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In Vitro Transcription Termination by *N,N'*-Bis(2-chloroethyl)-*N*-nitrosourea-Induced DNA Lesions

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SUMMARY

N,N'-Bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and its derivatives are chemotherapeutic DNA-damaging agents that generate a variety of monoadducts, intrastrand cross-links, and interstrand cross-links. The cytotoxic potential of the compounds has been linked to their ability to form DNA interstrand cross-links, which presumably inhibit subsequent DNA replication. To address the possibility that BCNU-induced lesions may also influence other DNA-directed actions such as transcription, and to identify the DNA lesions involved, a synthetic DNA template containing phage RNA polymerase promoters at both ends was incubated with BCNU and, after drug removal, transcribed *in vitro*. For comparison, similar studies were carried out with *cis*-diamminedichloroplatinum(II) and *trans*-diamminedichloroplatinum(II), which are known to induce defined transcription-terminating lesions. The results suggest that BCNU, like platinum compounds, can induce lesions resulting in termination of transcription *in vitro*, although the predominant transcription-terminating lesions, unlike those produced by *cis*-diamminedichloroplatinum(II), most likely represent interstrand DNA cross-links.

BCNU and its derivatives are clinically useful cancer chemotherapeutic agents. The cytotoxic potential of these compounds has long been associated with their ability to damage DNA and interfere with subsequent normal DNA-directed cellular processes (1). Of particular importance has been the ability of these compounds to form DNA lesions that block DNA replication. Such lesions have historically been considered to be double-stranded in nature, and indeed the ability of the compounds to generate interstrand DNA cross-links has been associated with the cytotoxic potential of the agents (2–4). The biological activity of these compounds might not, however, be explained solely by interstrand cross-link-mediated inhibition of DNA synthesis. Other BCNU-induced lesions may have biological significance, as suggested by studies indicating that BCNU generates single-stranded lesions, presumably guanine-guanine DNA intrastrand cross-links, that also terminate DNA replication *in vitro* (5). BCNU-induced lesions may also exert effects on DNA-directed processes other than DNA replication. The recent linkage of the processes of RNA transcription and DNA repair and the demonstration that lesion-induced stalling of RNA polymerase on a DNA template triggers the excision repair process in actively transcribed genes (6, 7) suggest that the effects of DNA-damaging agents on the process of transcription elongation may also have biological significance. The ability of a lesion to block RNA synthesis, as well as its ability to block DNA synthesis, not only may determine the immediate toxic potential of the damage but also may determine the duration of the lesion and the ultimate toxic potential of the damage. A number of chemotherapeutic agents, including Melphalan, nitrogen mustard (8, 9), *cis*-DDP (10), and *trans*-DDP (11), have been demonstrated to generate lesions that terminate transcription *in vitro*. In most cases these lesions included intrastrand as well as interstrand DNA cross-links. Although chloroethylnitrosoureas have been demonstrated to inhibit RNA synthesis, it remains unclear whether BCNU generates lesions that terminate transcription and what the nature of these lesions might be.

To assess whether BCNU can produce DNA lesions that block RNA polymerases and to determine the nature of these lesions, a synthetic DNA template containing phage RNA polymerase promoters at both ends was incubated with BCNU and, after drug removal, transcribed *in vitro*. Comparison of transcription termination sites identified on one strand of a given DNA region with those on the other strand of the same region then allowed for characterization of the lesions as single-stand or double-strand associated. For comparison, similar studies were carried out with *cis*-DDP and *trans*-DDP, which are known to induce transcription-terminating lesions (10, 11). The results suggest that BCNU, like platinum compounds, can induce lesions resulting in termination of transcription *in vitro*. The predominant transcription-terminating lesions, however,

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ABBREVIATIONS: BCNU, *N,N'*-bis(2-chloroethyl)-*N*-nitrosourea; DDP, diamminedichloroplatinum(II); PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

unlike those produced by *cis*-DDP, appear to be double-stranded in nature and in most cases likely represent inter-strand DNA cross-links.

