Biochemistry

 Volume 31, Number 40 October 13, 1992

Articles

In Vitro Transcription Analysis of DNA Adducts Induced by Cyanomorpholinoadriamycin†

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Received April 14, 1992; Revised Manuscript Received June 30, 1992

ABSTRACT: The reaction of cyanomorpholinoadriamycin (CMA) with DNA results in the formation of sequence-specific complexes with DNA. These complexes were revealed as blocked transcripts in an in vitro transcription assay—of 14 high-intensity blockages detected in the 120 bp probed in this assay, 12 were prior to GpG or CpC sequences. Slow read-through past the first few sites exhibited first-order kinetics, with half-lives of 25–200 min. Bidirectional transcription footprinting revealed nine high-intensity sites, eight of which were defined by a GpG element (nontemplate strand). Reaction of CMA with single-strand DNA, followed by a primer-extension assay, revealed four major blockages all of which were at GpG sites on the initial single-strand DNA. From a combination of these three experimental approaches, it appears that CMA yields dominantly intrastrand cross-links between adjacent guanine residues. Since CMA is also known to form interstrand cross-links, these appear to occur at GpC sequences but are minor in comparison to the extent of formation of intrastrand cross-links.

Adriamycin, an anthracycline antibiotic, has been one of the most successful chemotherapeutic agents in clinical use since its discovery in 1969 (Myers et al., 1988). Its success is largely due to its effects against a wide spectrum of neoplasia from solid tumours to leukaemias (Jones, 1982). Use of adriamycin, however, is limited primarily by its cardiotoxicity, which imposes a total drug dose limit due to potential irreversible heart damage (Fu et al., 1990), while other serious limitations of adriamycin therapy include acquired drug resistance and myelosuppression (Dorr & Jones, 1982).

The limitations in the use of adriamycin have prompted research into the development of adriamycin derivatives with higher therapeutic value and fewer limiting side effects. Of the hundreds of derivatives produced so far, one of the most promising is 3′-(3-cyano-4-morpholinyl)-3′-deaminoadriamycin, as CMA[1] has been demonstrated to be up to 1400 times more potent than adriamycin in vitro (Wassermann et al., 1986) and up to 1600 times more potent in vivo (Acton et al., 1984).

CMA has an increased affinity for the cell in comparison to adriamycin. The increased lipophilicity of CMA may result in rapid cellular uptake while efflux of the drug is much slower than adriamycin, perhaps due to a decreased affinity for the P-glycoprotein pump (Wassermann et al., 1986; Scudder et al., 1988). Once in the cell, CMA rapidly migrates to the nucleus where it forms DNA interstrand cross-links, the production of which has been demonstrated to correlate closely with the cytotoxicity of the drug (Acton et al., 1988).

Recently we have examined the sequence specificity of the CMA binding by an in vitro transcription assay, which demonstrated the rapid formation of apparent intrastrand and interstrand cross-links through GpG and GpC sequences, respectively (Cullinane & Phillips, 1991). The present work has further investigated the formation of CMA-induced transcriptional blockages. The size of the drug binding site has been defined to the 2-bp GpG, GpC, and CpG sequences, and there is a markedly different ability of RNA polymerase to be terminated at each of these sites.

† This work was supported by the Australian Research Council (D.R.P.), the Anticancer Council of Victoria (D.R.P.), and a Macfarlane Burnet Postgraduate Schlorship (C.C.).

[1] Abbreviations: CMA, 3′-(3-cyano-4-morpholinyl)-3′-deaminoadriamycin or cyanomorpholinoadriamycin; bp, base pairs; DMF, *N,N*′-dimethylformamide.

0006-2960/92/0431-9513$03.00/0

MATERIALS AND METHODS

Materials

CMA was a gift from Dr. E. M. Acton (NCI, Washington). The drug was dissolved in DMF and stored in the dark at −20 °C.

Dinucleotides GpA and ApU, ribonucleotide triphosphates, and 3′-*O*-methoxyribonucleotides, RNAse inhibitor (human placenta), nuclease-free BSA, and nuclease-free *Escherichia coli* RNA polymerase were purchased from Pharmacia. Ultrapure-grade agarose, urea, 'Instapage' (40% solution 19:1 acrylamide–bisacrylamide) were obtained from IBI, while molecular biology grade ammonium persulfate and dithiothreitol were from Bio-Rad. Radionucleotides [α-^{32}P]UTP and [γ-^{32}P]ATP and X-ray film were purchased from Amersham. Restriction enzymes were obtained from Boehringer Mannheim, and NA45 paper was obtained from Schleicher and Schuell. All other chemicals were of analytical grade, and all solutions were prepared using type 1 water from a Milli-Q four-stage water purification system (Millipore, MA).

pRW1 and pRW2 were as described previously (Skorobogaty et al., 1988; White & Phillips, 1988). Single-strand DNA was M13mp18 supplied as a control template from a Sequenase version 2.0 sequencing kit (U.S. Biochemicals).

