 to 2 mM. Lane F is the full-length transcript and lane I is the initiated complex. Lanes C and G are sequencing lanes using termination by 3′-*O*-methyl-CTP and 3′-*O*-methyl-GTP, respectively. The lengths of several of the major blocked transcripts are shown on the right-hand side of the autoradiogram.

amount of full-length transcript produced and a corresponding increase in shorter length transcripts. Further increases in the mustard concentration diminished the yield of all of the blocked transcripts. Maximal amounts of blocked 43-mers, 79-mers, and 103-mers were observed at 500 μM mustard.

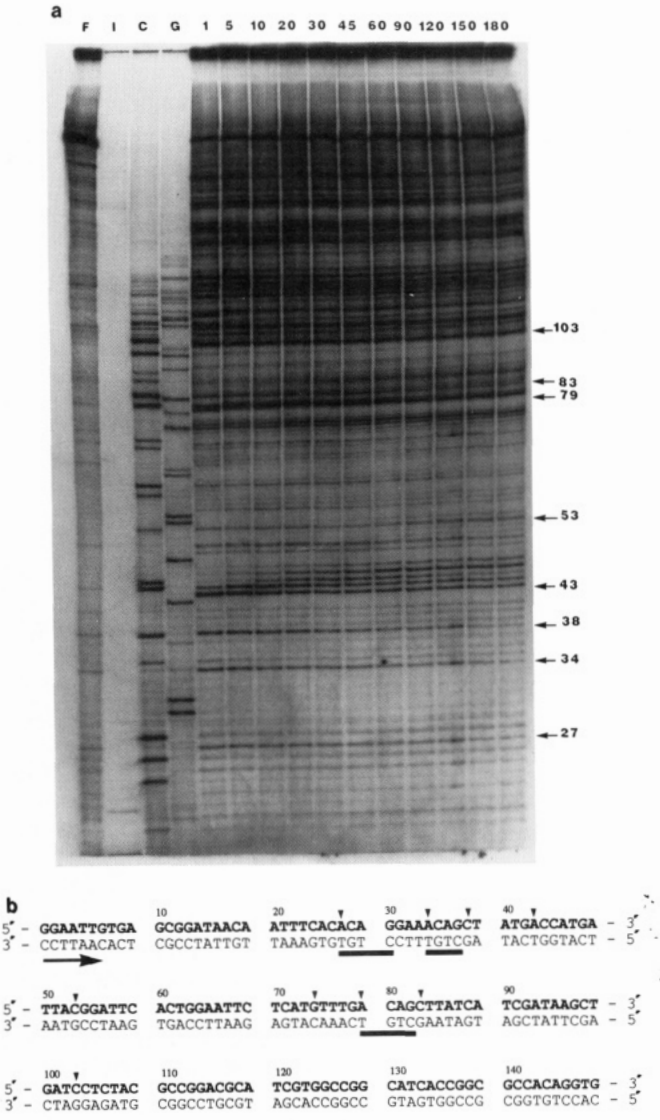

FIGURE 4: (a) Effect of elongation time. The autoradiogram shows the dependence of the appearance of transcriptional blockages on the elongation time. Nitrogen mustard (final concentration 200 μM) was reacted with the DNA fragment for 1 h at 37 °C (final volume 10 μL). Initiation was carried out as described for Figure 1. At the appropriate elongation time 7.5-μL aliquots were removed and added to the same volume of loading/termination buffer. The elongation time ranged from 1 to 180 min. Lane F is the full-length transcript, and lane I is the initiated complex. Lanes C and G are sequencing lanes using termination by 3′-*O*-methyl-CTP and 3′-*O*-methyl-GTP, respectively. The lengths of several of the major blocked transcripts are shown on the right-hand side of the autoradiogram. The reaction conditions for the left four lanes are as described in the legend to Figure 1. (b) Transcriptional blockage sites. Arrows above the non-template strand of the 497-bp fragment indicate the position on the RNA at which transcription is terminated by nitrogen mustard-induced transcriptional blockages after 1 min of elongation (panel a). Locations of 5′-CTGT sequences in the template strand are underlined. The large arrow indicates the direction of transcription, with the non-template strand shown in bold lettering. The numbers indicate the length of the RNA beginning from the G of GpA.