Materials and Methods

Drugs. BCNU was obtained from the Drug Development Branch of the National Cancer Institute. *cis*- and *trans*-DDP (Sigma Chemical Co.) were prepared as 25 μM, 50 μM, 100 μM, or 500 μM stock solutions in 0.5× PBS (1× PBS is 150 mM NaCl, 16 mM Na_2HPO_4, pH 7.3). *trans*-DDP solutions were sonicated (2.5 min in 15-sec bursts, Virsonic cell disruptor, setting 35) to aid dissolution.

Template preparation. After digestion with *Pvu*II (5 units/μg of DNA, 37°, 1 hr), pGEM-7Zf(+) DNA (1 ng; Promega) was added to a solution containing 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.01% gelatin, 200 μM levels each of dCTP, dGTP, dATP, and dTTP (Perkin-Elmer Cetus), 25 pmol each of two oligonucleotide primers complementary to the SP6 or T7 promoter regions of the plasmid, and 2.5 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus). The region of the plasmid bounded by the SP6 and T7 promoter regions was amplified for 25 cycles using PCR. Thermal cycling was accomplished in a heat block (DNA thermocycler; Perkin-Elmer Cetus) programmed to incubate the reaction at 95° for 30 sec, rapidly cool and incubate the reaction at 55° for 30 sec, and rapidly heat and incubate the reaction at 72° for 45 sec each cycle. An additional 5-min heat denaturation step at 95° was also included at the start of the 25 cycles. After amplification, the reaction mixture was extracted with phenol/chloroform and the PCR product was recovered by ethanol precipitation.

Drug treatment. For *cis*- and *trans*-DDP studies, the PCR DNA product (0.25 μg) was added to 0.5× PBS and drugs were added to a final concentration of 0, 25, 50, 100, or 500 μM (final volume, 50 μl). The mixtures were incubated for 1 hr at 37°. After incubation, drug was removed by passing the reaction through a Sephadex G-50 spin column that had been preequilibrated with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Nucleic acids were precipitated with ethanol, resuspended in TE buffer, and added to transcription reactions.

For BCNU studies, PCR product (0.25 ng) was added to BCNU buffer (80 mM NaCl, 40 mM sodium phosphate, pH 7.1, 0.1 mM EDTA) and BCNU (dissolved in ethanol) was added to a final concentration of 0, 25, 50, or 100 mM (final volume, 100 μl). The mixtures were incubated for 1 hr at 37° and the DNA was purified as described for platinum studies. Even the lowest drug concentration produced an average of >2 N^7-guanine adducts/template, as determined by modified Maxam-Gilbert sequencing of the template DNA (data not shown).

***In vitro* transcription.** Each 20-μl transcription reaction was composed of 1× transcription buffer (40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol), 1 unit of RNAsin (Promega), 500 μM levels each of ATP, CTP, GTP, and UTP, 10 μCi of [α-^{32}P]UTP (3000 Ci/mmol; NEN), 0.25 μg of control or drug-treated DNA template, and 6 units of SP6 or T7 RNA polymerase (Promega). Reactions were incubated at 37° for 60 min and extracted twice with phenol/chloroform. Nucleic acids were precipitated with ethanol, resuspended in TE buffer, and assayed for trichloroacetic acid-precipitable radioactivity.

RNA sequencing. Four transcription reactions containing control DNA were prepared as described above, except that each reaction contained less unlabeled UTP (38 μM), more labeled UTP (50 μCi of [α-^{32}P]UTP), and either 250 μM 3′-dCTP, 25 μM 3′-dUTP, 250 μM 3′-dGTP, or 250 μM 3′-dATP (Pharmacia).

Analysis of transcription reactions. The radiolabeled products of transcription reactions were analyzed on 6% denaturing polyacrylamide gels. Equal numbers of trichloroacetic acid-precipitable counts were analyzed for each transcription reaction on a given gel, although the numbers of counts analyzed on different gels varied from 1.5×10^4 to 8×10^4 cpm/lane. Before electrophoresis each sample was denatured by incubation at 65° for 2.5 min in an equal volume of loading buffer (89 mM Tris, 98 mM boric acid, 2 mM EDTA, 10 M urea, 0.01% xylene cyanol, 0.01% bromphenol blue). The samples were then rapidly chilled, loaded onto the gel, and electrophoresed (2 hr, 60 W). After electrophoresis the gel was dried and autoradiographed.