Methods

Unidirectional Transcription. A stable initiated transcription complex was formed as previously described (White & Phillips, 1988; Skorobogaty et al., 1988). This solution was divided into two aliquots—transcription buffer was added to one and 1 μM CMA (unless otherwise stated) in transcription buffer was added to the other and then incubated for 1 h. The transcripts were subsequently elongated and terminated as previously described (White & Phillips, 1988; Skorobogaty et al., 1988), unless otherwise stated.

Electrophoresis was as described previously (White & Phillips, 1988). Autoradiography was performed by the use of a Molecular Dynamics 400A PhosphorImager. Briefly, the dried gel was exposed to a storage phosphor screen overnight, the screen was then scanned and individual bands quantitated using the PhosphorImager.

Bidirectional Footprinting. The 315-bp fragment containing the counter-directed UV5 and N25 promoters was isolated from pRW2 as described previously (White & Phillips, 1989). Inactivation of one promoter was achieved by restriction digestion, and subsequent initiation of the desired promoter was as in White and Phillips (1989). The initiated complex was incubated with 2 μM CMA for 1 h before the transcripts were elongated; the reaction terminated and was subjected to electrophoresis and quantitation as described above.

Alkylation of Single-Strand DNA. Alkylation of single-strand DNA was carried out analogous to that of Pinto and Lippard (1985) for the platination of DNA. Single-strand M13mp18 DNA was incubated at 400 μM nucleotides with 5 and 10 μM CMA for 1 h in transcription buffer. The M13-40 primer was end-labeled using routine methods (Sambrook et al., 1989) prior to annealing to the drug-treated template. Extension of the labeled primer was achieved using Sequenase 2.0, with incubation of the reaction in the presence of 2 mM deoxynucleotides and 20 mM NaCl at 37 °C for 5 min prior to the addition of formamide termination buffer.

Reactions were subjected to extended electrophoresis on an 8% denaturing acrylamide gel and autoradiographed as described above.

RESULTS

Effect of Elongation Time. Initiated transcription complexes were exposed to 1 μM CMA for 60 min prior to elongation for up to 4 h. Figure 1 shows an autoradiogram representing CMA-induced blockages not evident in the control lanes, at elongation times from 1 min to 4 h.

In the 120-bp region resolved in this assay, 21 drug-induced blockages were identified after 1 min of elongation, and the relative intensity of these sites are shown in Figure 2. Of the 14 "high-intensity" sites, 12 are prior to GpG or CpC sequences of the nontemplate strand (sites 1, 3–5, 9, 11, 12, and 16–20), essentially the same as noted previously after 4 min of elongation (Cullinane & Phillips, 1991). Of the remaining well-resolved blockages, four appear to be prior to GpC sequences (sites 2, 6, 8, and 14) but are generally of only minor intensity compared to the 12 strong blockage sites.

The relative intensity of each blockage site as a function of elongation time is illustrated in Figure 1. Apart from site 1, the remaining blockages appear essentially constant for elongation times up to 1 h, as noted previously (Cullinane & Phillips, 1991). However, at longer elongation times (2–4 h) read-through past some sites is apparent (e.g., 1–5), and an increase of full-length transcript is also observed. The blocked transcripts were quantitated as the mole fraction relative to all transcripts in each lane and analyzed in terms of a first-order plot (Figure 3). Over the 4-h elongation time course it is apparent that each of blockage sites 1–3 decayed as a first-order process, with apparent half-lives of 25, 91, and 200 min, respectively. Because of the effect of read-through of RNA polymerase from one site to another, the accumulative effect is such that a rigorous statistical analysis is required to yield meaningful kinetic parameters for additional downstream drug sites (Phillips et al., 1990a).

Effect of Drug Concentration. Incubation of the initiated transcription complex with increasing concentrations of CMA (for 1 h in 5% DMF and transcription buffer) results in an increased inability of the RNA polymerase to progress through the templates (not shown). Drug-induced blockages were evident at concentrations as low as 0.5 μM CMA, and at 15 μM the extension of the polymerase was restricted to 60 nucleotides, with almost complete termination of transcription occurring at the first two major blockage sites.

