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The use of bidirectional transcription footprinting to detect platinum-DNA crosslinks by acridine-tethered platinum diamine complexes and cisplatin

Carleen Cullinane, Geoffrey Wickham[1], W.David McFadyen[2], William A.Denny[3], Brian D.Palmer[3] and Don R.Phillips*

Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083, [1]School of Pharmaceutical Chemistry, Victorian College of Pharmacy, Parkville, Victoria 3052, [2]Division of Chemistry and Physics, School of Science and Maths Education, Institute of Education, University of Melbourne, Carlton, Victoria 3053, Australia and [3]Cancer Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand

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ABSTRACT

Bidirectional transcription footprinting has been used to probe the platination of DNA by cisplatin, and to examine the modulation of these interactions by (a) cyclisation of the non-reactive amino group by either ethyl or propyl groups, and (b) the further addition of a pendant intercalator (9-amino acridine) linked by either phenylethyl or phenylpentyl groups. Intrastrand crosslinking was detected for all derivatives at all 5′-GG and 5′-AG sequences on the template strand, but the same sites did not result in transcriptional blockages when on the non-template strand. There was little effect of cyclysation of the amino groups, but the further addition of an intercalator resulted in three responses: a time-dependent increase of the blocked transcript by one and three nucleotides; a reduction of the sequence selectivity of platination; a decrease of apparent interstrand crosslinking for these derivatives with a pendant intercalator tethered to the amino moiety of cisplatin.

INTRODUCTION

The biological effects of the important clinical antitumor agent cis-diamminedichloroplatinum (II) cisplatin 7 (Figure 1) are due to its formation of coordination complexes with DNA. The majority of these adducts are intrastrand crosslinks with a smaller proportion of interstrand crosslinking (1). Estimates of the frequency of formation of interstrand crosslinks range from 1–7% (1). Intrastrand crosslinks are considered to be relatively difficult to repair (2) and this may be the reason for their marked effectiveness of cisplatin against tumours of germ cell origin (e.g. testicular and ovarian tumors). Although widely used in cancer chemotherapy, cisplatin and its analogues suffer from the drawback that tumour cells develop resistance to these drugs. This can occur by a number of mechanisms, among them deactivation of platinum by over expression of thiols and thiol transferring enzymes, by alterations to transport mechanisms and by enhancement of DNA repair mechanisms that excise DNA platinum adducts (1).

One focus of our work on the development of platinum complexes with improved activity against cisplatin resistant cell lines has been to target platinum to DNA by its attachment to suitable DNA—affinic carrier ligands. In this way, mechanisms of resistance may be circumvented by minimizing exposure of platinum to other cellular components such as thiols and by the formation of platinum DNA adducts which are sufficiently novel to escape repair by the normal mechanisms. Examples of such DNA-targeted platinum complexes include the series of 9-anilinoacridine complexes 2, 3, 5 and 6 (3). Measurements of DNA unwinding of supercoiled plasmid pBR322 DNA by these complexes show that in binding to DNA the platinum atom is covalently bound with the acridine chromophores intercalated adjacent to the metal binding site, or in the case of the longer chain homologues, one base pair removed from this site (4). Experiments with linearized pBR322 fragments indicate that the complexes form DNA interstrand crosslinks although no more effectively than the monomers $PtenCl_2$, 1, or $PtpropCl_2$, 4, (4). Although these measurements reveal the gross features of DNA interaction they provide no information on the sequence preferences of drug binding.

The sequence selectivity of the platination of DNA by cisplatin and its analogues has been investigated through a number of approaches, all of which indicate a preference for binding to GG sequences. A polymerase stop assay, using *Taq* DNA polymerase has been used by Ponti *et al.* (5) to reveal blockages almost exclusively at G_n ($n>2$) sequences. Lemaire *et al.* (6) have demonstrated that interstrand crosslinks are preferentially formed at 5′-GC sites—these sites were mapped from the ability of

* To whom correspondence should be addressed

cisplatin adducts with DNA to terminate transcription of the T7 and SP6 RNA polymerases on double-stranded DNA. The binding site preference of complex 8 has been mapped by exonuclease III digestion of restriction fragments from pBR322 DNA (7). Again, oligo (dG) sequences are the most prevalent sites but the presence of the tethered intercalator leads to secondary binding, mainly at AG sites. The *in vitro* sequence selectivity of DNA adducts formed by the complexes 9 and 10 has also been probed (8). Utilizing plasmid DNA and the Taq polymerase amplification system it was found that runs of two or more consecutive G's were the major site of adduct formation with lesser damage at GA AG and GC sites (8). This method has also shown that a similar pattern of damage was caused to the DNA in human cells for the complex 9 whereas damage to intracellular DNA was not detected for the complex 10 (9).

An *in vitro* transcription assay has been used previously to probe a variety of drug-DNA interactions (10) and has been utilised for bidirectional transcription footprinting of drug sites on DNA (11,12). These procedures offer advantages over other footprinting methods in that relative affinity for individual sites is readily apparent, as is the kinetics of drug-DNA interaction at each site. We report here the application of bidirectional transcription footprinting to detect sites of platination by the complexes 2, 3, 5, and 6, their selectivity, absolute size of the blockage unit, relative occupancy at preferred sites as well as the irreversibility of each complex with DNA. The effects of the intercalating chromophore on platinum binding are probed by including cisplatin, 7, $PtenCl_2$, 1 and $PtpropCl_2$, 4, as monomeric comparators.