Results

To assess the ability of BCNU to generate DNA lesions that terminate transcription and to identify these lesions, a relatively simple *in vitro* transcription system was used (9). In this system either strand of a small fragment of a commercially available plasmid can be transcribed using bacteriophage RNA polymerases that recognize promoter sequences near the ends of the DNA fragment (Fig. 1). If the DNA fragment is first incubated with a DNA-damaging agent such as the chemotherapeutic compounds *cis*-DDP, *trans*-DDP, or BCNU, subsequent transcription of the DNA in the presence of [^{32}P]UTP generates full length radiolabeled RNA molecules as well as truncated radiolabeled RNA molecules. The size of the truncated RNAs is an indication of where, along the template, transcription was terminated. Transcription stop sites identified in drug-treated templates but not in control templates are indicative of the presence of drug-induced transcription-terminating lesions. The identity of these lesions can be inferred from the sequence of the DNA, as well as by comparison of sites of transcription termination in a given region in which both strands have been transcribed. Single-stranded lesions (primarily guanine and adenine monoadducts, guanine-guanine and adenine-guanine 1,2-intrastrand cross-links, and guanine-X-guanine 1,3-intrastrand cross-links for *cis*-DDP, guanine and adenine monoadducts and guanine-X-guanine 1,3-intrastrand cross-links for *trans*-DDP, and N^7-guanine and O^6-guanine monoadducts and presumably guanine-guanine 1,2-intrastrand cross-links for BCNU) would be expected, if they are able, to terminate

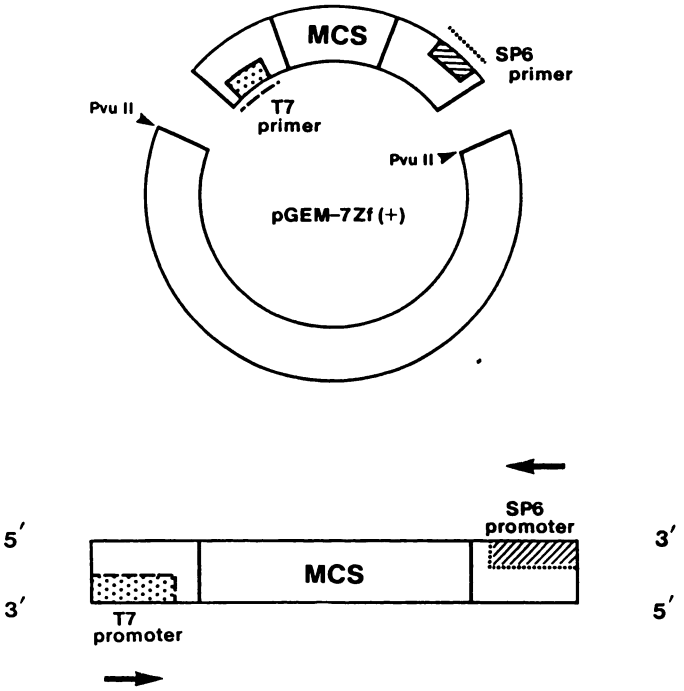


Fig. 1. DNA molecules used in transcription termination studies. The PCR-generated molecule allows for transcription of either DNA strand by T7 or SP6 RNA polymerase, in the directions indicated by the *arrows*. MCS, multiple cloning site.

transcription in only one DNA strand and not the other. Double-stranded lesions (N^7-guanine-N^7-guanine interstrand cross-links for *cis*-DDP, N^7-guanine-cytosine interstrand cross-links for *trans*-DDP, and N^1-guanine-N^3-cytosine interstrand cross-links for BCNU), however, would be expected to terminate transcription in both DNA strands at approximately the same location.