The mole fraction of blocked transcripts is shown for sites 1–3 in Figure 4 at increasing CMA concentrations. Each shows a linear concentration dependence, plateauing upon saturation at that site. At low concentrations (e.g., <5 μM) the relative occupancy of these sites is $3 > 1 > 2$. However, at higher concentrations of CMA (or longer reaction times) the apparent order of occupancy can change as earlier sites become increasingly occupied and RNA polymerase becomes less likely to reach downstream drug sites—downstream drug sites therefore become increasingly underestimated as has been noted previously (Phillips et al., 1990b).

Bidirectional Transcription Footprinting. The transcriptional blockages induced by CMA (Figures 1, 2, and 5) indicate the site at which a drug is bound, thereby preventing progression of RNA polymerase past that site. This assay does not provide any indication as to the physical size of the blocking unit downstream of the actual block site itself (i.e., it does not distinguish between a drug blockage unit localized to one discrete nucleotide or whether the blockage unit physically occupies many bp).

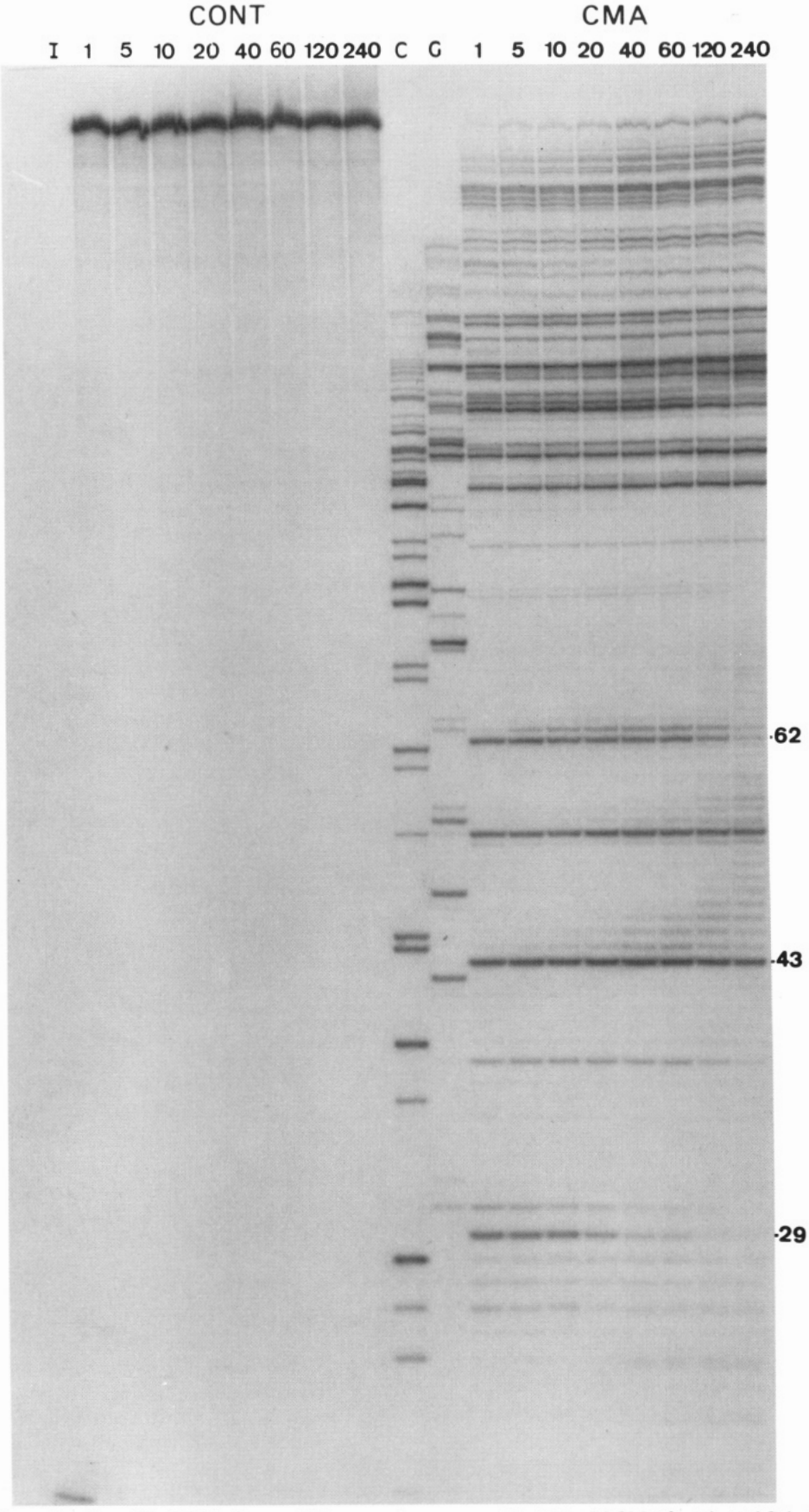

FIGURE 1: Effect of elongation time. The initiated transcription complex was reacted with 1 μM CMA for 1 h, 37 °C, in transcription buffer, pH 8.0, and then elongated for 1–240 min prior to separation of transcripts by sequencing gel electrophoresis. The lane representing the initiated transcript is shown as I, and the 3′-methoxy-CTP and 3′-methoxy-GTP sequencing lanes denoted as C and G. Control lanes of DNA not subjected to reaction with CMA, but elongated for 1–240 min, are denoted as CONT.