MATERIALS AND METHODS

Materials

Established methods were used to prepare cisplatin, 7 (13) and $PtenCl_2$, 4 (14). The complexes 2, 3, 5, and 6 were synthesised in our laboratories and have been described previously (3). All drugs were dissolved in dimethylformamide to 2 mM solutions and stored in the dark at −20°C.

Urea, TEMED and 'Instapage' (40% solution of 19:1 acrylamide:bisacrylamide) were supplied by IBI (Connecticut, USA) as ultrapure reagents. Nucleotides (including 3'-O-methylnucleotides, primers; ApU and GpA and ribonucleotides), *E.coli* RNA polymerase (nuclease free), BSA (nuclease free) and ribonuclease inhibitor (human placenta) were purchased from Pharmacia. [α-^{32}P]UTP (specific activity 3000 Ci/mmol) and X-ray film (Hyperfilm-β-Max) were obtained from Amersham while restriction enzymes were from New England Biolabs. NA45 membranes were purchased from Schleider and Schuell and heparin was obtained from Sigma.

All other reagents were of analytical grade and all solutions were prepared using water purified through a Milli-Q four stage system (Millipore, MA).

Methods

Isolation of pRW2 and subsequent restriction digestion with Xho I and Pvu II to yield the 315 bp fragment containing the N25 and UV5 counter directed promoters was as previously described (12,15).

Inactivation of the N25 or UV5 promoters was carried out by restriction digestion with Dra 1 or BstN 1 respectively, as previously described (12,15).

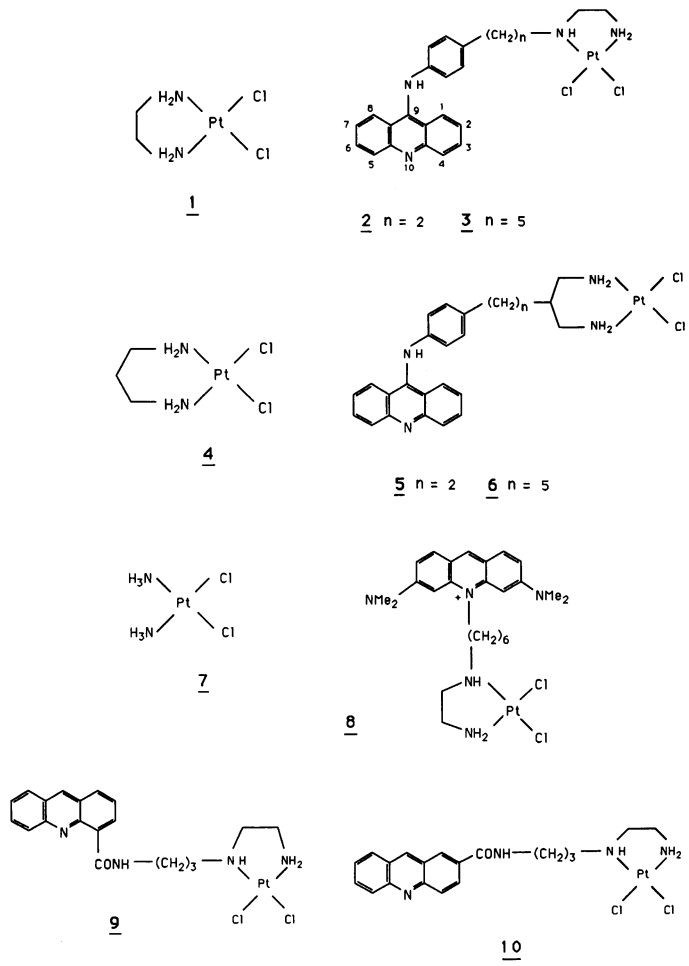


Figure 1. Structures of cisplatin and derivatives referred to in this work.

Drug reactions

100 μM bp of the appropriate promoter-containing DNA fragment was incubated for 18 hours with platinum compounds at 20 μM for compounds 1, 4 and 7 and 50 μM for compounds 2, 3, 5 and 6 in 50 mM HEPES, pH 7.0. Drug reactions were performed in the dark at 37°C for 18 hours. Control reactions, in the absence of drug, were performed identically in buffer containing 2.5% DMF.

Transcription

Transcription conditions employed were as previously described (12). Briefly, drug treated promoter was incubated with *E.coli* RNA polymerase at 37°C in a transcription buffer (40 mM Tris−HCl pH 8.0, 100 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.125 μg/ml BSA, 2 u/μl RNAse inhibitor) to form an open ternary complex. Non-specifically bound polymerase was removed by the addition of heparin to a final concentration of 400 μg/ml.

A labelled initiated complex was formed in the presence of 200 μM of GpA (for the UV promoter) or ApU (for the N25 promoter) together with 5 μM ATP, GTP and UTP and [α-^{32}P]ATP. Elongation of the transcript was carried out for 1,5 or 15 min, and was initiated by adjusting conditions to 2 mM of all four nucleotides and 400 mM KCl. Reactions were