To verify the ability of the system to identify transcription-terminating lesions, initial experiments were performed with the cancer chemotherapeutic compounds *cis*- or *trans*-DDP, which are known to induce transcription-terminating lesions. In the present studies, both *cis*- and *trans*-DDP produced lesions that terminated SP6 transcription in a dose-dependent fashion across the exposure range of 25–500 μM (Fig. 2, A and B). The location of the lesions, however, was independent of the concentration of the drug used. *cis*-DDP produced transcription-terminating lesions not at single guanines in the template but rather at locations containing multiple guanines, adenine-guanine dinucleotides, or potential sites of interstrand N^7-guanine-N^7-guanine cross-linking (guanine-cytosine). *trans*-DDP also induced transcription-terminating lesions, although these lesions were less numerous and less well defined than those generated by *cis*-DDP. These lesions occurred at locations containing multiple guanines, guanine-X-guanine sequences, or sites of potential intrastrand cross-linking, rather than at single guanines. These results are consistent with previous studies (10, 11) and suggest that transcription-terminating lesions induced by platinum compounds are bifunctional in nature. A more exact definition of the platinum-induced transcription-terminating bifunctional lesions can be obtained by comparing sites of drug-induced lesions that terminate SP6-mediated transcription and those that terminate T7-mediated transcription (Fig. 2C). *cis*-DDP has been demonstrated to form interstrand cross-links at guanine-cytosine sequences (10, 12). Although the DNA template used in the present study contains numerous guanine-cytosine dinucleotides (e.g., nucleotides 22/23, 26/27, 74/75, 89/90, and 98/99), transcription was terminated in both strands at only one of these sites (nucleotides 89/90). Most transcription-terminating lesions induced by *cis*-DDP occurred at sequences (guanine-guanine, adenine-guanine, or guanine-X-guanine) consistent with known *cis*-DDP-induced intrastrand cross-links (10) (e.g., nucleotides 42/43, 78/79, and 85/86 for SP6 and nucleotides 53–55, 81–83, and 92–94 for T7).

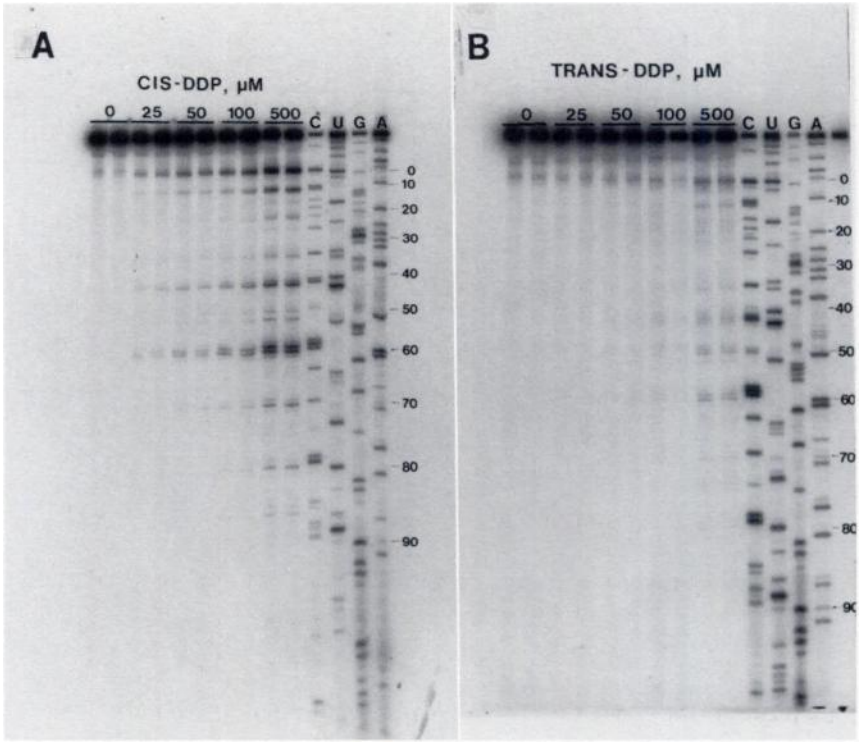
In contrast to bifunctional transcription-terminating adducts induced by *cis*-DDP, those induced by *trans*-DDP appear to be more frequently double-stranded in nature. *trans*-DDP has been demonstrated to form interstrand N^7-guanine-cytosine cross-links at cytosine-guanine sequences (11, 13), and transcription appeared to be terminated in both strands at several of these sequences in the DNA template studied (e.g., nucleotides 48, 63, 69, and 84). Single-stranded transcription-terminating lesions were also, however, noted at sequences consistent with known intrastrand bifunctional guanine-X-guanine lesions induced by *trans*-DDP (e.g., nucleotides 90–92 and 92–94 for T7). The distribution of *trans*-DDP-induced transcription-terminating lesions has not been reported, but the previously reported increased ability of *trans*- versus *cis*-DDP to form interstrand cross-links (11) and the known ability of *trans*-DDP-induced interstrand cross-links to terminate transcription are consistent with the present findings that transcription-terminating lesions induced by *trans*-DDP are more likely to be interstrand cross-links than those induced by *cis*-DDP. These studies suggest that the system used can differentiate between single-stranded and double-stranded transcription-terminating lesions and, given the spectrum of adducts produced by an agent, can narrow the identification of the lesion.