In order to establish the number of bp over which the CMA-induced blockages extend, a bidirectional transcription footprinting assay was employed (White & Phillips, 1989; Cullinane & Phillips, 1990). Selective initiation of each

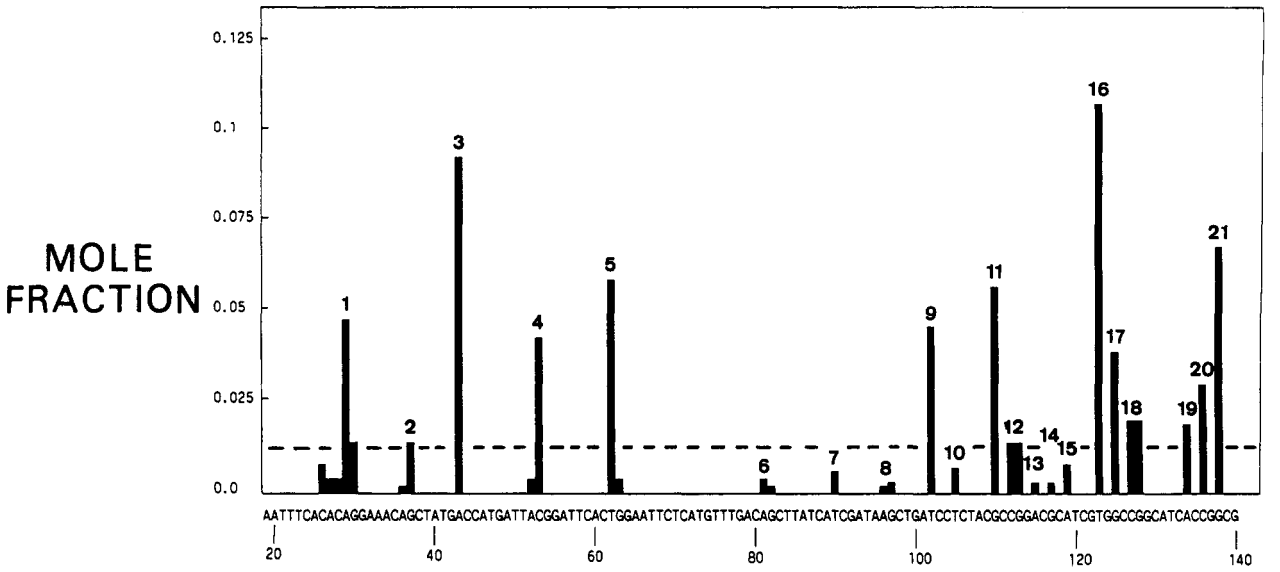

FIGURE 2: Sequence specificity of CMA. The mole fraction of blocked transcripts was determined from the 1-min elongation lane of Figure 1. Numbering is from G of the GpA dinucleotide used to initiate transcription.

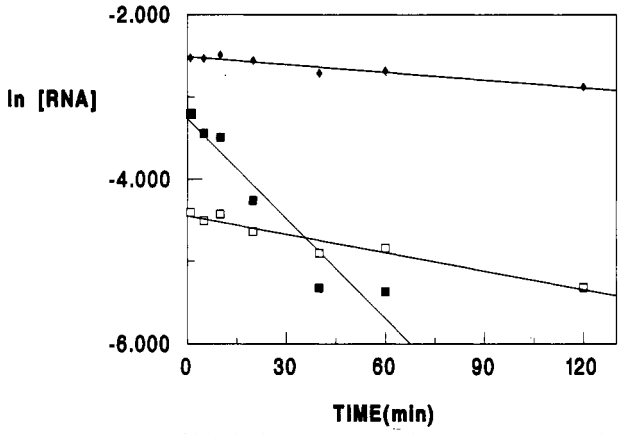

FIGURE 3: Decay of blocked transcripts. The RNA concentration is represented as the mole fraction of blocked transcripts (after 1-min elongation time) at sites 1 (■, 29/30-mer), 2 (□, 37-mer), and 3 (♦, 43/44-mer).