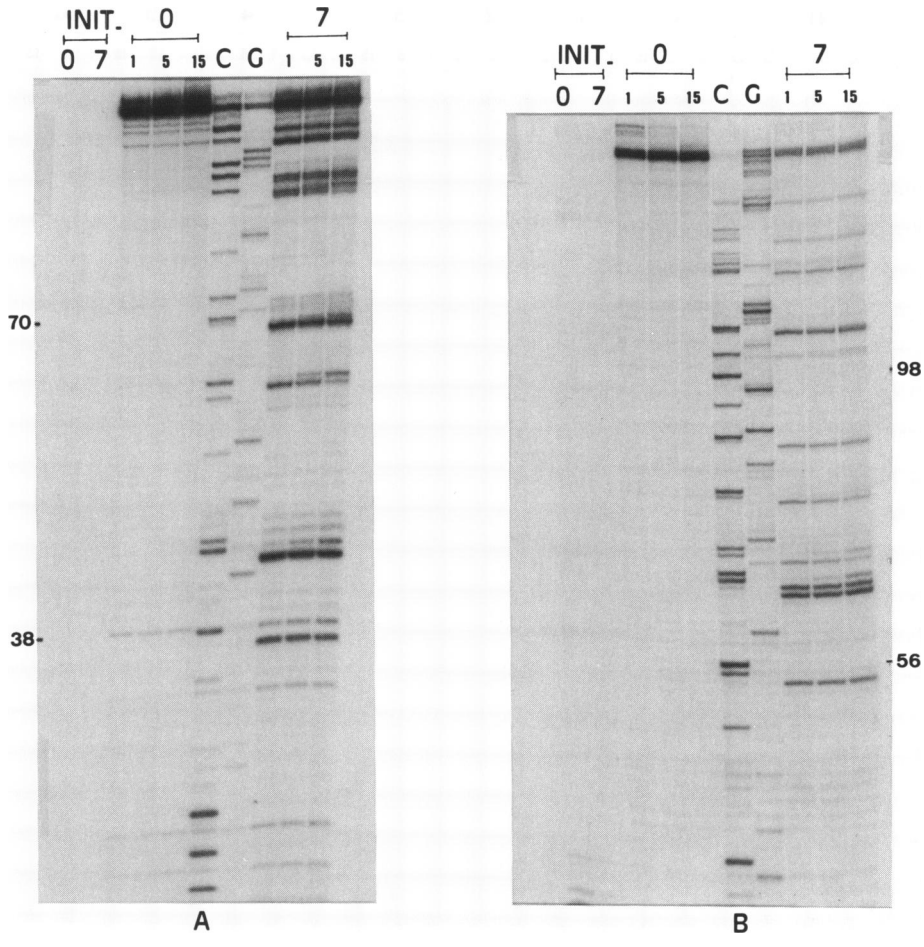


Figure 2. Bidirectional footprinting of cisplatin (7). 100 μM bp of the 315 bp fragment (restriction digested to inactivate one of the two counter-directed promoters) was incubated in the absence (○) or presence of 7 for 18 hours. Transcription of treated DNA was initiated from the UV5 (panel A) and N25 (panel B) promoters and subsequently elongated for 1,5 and 15 min. 'INIT' lanes show initiation products prior to elongation while C and G represent sequencing lanes obtained using 3-O-methyl CTP and 3-O-methyl GTP respectively. The length of transcript relative to the −1 site of transcription is indicated at the side of each autoradiogram.

terminated by the addition of an equal volume of loading dye (8 M urea, 10% sucrose, 40 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol).

Electrophoresis and autoradiography

Reactions were electrophoresed through a 10% denaturing gel and the gel was subsequently dried and autoradiographed using standard procedures (12). Quantitation was performed using a 300A Computing Densitometer (Molecular Dynamics, CA, USA).

RESULTS

Transcriptional blockages

The effect of cisplatin in inducing transcriptional blockages is shown in Figure 2. In this assay the initiated transcription complex contains nascent RNA synchronised to a length of mainly 10 nucleotides (10). When elongated in the presence of all four ribonucleotides, the full-length transcript is obtained within one minute, with no variation observed if elongation is continued for longer times. However, with DNA treated with cisplatin major transcriptional blockages are apparent at RNA lengths of 37, 43, etc. following transcription from the UV5 promoter (Fig. 2a), as are blocked transcripts with lengths of 55, 63, 64, etc. from the N25 promoter (Fig. 2b). The blockages are largely independent of elongation time, since only minor changes of the blocked transcripts are apparent after a 15-fold increase of elongation time (i.e. 15 min compared to 1 min) for transcription from either promoter. Additional low intensity bands after major blockages, such as at 43 (Fig. 2A), probably represents the ability of RNA polymerase to transcribe weakly for 3 or 4 nucleotides past stereochemically small blockages by a partial polymerase dissociation mechanism, and has been observed with other small DNA adducts (16).

Such long-lived transcriptional blockages are characteristic of covalent or essentially irreversible complexes such as adducts with nitrogen mustard (17) and are as expected for cisplatin which has been extensively documented to form coordination complexes with DNA. The control lanes show mainly full-length transcripts arising from elongation of the initiated transcription complex which had not been subjected to drug treatment. The background is minimal, with only minor natural pausing apparent using the UV5 promoter (e.g. 38-mer in Fig. 2a).

Similar sequence dependent blockages are also apparent for the platinum complexes 1-6 for transcription from the UV5 promoter (Figure 3) and from the N25 promoter (Figure 4).