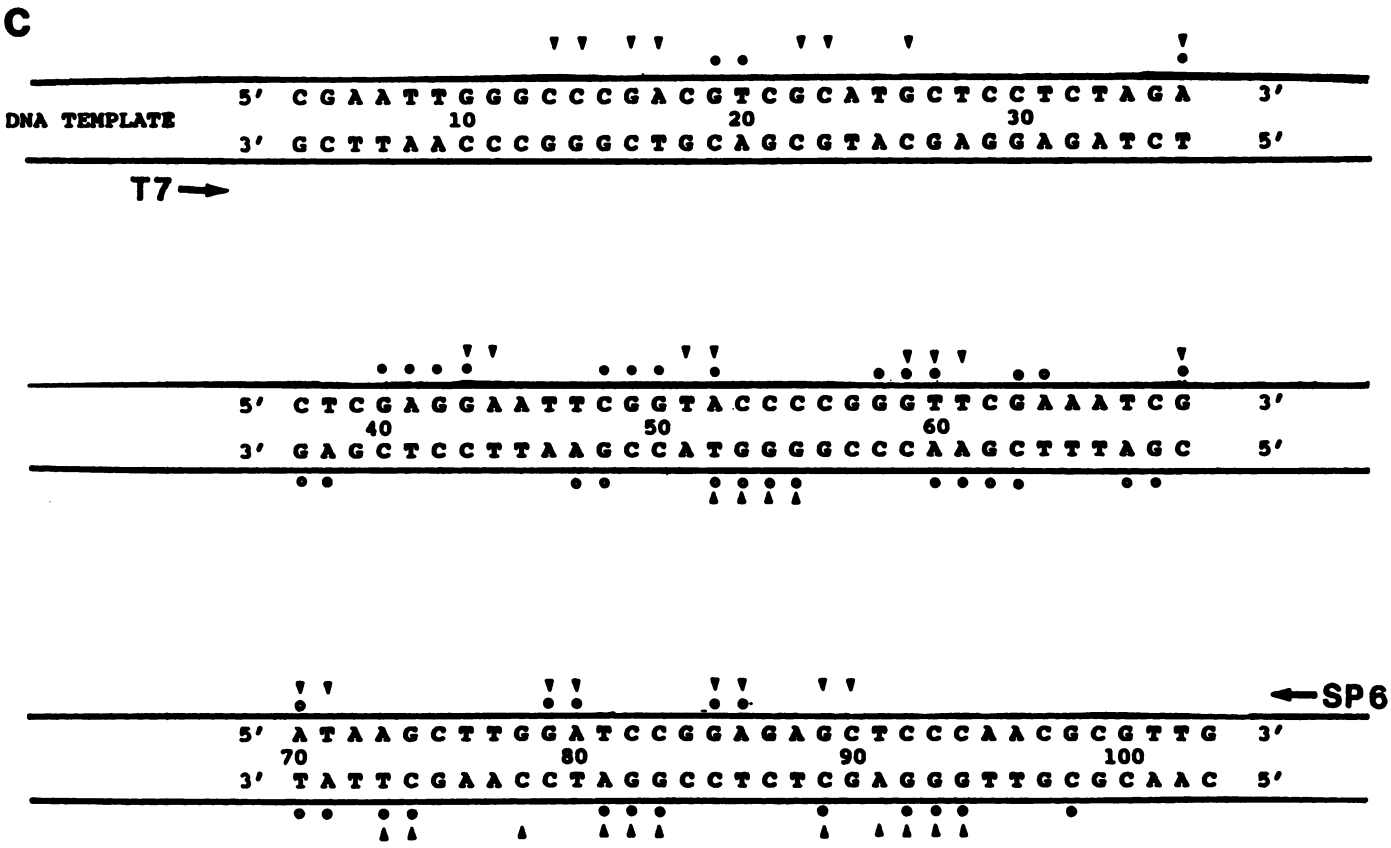
Studies similar to those performed with platinum compounds were also performed with BCNU. The results of these studies are presented in Fig. 3. BCNU produced lesions that terminated transcription by both SP6 and T7 RNA polymerases and did so in a weakly dose-dependent fashion across the exposure range of 25–100 mM. The location of the lesions, however, as with the platinum compounds, was independent of the concentration of drug used. BCNU-induced transcription-terminating lesions occurred at sites containing no guanines, one guanine, or multiple guanines. In distribution of sites of transcription termination, BCNU most resembled *trans*-DDP, although there were several sites of transcription termination unique to the two compounds. The nature of the BCNU-induced transcription-terminating lesions was more apparent in comparisons of the pattern of SP6 transcription termination with that of T7 transcription termination (Fig. 3C). In the majority of instances (seven of 11), BCNU-induced lesions that terminated transcription from one direction on one strand also terminated transcription from the other direction on the other strand (e.g., nucleotides 62/63, 68/69, 79/80, and 99/100). The sites of transcription termination were predominantly at guanine-cytosine dinucleotides, consistent with known sites of BCNU-induced interstrand cross-linking (14). These results suggest that, whereas *cis*-DDP, *trans*-DDP, and BCNU all induce transcription-terminating lesions predominantly at guanine-containing sequences, the nature of the lesions differs for each compound.