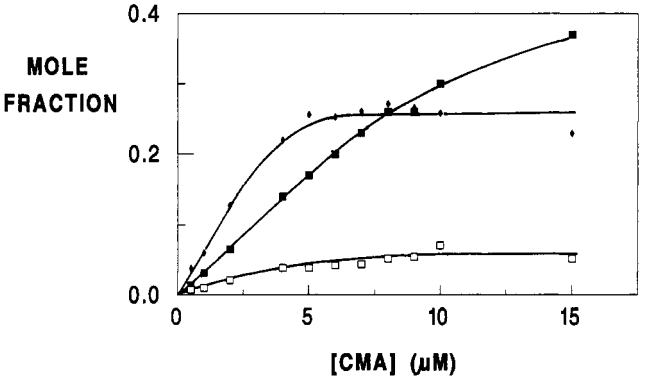

FIGURE 4: Profile of CMA concentration dependence. The mole fraction of blocked transcripts were determined (after 1-min elongation time) for sites 1 (■, 29/30-mer), 2 (□, 37-mer), and 3 (♦, 43/44-mer) for CMA concentrations of 0–15 μM.

counter-directed promoter, followed by reaction with 2 μM CMA for 1 h, yielded a range of sequence-specific blockages (Figure 5). These blockages have been quantitated in terms of the mole fraction at each site and are summarized in the bidirectional transcription footprint in Figure 6. Nine-intensity blockages are apparent when probed from either promoter, and the eight highest intensity sites all 'footprint' a GpG sequence, with the blockage occurring one nucleotide prior to this sequence. One GpC sequence was also footprinted by this procedure (site 2), but occupancy at this site was significantly lower than that occurring at isolated GpG sequences. Although other GpC sequences were present on the DNA fragment probed, binding at one such site was masked by blockage associated with flanking GpG sequences, while the other was masked by the initiation complex of the N25 promoter region.

Adducts on Single-Strand DNA. CMA was reacted with single-strand DNA and adducts detected by a primer-extension assay. In this procedure, progression of T7 DNA polymerase is blocked by adducts on the single-strand DNA template and results in discrete lengths of the newly synthesized DNA strand, extending from a primer annealed to the 3′ end of the template and ending at (or slightly prior to) the nucleotide containing the adduct on the template strand.

In the 230 bp resolved by this assay, CMA induced four major blockages with lengths of newly synthesized DNA corresponding to 33–35, 42, 145, and 200–201 (Figure 7). All four of the major blockages occur one nucleotide prior to a GpG sequence on the template strand. Furthermore, where a "staggered" blockage is apparent (33–35 and 199–200), the template blockage site comprises multiple adjacent G residues. The sequences flanking these sites have been summarized in Table I, together with eight other strong, but lesser intensity, blockage sites. Of the lesser intensity blockages, another four are prior to GpG of the template strand, and there is no obvious consensus of possible adduct sites from the remaining four blockage regions.

DISCUSSION

Sequence Specificity and Size of Adducts. Most of the high-intensity transcriptional blockages observed were at GpG or CpC sequences. From this result alone it is not possible to deduce whether adducts are associated with adjacent G residues, adjacent C residues, or an interstrand G–C cross-link. However, since all of the major adducts detected by the primer-extension assay (after reaction with single-strand DNA) were at GpG sites of the template strand, this provides convincing evidence that the transcriptional blockages associated with GpG and CpC sequences of duplex DNA are due to adduct formation with the GpG site. Furthermore, since only low levels of transcriptional or primer-extension blockages