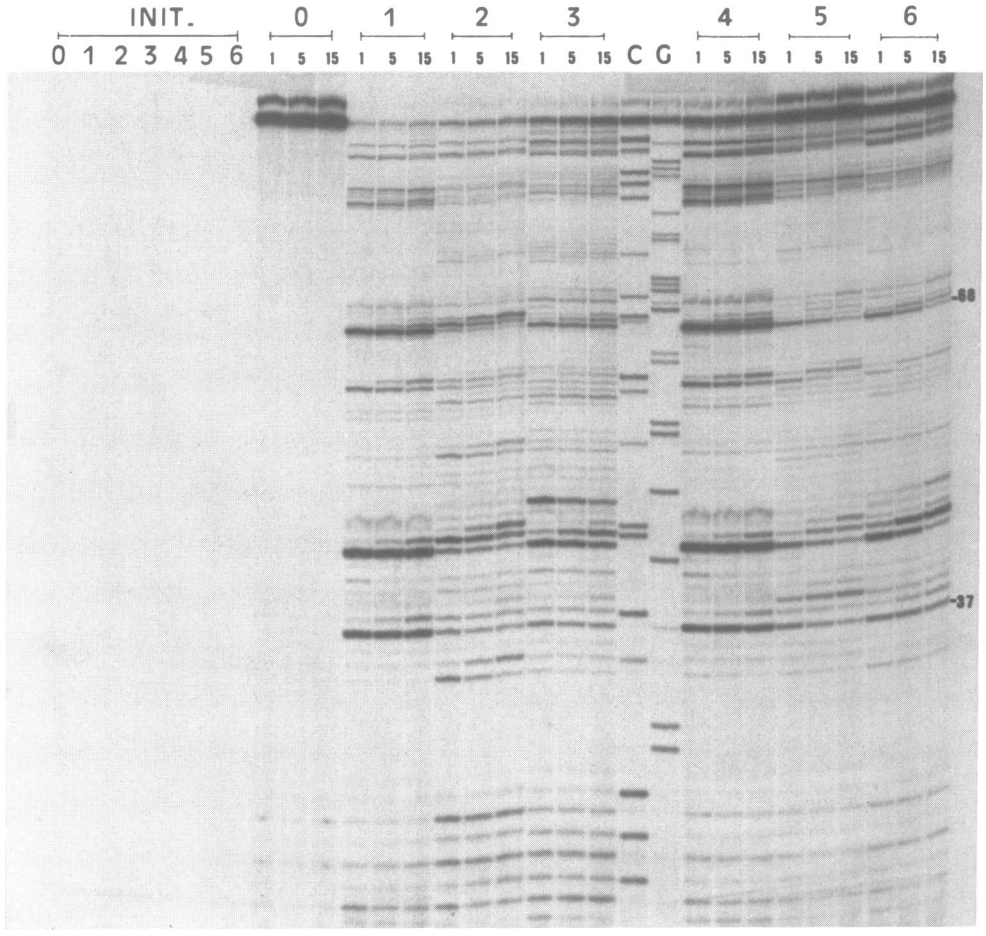


Figure 3. UV5 transcription detection of platinum derivatives. The autoradiogram shows transcriptional blockages detected by RNA polymerase, initiated from the UV5 promoter of DNA previously treated with 20 μM 1 and 4 and 50 μM 2, 3, 5 and 6.

The intensity of transcriptional blockages is proportional to the number of RNA molecules comprising each band on the sequencing gel. This results from the nature of the elongation conditions which ensures that each transcript contains the same amount of radiolabel incorporated into the nascent RNA during initiation of the ternary transcription complex, and no further label is incorporated during the elongation phase.

The autoradiograms were scanned by laser densitometry to quantitate the fractional number of moles of RNA of each length in a lane. The mole-fraction of each RNA band in the 15 min elongation time lanes has been shown in Figure 5 in terms of a histogram which summarises all transcriptional blockages shown in Figures 2–4 up to transcript lengths of approximately 110.

Simple platinum complexes

Transcription from the UV5 promoter revealed eight well-resolved regions (up to 110 nucleotides) of blockage induced by cisplatin (7) (Fig. 2), with the strongest (43, 68 and 106) occurring one nucleotide prior to 5′-CC sequences and two blockages at G of 5′-GC sequences (37 and 72). Other lower intensity blockages were prior to 5′-CT sequences at 61, 94 and 98. Seven discrete blockages were seen (up to 120 nucleotides) with the N25 promoter (Fig. 2), of which six were one nucleotide prior to 5′-CC sequences, with the other at G of 5′-GC. All sequences refer to the non-template strand, with the numbering referring to the transcript length from the −1 site of each promoter (since initiation of transcription was forced to commence from the −1 position by high levels of GpA and ApU for the UV5 and N25 promoters, respectively).

Overall, fourteen high intensity blockage sites (mole fraction greater than 0.025) were identified in the regions probed from the UV5 and N25 promoters and twelve of these are summarised in Fig. 5 in the form of a bidirectional transcription footprint (note that sites at 106 and 110 from the UV5 promoter are not represented on this summation since they are outside of the region probed by the N25 promoter). Of these fourteen high intensity sites, ten are prior to 5′-CC sequences of the non-template strand, and none of these were detected as blockages from both promoters. The only site which yielded an unambiguous, isolated footprint (i.e. blocked transcripts from both promoters) was the 5′-GC sequence at 37 from the UV5 promoter.

Moving from cisplatin, 7, to $PtenCl_2$, 1 and $PtpropCl_2$, 4, where the coordinated nitrogens are linked by two and three carbons respectively in a chelate ring, produces essentially the same UV5 or N25 transcriptional blockage patterns (Fig. 3 compared to cisplatin Fig. 2). The blockages have been summarized in Fig. 5. The same six high intensity sites are apparent from both the UV5 and N25 promoters for both chelates, as compared to cisplatin. One difference to emerge among these complexes was the observation that the isolated footprint (37 from the UV5 promoter) was absent in $PtpropCl_2$, 4.