Effect of Elongation Time. The transcription system employed yields a synchronized population of initiated transcripts that are mainly 10-mers (Phillips & Crothers, 1986; Phillips et al., 1990). The rate of movement of the RNA polymerase can therefore be measured from the rate of elongation of the initiated transcripts. Figure 4a shows mustard-induced transcriptional blockages that exhibit two types of effects with increasing elongation time. At some sites, transcription is able

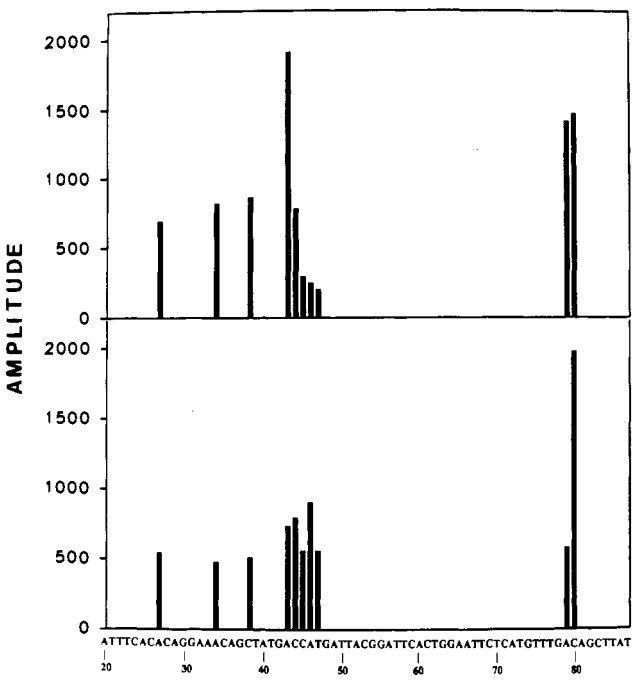

FIGURE 5: Quantitation of the blocked transcripts. The mole fraction of the RNA transcripts is shown for the major blockages together with the sequence of the transcribed RNA. The upper diagram shows the intensity of the major bands at an elongation time of 5 min. The intensity of the same bands after elongation for 120 min is shown in the lower diagram. The numbering represents the length of each transcript, beginning from the G of GpA.

to continue past the initial blockage, as if the blockage is removed or bypassed—this is clearly shown for the blockages close to the promoter (27-mer, 34-mer, 38-mer). At other points, the RNA polymerase was able to transcribe several additional nucleotides with increasing elongation time, as seen in the vicinity of transcripts initially blocked at the 43–44-mers. Figure 5 shows the quantitation of the major blocked transcripts at elongation times of 5 and 120 min and shows clearly the forward movement of the RNA polymerase several nucleotides in the vicinity of the 43-mer and one nucleotide in the vicinity of the 79-mer.

Sequence Dependence. The autoradiogram (Figure 4a) allows the identification of nine major blockage sites, and these are summarized in Figure 4b. Four of these sites occur at a T preceding a G (27-mer, 34-mer, and 79-mer) or a GG sequence (43-mer) in the template strand (i.e., reading in the 3′–5′ direction). Four of the other five occur at adjacent G·C bp (38-mer, 53-mer, 83-mer, and 103-mer) with at least one of the G residues in the template strand. Only one significant blockage was located at a lone G (74-mer) on the non-template strand. The exact site of the blockages in the vicinity of the 43-mer is uncertain because of the progression of the enzyme with increasing elongation time.

Discussion

Reaction Conditions. This study demonstrates the feasibility and simplicity of examining the effect of nitrogen mustard on the termination of transcription without the necessity of a DNA purification step between the alkylation and transcription processes. Furthermore, since the half-life of the initiated complex at 37 °C is 23 h (Cullinane & Phillips, 1990), dissociation of the initiated complex is small (<10% over 2 h) and permits studies of the time course of movement of RNA polymerase at individual alkylation sites. The dramatic decrease of elongated transcripts with increasing mustard concentration is presumably due to increasing alkylation of the promoter region prior to subsequent initiation with RNA polymerase. This is to be expected since there are seven G residues that could serve as potential alkylation sites on the template strand in the major contact regions (−42 to −24 and −18 to +3) of the *lac* UV5 promoter (von Hippel et al., 1984). This effect may also explain the decay of the 43–47-mer blocked transcripts (Figures 1 and 2) at long alkylation times. On the basis of this effect, exposure of the promoter region to this alkylating agent would result in total inhibition of initiation of transcription in a period of 10–20 h under the present conditions. An additional effect may also arise from the reaction of mustard directly with the RNA polymerase at high mustard concentrations. This apparent role of nitrogen mustard in alkylating RNA polymerase is now the subject of a separate investigation.