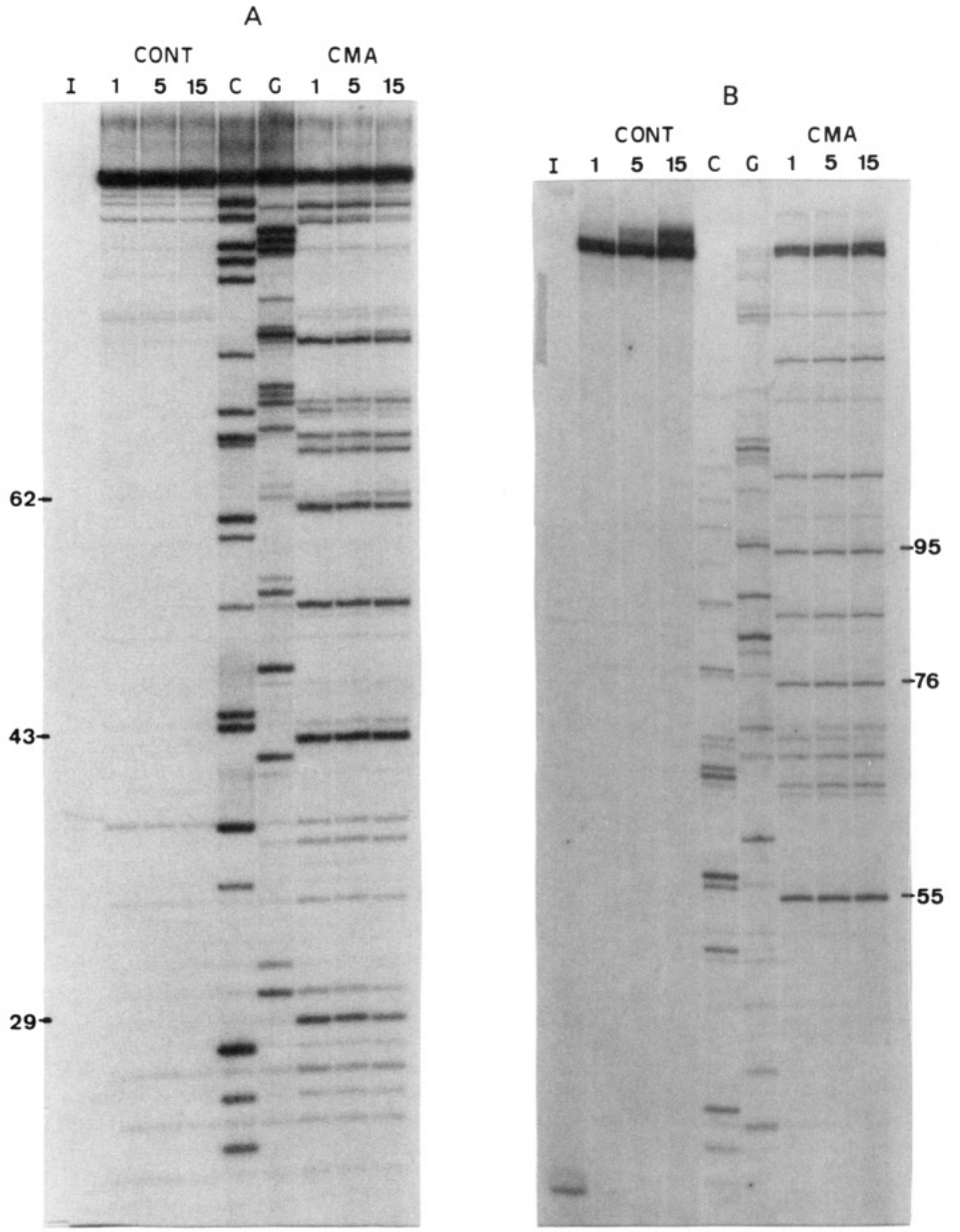

FIGURE 5: Bidirectional transcriptional blockages. The restriction digested (modified) 315-bp DNA fragment was initiated from either the UV5 or N25 promoter (panels A and B, respectively), and the initiated transcription complex then reacted with 2 μM CMA for 60 min and was then subjected to elongation for 1–15 min.

were observed at isolated G residues, it appears that the blockages at GpG sites are most likely due to an intrastrand cross-link between the adjacent G residues. An intrastrand cross-link of this type is also consistent with the size of the blocking region detected by bidirectional transcription footprinting, where eight of nine footprints were restricted to the 2-bp element defined by GpG.

A single, clearly defined GpC footprint was detected by bidirectional transcription footprinting. Since CMA is known to form interstrand cross-links and GpG sequences are associated with intrastrand cross-links, GpC is the most likely sequence at which such binding would occur and probably involves guanine residues on each strand. If GpC sequences are sites of interstrand cross-linking, then alkylation of single-strand DNA would not be expected to yield significant amounts of adducts at these sites. Of the 10 isolated GpC sequences on the template strand, only one (72-mer, Table I) was associated with significant intensity of blockage detected by the primer-extension assay. In principle, this single GpC blockage could also be due to alkylation of the isolated G, but at this stage there is insufficient data to comment further on this possibility. Overall, the GpC footprint has the characteristics of an interstrand cross-link as adducts are readily observed when CMA is reacted with duplex DNA (all eight GpC sites are associated with transcriptional blockages, Figure 1) whereas only one GpC site (out of a possible 10 isolated GpC sequences) exhibited a primer–template blockage following reaction with single-strand DNA (Figure 7 and Table I).