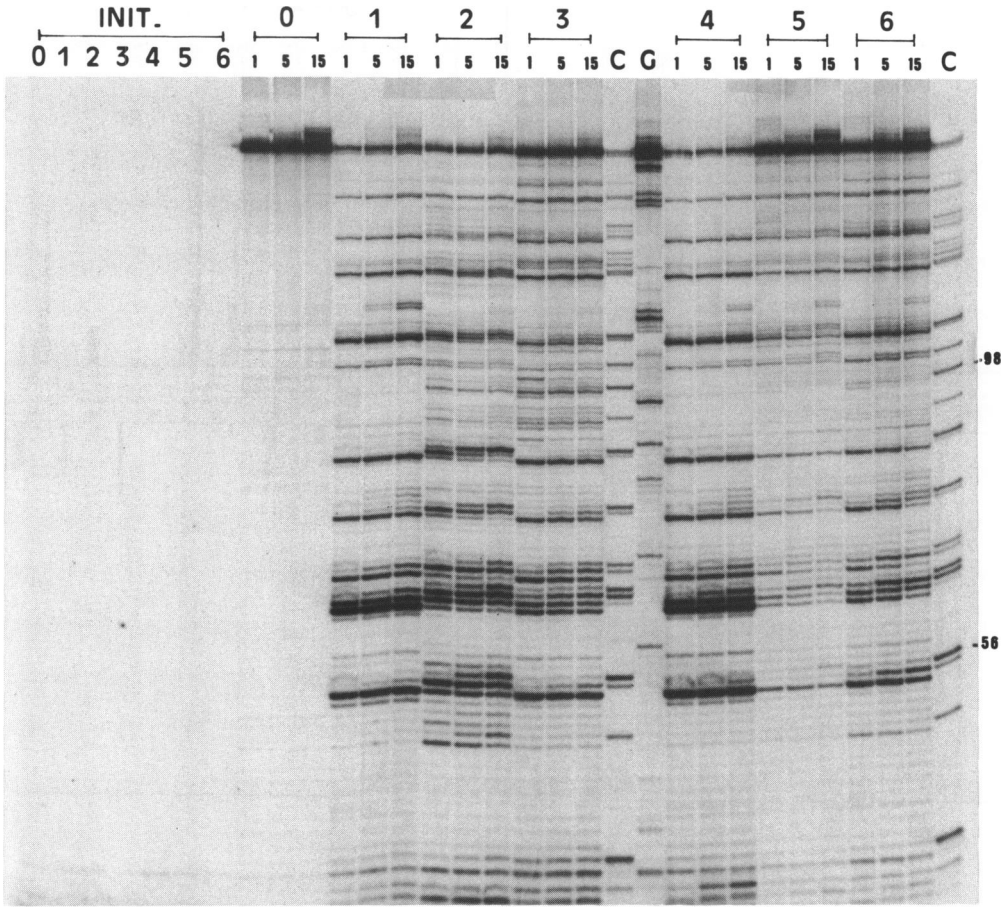


Figure 4. N25 transcription detection of platinum derivatives. The autoradiogram shows transcriptional blockages detected by RNA polymerase initiated from the N25 promoter of DNA previously treated with 20 μM 1 and 4 and 50 μM 2, 3, 5 and 6.

Intercalator-tethered complexes

For the complexes 2 and 3 (which are formally derived from $PtenCl_2$, 1) the pattern of transcriptional blockages is broadly similar to that observed for $PtenCl_2$, 1. However, some notable differences are apparent. For the complex 2, in which the anilino ring is tethered to the platinum moiety by a $-(CH_2)_2-$ chain, some 5′-CC blockages occurred one nucleotide downstream of those observed for $PtenCl_2$, 1 (e.g. 44 and 69 for the UV5 promoter (Fig. 3) and at 56, 64 and 76 for the N25 promoter (Fig. 4)). Additional blockages were also observed at 34/35 for the UV5 promoter (Fig. 3) and at 49 and 97 from the N25 promoter. The homologue, 3, which contains the longer $-(CH_2)_2-$ linker also shows additional blockages at 47 for the UV5 promoter and 91 and 97 from the N25 promoter. For both complexes the additional blockages all occur at, or one nucleotide prior to isolated G residues on the template strand. It is also significant that the appearance of blockages from both directions at 5′-GC (37 of UV5) observed for 1 is missing (102 of N25) in the tethered complexes.

When compared with $PtpropCl_2$, 4, the complex 5 shows additional blockages at 25 and 27 for the UV5 promoter and 75, 77, 88, 102, 108 and 116 for the N25 promoter with almost total loss of the UV5 blockage at 93. Likewise, this blockage is lost in 6 and several new blockages emerge at 25 and 27 for the UV5 promoter and 49, 88 and 102 for N25. It is noteworthy that $PtpropCl_2$, 4, shows no evidence of the blockage on both strands (at 37 of UV5) but that this is observed in the tethered complexes 5 and 6.