Termination Sites. The location of the mustard-induced transcription termination sites is consistent with the expected location of the most damaging types of alkylation. Eight of the nine sites discussed are located at or immediately prior to a G or GG sequence in the template strand. These eight sites may be divided into two main groups. Five of these locations (38-mer, 43-mer, 53-mer, 83-mer, 103-mer) allow the formation of either GG interstrand of GG intrastrand cross-links between adjacent G·C bp (Figure 4b). The remaining three sites (27-mer, 34-mer, and 79-mer) are at lone G residues in the template strand but always occur in the sequence 5′-CTGT (Figure 4b). The termination is at the T preceding (i.e., transcribed prior to) the G in all three cases. This sequence has potential for forming a GG interstrand cross-link spanning the intervening base-pair (Figure 4b). At the mustard concentration and alkylation time used, alkylation of most of the G residues would be expected to occur (Pieper et al., 1989). However, except for one site (74-mer), the RNA polymerase did not detect such adducts on the non-template strand, and this is most evident by the complete absence of transcriptional blockages at adjacent G's on the non-template strand at positions +30 and +63 (Figures 1 and 3).

The transcriptional blockages all occurred at one bp prior to the G on the template strand. The transcriptional blockages were at the G of a G·C bp (G on the template strand) in four instances (38-mer, 53-mer, 74-mer, 83-mer). Blockages occurred one bp prior to a G·C bp in all four cases where there were 3′-TG sequences on the template strand (27-mer, 34-mer, 43-mer, 79-mer). This suggests that T prior to an alkylated G (template strand) results in termination of transcription at the T preceding the alkylation site. These results are consistent with previous observations that small molecules result in transcriptional blockages at, or one nucleotide prior to, drug-binding sites [White & Phillips, 1988; 1989; Cullinane & Phillips, 1990].

The series of elongation time-dependent transcriptional blockages beginning at the 43-mer and extending to a 47-mer is puzzling (Figure 4). The 43-, 44-, and 45-mers are presumably due to alkylation of G at 44 and 45, resulting in dominantly 43- and 44-mer blocked transcripts at early elongation times. In this instance the polymerase is able to "read through", past these apparent sites of alkylation, to yield blockages at 46 and 47. The reason for this behavior may be associated with the formation of an intrastrand cross-link between adjacent G's at 44 and 45. A complete understanding of this phenomenon is likely to emerge from bidirectional transcription footprinting studies (White & Phillips, 1988) currently under way.

The transcription termination studies of Pieper et al. (1989) and Pieper and Erickson (1990) involved a DNA purification

step between alkylation of the DNA by nitrogen mustards and transcription. In the transcription system described here, we have shown that transcription proceeds in the presence of quite high levels of HN2, thus obviating the need for the purification step. This is probably due to the scavenging of free mustard by the components of the transcription buffer. In addition, use of a synchronized transcription system has provided information of the nature of the inhibition kinetics of RNA polymerase at the blockage site. The current study also shows blockage at isolated G's of the template strand (27-mer, 34-mer, 79-mer), in contrast to the results of Pieper et al. (1989) and Pieper and Erickson (1990), where blockages were observed only at multiple G's of the template strand. This difference may be due to different sensitivities of the different RNA polymerases employed in the two systems, the higher mustard concentration used in our study, or the different sequences within which the G residues are embedded. This latter explanation is supported by the demonstration by Mattes et al. (1986) that alkylation depends on the electronegativity of the environment of the alkylated nucleotide. In addition, blockages detected at isolated G residues all occurred in the same sequence (5'-CTGT of the template strand) and were all high-intensity regions of transcriptional blockage. Since this sequence was not present in the sequences probed by Pieper et al. (1989) or Pieper and Erickson (1990), it is not yet possible to establish whether this sequence, or adjacent G's on the template strand, provides the greater determinant or specificity for the alkylation of DNA by nitrogen mustard.

Several questions remain to be answered about the nature of the transcriptional blockage produced by the alkylation of DNA by compounds containing the chloroethyl group. The use of monofunctional alkylating agents and bidirectional transcription footprinting (White & Phillips, 1989; Cullinane & Phillips, 1990) will help answer the question of the relative importance of mono- and difunctional alkylations in terminating transcription. In addition, once a greater range of nucleotide sequences has been probed, the exact nature of transcription-terminating lesions will be better defined. The full characterization of these blockage sites by the present in vitro transcriptional studies will therefore pave the way for the analysis of such adducts in vivo.

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