Nature of Transcriptional Read-Through. There is a clear ability of RNA polymerase to transcribe past some (or all) of the blockage sites shown in Figures 1–3. This is surprising given the well-known ability of CMA to form adducts and cross-links with DNA (Jesson et al., 1989). There are two possible explanations for this phenomenon. Since RNA polymerase tracks essentially in the minor groove (White & Phillips, 1988), adducts attached to appropriate sites on the nucleotides, such that they protrude into the major groove (e.g., N7 of guanine or adenine), are less likely to hinder transcription than adducts which protrude into the minor

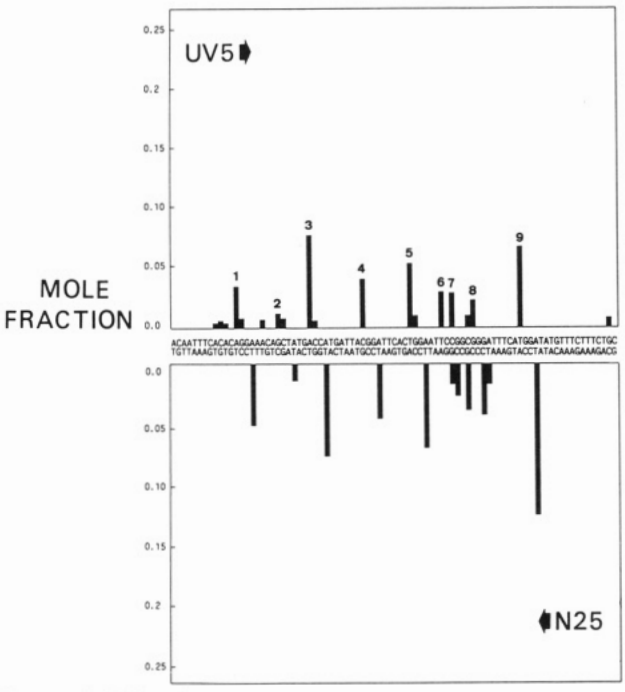

FIGURE 6: Bidirectional transcription footprint of CMA. The mole fraction of blocked transcripts from either the UV5 promoter (upper sequence, which represent the 5′–3′ nontemplate strand) or N25 promoter were determined from the data shown in Figure 5.

groove. Such selectivity of an enzyme to detect drug binding has been noted previously with λ-exonuclease, which is insensitive to N7 alkylation (major groove) but sensitive to perturbed DNA structures (kinked) induced by platinum or N2 minor groove alkylations (Mattes, 1990). It is therefore conceivable that an adduct in the major groove may result in a transcriptional blockage, but read-through of the polymerase past that site would be possible if such a group had sufficient flexibility to move out of the path of the polymerase and would therefore yield the first-order kinetics of read-through observed (Figure 3, sites 1 and 3). Alternatively, it is possible that RNA polymerase has an inherent ability to slowly bypass small, permanent drug blockages by means of a two-stage dissociation process. In this process the leading part of the polymerase may dissociate from DNA, move over the drug site, and reattach to DNA, with the trailing part of the polymerase following behind in a similar manner. Experimental analysis of these possibilities are currently in progress.

Structure of Cross-Links. Both the intrastrand cross-link at GpG and the interstrand cross-link at GpC appear to involve attachment at G residues which have many possible reactive centres (N7, O6, N2-NH_2 etc) (Pullman & Pullman, 1981). In the light of known drug reactivities at guanine N2 and N7 sites (Blackburn, 1990; Nielsen, 1990; Pullman & Pullman, 1981) and the known accessibility at N7 (Pullman & Pullman, 1981), the intrastrand cross-link is most likely to involve either one (or both) of these sites.

One site of reactivity on CMA is the cyano moiety which exhibits facile exchange (Peters et al., 1988) and has been shown to be released during reaction with DNA (Westendorf et al., 1989). The chemistry of this interaction has recently been reviewed (Acton et al., 1988), and the most convincing evidence for the role of this group is that such adducts are not detected with the analogous morpholino derivative lacking the cyano functionality (Wassermann et al., 1986). Either metabolic activation or a β-elimination of the cyano group leads to a reactive cyano iminium derived from the cyano-morpholino ring system (Acton et al., 1988), and such a species