Permanence of blockages

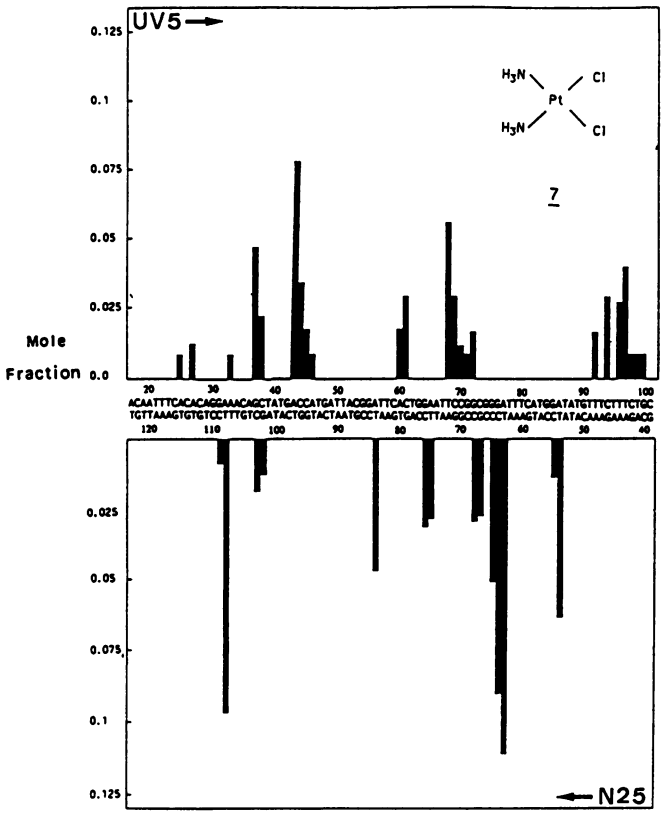
The majority of all blockages detected were independent of elongation time up to 15 min. This indicates that the cause of the blockage is essentially irreversible with time, as expected for coordination complexes, compared to drugs which interact reversibly with DNA (10).

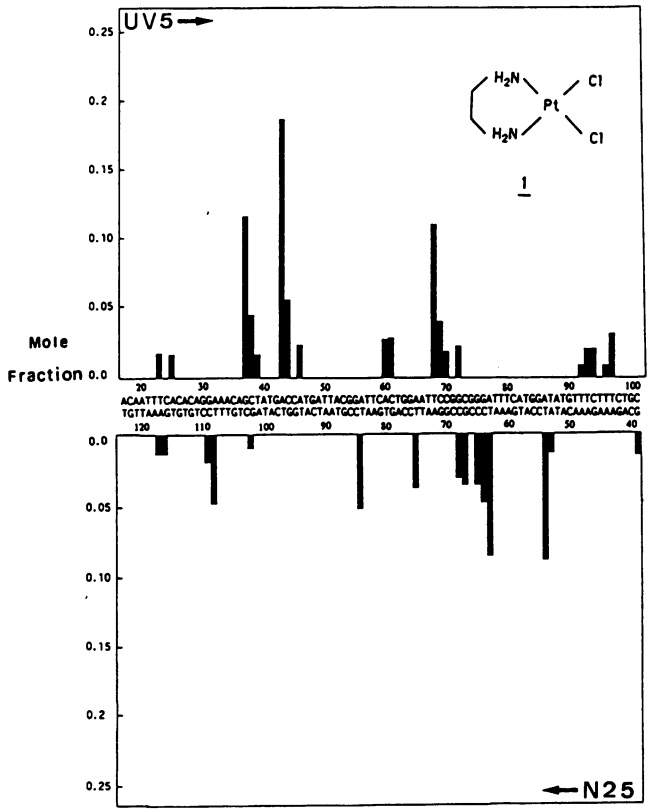
The only sites where some time dependence of the blockages was apparent was for those derivatives with a pendant intercalator (2, 3, 5 and 6; Figures 2 and 3). In these cases a one nucleotide 'creep' of the blocked transcript was observed over the 15 min elongation time studied, as has been noted previously with other intercalators (11).