Discussion

The cytotoxic effects of DNA-damaging cancer chemotherapeutic agents have long been associated with the ability of these agents to damage DNA. Although much attention has been focused on DNA damage in the form of DNA replication-inhibiting interstrand cross-links, the recent appreciation of the linkage between DNA excision repair and transcription suggests that effects of DNA damage on other processes, most notably transcription, warrant examination. The present study suggests that BCNU, like other DNA-damaging cancer chemotherapeutic agents, induces lesions that terminate transcription *in vitro*.

BCNU induces a variety of DNA adducts, both monofunctional and bifunctional, that could inhibit RNA polymerases. In the present study, single guanines were not consistently found to be sites of transcription termination, despite the fact that the template contained an average of at least 2 N^7-guanine adducts/molecule. These results suggest that monofunctional lesions produced by BCNU (primarily N^7-guanine and O^6-guanine) are not inhibitory to RNA polymerases in this system. These results are consistent with those derived from studies of other N^7-guanine-alkylating agents [*cis*- and *trans*-DDP (10, 11), Melphalan, nitrogen mustard, and chlorambucil (8, 9)], which suggest that N^7-guanine monofunctional adducts, regardless of the causative drug, rarely terminate transcription. In addition to monofunctional lesions, BCNU has also been





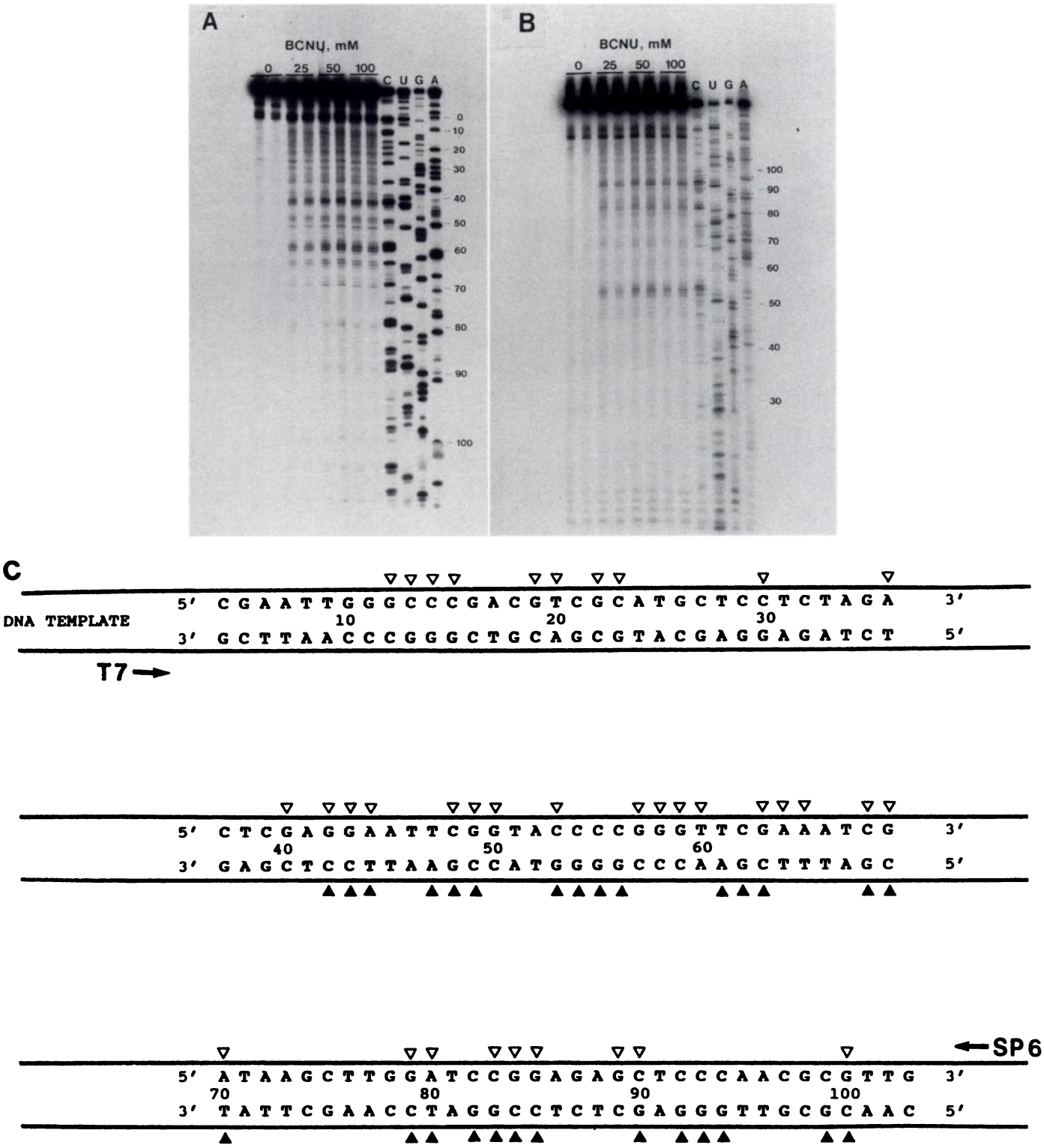


Fig. 3. Termination of RNA polymerase-mediated *in vitro* transcription by BCNU-induced DNA adducts. DNA template was incubated in duplicate with 0–100 mM BCNU. After drug removal, the DNA was transcribed *in vitro* in the presence of [^{32}P]UTP, using either SP6 or T7 RNA polymerase. Products of the transcription reactions were electrophoresed on denaturing polyacrylamide gels, next to the products of RNA sequencing reactions (*lanes C*, *U*, *G*, and *A*), and were autoradiographed. A and B, Autoradiographs of RNA products generated by SP6 (A) or T7 (B) transcription of drug-treated template. C, Complete listing of BCNU-induced sites of SP6 (▽) or T7 (▲) transcription termination. Numbering between DNA strands corresponds to that in A and B. Sites of transcription termination were determined between nucleotides 35 and 103 for T7 RNA polymerase and between nucleotides 10 and 103 for SP6 RNA polymerase. Autoradiographs are representative of three experiments.