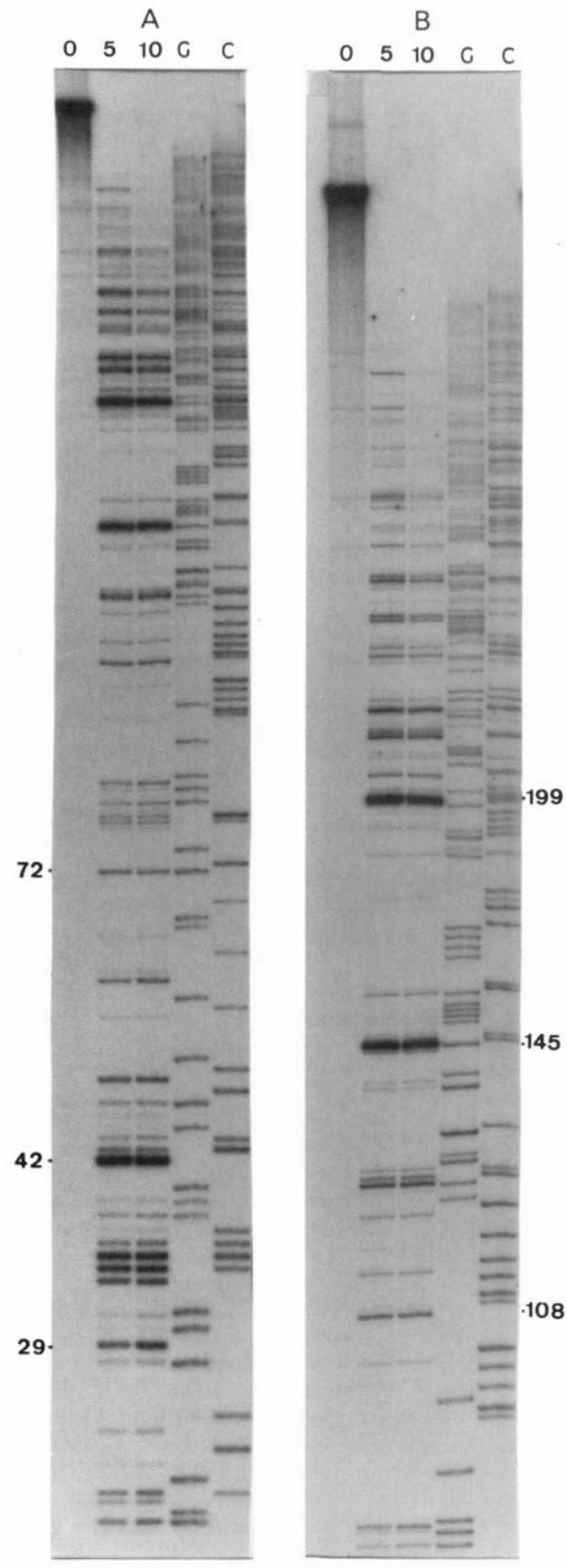

FIGURE 7: Single-strand DNA adducts with CMA. Single-strand DNA was reacted with 0, 5, or 10 μM CMA for 1 h prior to annealing of an end-labeled primer and extension by Sequenase 2.0. The required sequence resolution for DNA of 20–100 nucleotides is shown in panel A. Enhanced sequence resolution for the region 100–200 nucleotides (panel B) was achieved by electrophoresis for double that of panel A.

Table I: Adduct Sites on Single-Strand DNA

intensity	DNA length	flanking sequence[a]
strong	33–35	ATCCCGG TAGGGCC
	42	TACCGA ATGGCT
	145	AGCCTG TCGGAC
	199–200	TGCCCGC ACGGGCG
moderate	29	GAGGAT CTCCTA
	49	CTCGAA GAGCTT
	59	GTAATC CATTAG
	72	TAGCTG ATCGAC
	108	TTCCAC AAGGTG
	124	AGCCGG TCGGCC
	218	AACCTG TTGGAC
	227	TGCCAG ACGGTC

[a] The upper strand represents the sequence of newly synthesized DNA (5′–3′), while the lower strand is the template sequence. Nucleotides underlined indicate sites of polymerase blockage. Low-intensity blockages have not been indicated. The flanking sequence shown is 2 bp either side of the apparent site of reaction with CMA.

would be expected to be well-suited to reaction with the N7 or N2 nucleophilic centers of guanine.

The second end of a CMA-induced cross-link is not defined at this stage. Analysis of space-filling models of DNA and CMA reveal a close proximity of the C-9 side chain of CMA (i.e., $-(CO)-CH_2OH$) with potentially reactive $-NH_2$ groups of a neighboring G (or in principle also C or A) residues. This hypothesis is attractive since the resulting Schiff base would yield a reversible complex in vitro and may well explain why apparent interstrand cross-links at GpC sequences do not result in termination of transcription in vitro. Such complexes could be further reduced in vivo to yield completely stable covalent cross-links, as observed previously using alkaline elution assays (Wassermann et al., 1986).

Conclusions. A combination of an in vitro transcription assay, bidirectional transcription footprinting, and a primer-extension assay has revealed that the reaction of CMA with DNA in vitro yields dominantly intrastrand cross-links between adjacent guanine residues. The formation of interstrand cross-links by CMA have been well-documented by others and appear to occur at GpC sequences, but are relatively minor (at least 1 order of magnitude lesser occurrence) compared with intrastrand cross-links.

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