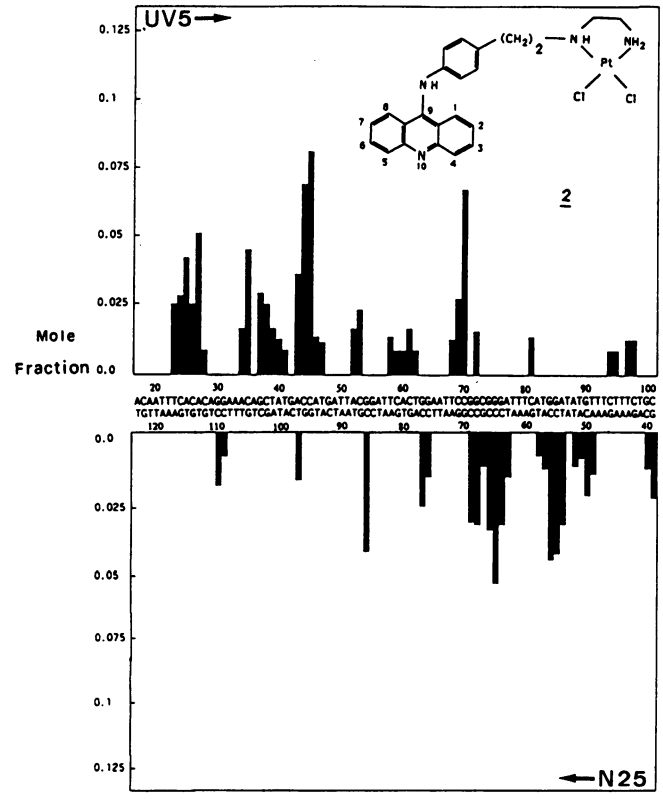
DISCUSSION

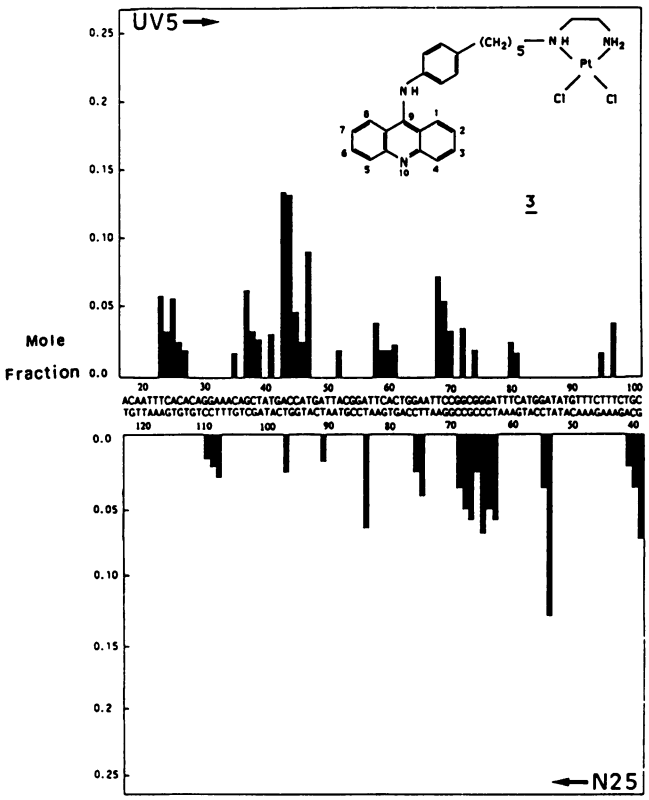
Intrastrand crosslinks

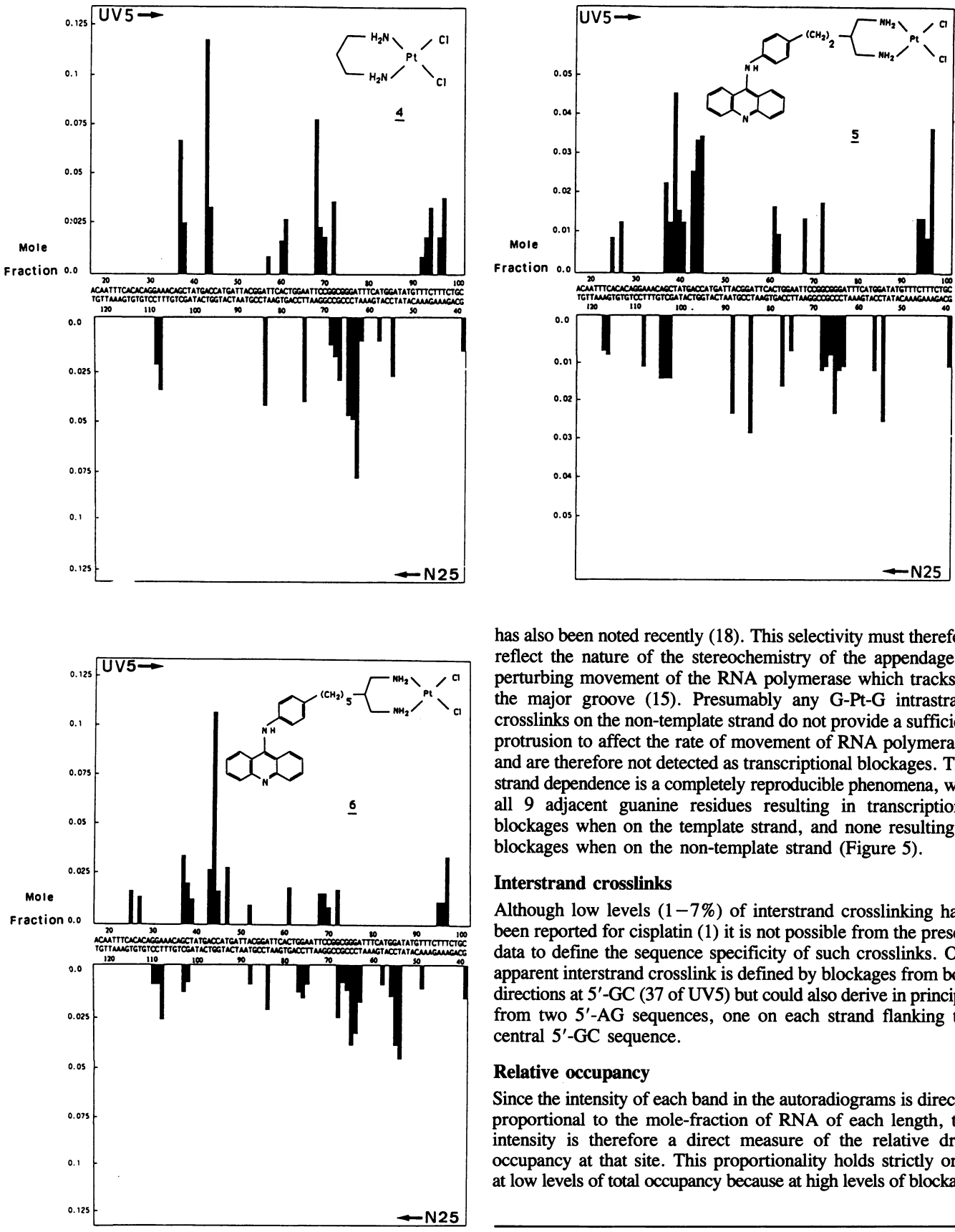
For both the tethered and simple complexes, transcriptional blockages were detected at all 5′-CC sequences on the non-template strand. This phenomena is consistent with the formation of intrastrand crosslinks between adjacent guanine residues on the template strand. Such intrastrand crosslinks have been well documented for cisplatin (1). These blockages only induce transcriptional blockages when on the template strand and this


UV5
Mole Fraction
7
N25


UV5
Mole Fraction
1
N25


UV5
Mole Fraction
2
N25


UV5
Mole Fraction
3
N25



has also been noted recently (18). This selectivity must therefore reflect the nature of the stereochemistry of the appendage in perturbing movement of the RNA polymerase which tracks in the major groove (15). Presumably any G-Pt-G intrastrand crosslinks on the non-template strand do not provide a sufficient protrusion to affect the rate of movement of RNA polymerase, and are therefore not detected as transcriptional blockages. This strand dependence is a completely reproducible phenomena, with all 9 adjacent guanine residues resulting in transcriptional blockages when on the template strand, and none resulting in blockages when on the non-template strand (Figure 5).