demonstrated to form N^1-guanine-N^3-cytosine interstrand cross-links (14). The existence of guanine-guanine intrastrand cross-links has also been inferred from the identification of diguanylethane from BCNU-treated DNA (15). These bifunctional adducts appear to be relevant in BCNU-induced transcription termination, because most sites of termination in the present study involved either multiple guanines or GC/CG sequences. Differentiation between interstrand and intrastrand lesions was made possible by the comparison of transcription termination sites in one strand with those in the opposite strand. Although the presence of transcription-terminating lesions at multiple guanines suggests that BCNU-induced intrastrand guanine-guanine adducts may terminate transcription, a closer examination suggests that transcription termination at these multiple guanines usually involves both DNA strands. BCNU-induced lesions that terminate transcription therefore appear to be interstrand cross-links and to differ from those BCNU-induced intrastrand guanine-guanine lesions that block DNA polymerases in a similar *in vitro* system (5). Given, however, that there are sequences at which BCNU-induced transcription-terminating lesions are likely to be guanine-guanine intrastrand lesions (e.g., nucleotides 92–94), it appears likely that BCNU-induced guanine-guanine intrastrand lesions can block both DNA and RNA polymerases.

A comparison of results obtained from studies using BCNU-treated templates and those using *cis*- or *trans*-DDP-treated templates suggests that effects of various compounds on transcription depend on the initial adduct formed as well as on the final distribution of adducts. *cis*-DDP initially induces primarily guanine monoadducts (16) that, while having no effect on transcription by themselves, can subsequently form inter- or intrastrand transcription-terminating lesions. The identification of most *cis*-DDP-induced transcription-terminating lesions as intrastrand cross-links is consistent with the fact that 90% of all *cis*-DDP bifunctional adducts are intrastrand (10, 17). *trans*-DDP also initially forms non-transcription-terminating guanine monoadducts. The inability of these lesions to rearrange to form 1,2-intrastrand cross-links, along with the increased propensity of *trans*-DDP to form interstrand cross-links (11), is consistent with the finding that most *trans*-DDP-induced transcription-terminating lesions are interstrand cross-links. BCNU, unlike platinum compounds, induces two different types of guanine monoadducts (N^7- and O^6-guanine adducts) (2, 17). The N^7-guanine monoadduct can subsequently form N^7-guanine-N^7-guanine DNA intrastrand cross-links, whereas the O^6-guanine monoadduct rearranges to form N^3-cytosine-N^1-guanine interstrand cross-links. The finding that most BCNU transcription-terminating lesions are DNA interstrand cross-links at guanine-cytosine sequences suggests either that O^6-guanine lesions outnumber N^7-guanine lesions, that guanine-cytosine cross-links form more readily than guanine-guanine cross-links, or that guanine-cytosine interstrand cross-links are more effective at blocking RNA polymerases than are guanine-guanine cross-links. Given that N^7-guanine lesions have been demonstrated to be significantly more numerous than O^6-guanine lesions after BCNU exposure (2), the latter possibilities appear more likely.

The cytotoxic potential of BCNU- and platinum-induced transcription-terminating lesions remains uncertain. Blockade of RNA synthesis in genes critical for cell survival could be detrimental, although it should be noted that transcription-terminating lesions generated by the noncytotoxic agent *trans*-DDP must be of little consequence to cell viability. It may, however, be that the cellular response to transcription termination may influence cell viability. The identification of BCNU-induced transcription-terminating lesions as likely interstrand DNA cross-links suggests that BCNU-induced interstrand cross-links, which are considered to be cytotoxic, may also, by their ability to block RNA polymerase, trigger their own rapid repair in transcribed regions. Transcription-terminating lesions may thus be of biological consequence in their direct effects on RNA synthesis as well as their indirect effects on DNA repair. Although the present studies have used bacteriophage RNA polymerases and purified, histone-free DNA and may not perfectly mirror the transcription/repair systems noted *in vivo*, the results of these studies suggest that BCNU-induced effects other than those on DNA replication may be of biological significance.

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