Interstrand crosslinks

Although low levels (1−7%) of interstrand crosslinking have been reported for cisplatin (1) it is not possible from the present data to define the sequence specificity of such crosslinks. One apparent interstrand crosslink is defined by blockages from both directions at 5′-GC (37 of UV5) but could also derive in principle from two 5′-AG sequences, one on each strand flanking the central 5′-GC sequence.

Relative occupancy

Since the intensity of each band in the autoradiograms is directly proportional to the mole-fraction of RNA of each length, the intensity is therefore a direct measure of the relative drug occupancy at that site. This proportionality holds strictly only at low levels of total occupancy because at high levels of blockage

Figure 5. Quantitation of platinum-induced blocked transcripts. The mole-fraction of blocked transcript from each promoter was quantitated by densitometry and is shown with respect to the sequence and transcript length of the non-template strand.

less of the RNA polymerase probe is able to reach the more downstream sites. For this reason, the band intensity should be considered as a good approximation of relative occupancy as long as the accumulative mole fraction of blocked transcripts is less than approximately 50%, otherwise sites further downstream are underestimated (10). Quantitation of relative blockages is therefore completely valid for compounds 7 (Fig. 2), 4–6 (Fig. 3), 3, 5 and 6 (Fig 4) but leads to progressive underestimation of downstream sites for compounds 1–3 (Fig. 3) and 1, 2 and 4 (Fig. 4).

The single highest affinity, isolated, intrastrand blockage sites for cisplatin are immediately prior to 5′-CC (nontemplate strand) and are at 43 of UV5 and 54 and 108 of N25 (Fig. 5). Although the flanking sequence common to two of these sites is 5′-CCAT, there are insufficient isolated sites with this motif to establish the significance of this sequence.

Only six 5′-AG sequences were present in the sequence probed, and all six exhibited significant transcriptional blockages at (or one nucleotide prior to) G of 5′-AG sites of the template strand (blockages at 37, 60, 93 and 97 from UV5, and 103 and 110 from N25). For the purpose of a quantitative comparison of relative occupancy at different sites, only those binding sites which are clearly isolated from neighbouring sites were utilised. By this means, the relative occupancy of the blockage at 42 (prior to 5′-GG of the template strand of UV5) compared to the blockage at 60/61 (i.e., the total blockage prior to 5′-AG of the template strand of UV5) is 2.9:1, consistent with the ratio of 2.6:1 reported previously for GG compared to AG intrastrand crosslinking (19).

Modulation of transcription by tethered complexes

Several major points are evident in comparison of the tethered complexes with the simple platinum compounds. The first was an increase of the length of the blocked transcript by one and two nucleotides for many of the blockages exhibited by derivatives 2, 3 and 6 (eg. 44 and 45 for the UV5 derived transcripts) accompanied by a time-dependent extension to the longer transcript over 5–15 min. Since there is no net escape of RNA polymerase past the identifiable platination site, the most likely explanation of this effect is that the intercalator perturbs the geometry of the coordination complex sufficiently to enable the catalytic site of the RNA polymerase to move slowly an additional one or two bp along the DNA. This suggests that the overall effect of the intercalator is to alter the stereochemistry of the intrastrand coordination crosslink to permit the RNA polymerase to track more freely in the major groove for an additional one or two bp prior to some part of the polymerase being terminally blocked.

In the second instance, in spite of the presence of the intercalating chromophore and linker chain, both of which lead to an increase in binding site size, the blockages induced by the tethered complexes are broadly similar to those caused by cisplatin, 7, $PtenCl_2$, 1, and $PtpropCl_2$, 4 as most high intensity blockages occurred at runs of two or more G's on the template strand. The presence of the intercalator has also caused a larger number of blockages compared with the more discrete damage caused by the simple complexes. This reduction in the selectivity of platination can be ascribed to the presence of the highly DNA-affinic acridine chromophore which enhances overall levels of background binding on the DNA helix. Studies with the related complexes 9 and 10 reveal a similar pattern of behaviour (8). The behaviour of the platinum complexes stands in contrast to DNA-targeting of alkylating groups by intercalating chromophores. The normal preference for alkylation at runs of G's is significantly modified in acridine-tethered aniline mustards (20) and phenanthridinium alkyl bromides (21) where greatly enhanced reaction occurs at isolated guanines located in 5′-GT sequences.

Differences also emerge between the two sets of acridine-tethered complexes. If the presence of a blockage from both directions at 5′-GC (37 of UV5) can be ascribed to an interstrand crosslink, then the absence of a dual blockage with both 2 and 3, which is present for 5 and 6 suggests that the complexes derived from $PtpropCl_2$, 4, have much greater crosslinking efficiency than their ethylenediamine counterparts.

CONCLUSIONS

Bidirectional transcription footprinting has revealed that the DNA sequence selectivity of platinum diamine complexes is modulated by a pendant intercalating acridine group. The apparent decrease of interstrand crosslinking when the intercalator is tethered to the amino moiety of cisplatin offers the prospect of a new series of derivatives with constant potential for platination, but varying hydrophilicity and varying capacity to induce interstrand crosslinking.

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