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THE STRONG BINDING OF LUZOPEPTIN TO DNA

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Abstract—The effect of luzopeptin on the mobility of DNA in polyacrylamide gels has been determined. Experiments on a mixture of DNA fragments of various lengths have shown that the drug does not form intermolecular cross-links. Gel analysis of complexes of the drug with short DNA fragments (15–35 base pairs) reveals a ladder of discrete bands in which each band appears to correspond to the addition of a further drug molecule. The results suggest that luzopeptin binds very strongly to DNA, occupying about four base pairs and displays little or no sequence selectivity. Luzopeptin renders certain adenine residues hyperreactive to diethylpyrocarbonate, these occur in different positions to those affected by echinomycin.

The luzopeptins, formerly BBM928 (Fig. 1), are antitumor antibiotics which contain two substituted quinoline chromophores linked by a cyclic decadepsipeptide [1]. They are structurally similar to the quinoxaline group of antibiotics such as echinomycin, which possess an octadepsipeptide ring bridged by a disulphide or thioacetal linkage. Luzopeptin has been shown to bind to DNA by the mechanism of bifunctional intercalation [2] with one binding site for every five to six base pairs, consistent with a crystal structure and NMR data which separate the chromophores by sufficient distance to accommodate two or three base pairs [3, 4]. Luzopeptin binds to DNA at least ten times more strongly than echinomycin [1], and retards the mobility of DNA on non-denaturing polyacrylamide gels; it has been suggested that its interaction with DNA may be covalent [2, 5]. In contrast to other bisintercalators it has been reported to form intermolecular DNA cross-links [6].

The two compounds differ as regards their sequence recognition properties. In footprinting experiments echinomycin has been shown to bind almost exclusively to the dinucleotide step CpG [7, 8], while luzopeptin interacts with many DNA sequences and appears to bind best to regions of high AT content [5]. A recent NMR study of the interaction of luzopeptin with the hexanucleotide GCATGC suggests that the antibiotic spans the central two AT base pairs, forming hydrogen bonds to the thymine residues [9].

In this paper we examine the binding of luzopeptin to several short DNA fragments (16–160 base pairs) so as to assess the nature of the interaction. We find that the drug does not form cross-links between DNA duplexes, and can interact with many different sites saturating the DNA at one drug molecule for every four base pairs.

MATERIALS AND METHODS

Drugs and enzymes. Luzopeptin A was a gift from Dr M. Konishi, Bristol-Banyu Research Institute, Tokyo. Echinomycin was obtained from Dr M. J. Waring, University of Cambridge, U.K. Because of their low aqueous solubility, 1 mM stock solutions were prepared in dimethylsulphoxide (DMSO) and stored at 4°. The antibiotics were diluted into 10 mM Tris–HCl pH 8.0 containing 10 mM NaCl immediately before use; the final concentration of DMSO never exceeded 3% (v/v). Since the drugs slowly precipitate from these aqueous dilutions the concentrations recorded may not be exact, but represent upper limits. Drug–DNA complexes were equilibrated for 30 min before subsequent treatment so as to allow complete reaction. In the cross-linking experiments the DNA fragments were mixed before adding the drug to avoid any complications arising from possible irreversible complex formation with one DNA before adding the other.

DNA fragments. The 70-, 130- and 135- base pair DNA fragments and TyrT DNA (160 base pairs) whose sequences are shown in Fig. 2 were prepared as previously described [10]. The short DNA fragments used in the gel retardation experiments were prepared as follows. Plasmid pBR322 was digested with EcoR1, labelled with α-^{32}P-dATP using reverse transcriptase and cut again with HaeIII. The 3′-end labelled 175 base pair DNA fragment was isolated from a 6% polyacrylamide gel. Shorter DNA fragments of 16, 24, 29, 31 and 34 base pairs were generated from this by cutting with restriction enzymes AluI, TaqI, HindIII, AluI and MseI. These short fragments were not purified from the longer unlabelled material so as to maintain a constant DNA concentration in each experiment. The 18 base pair fragment corresponds to the HaeIII (4344)-EcoR1 fragment from pBR322. The exact DNA concentration in each experiment is difficult to estimate rigorously and is about 0.2–1 μM base pairs. The concentration of different DNA fragments between experiments was maintained approximately constant by using a fixed quantity of radiolabelled DNA.

Reaction with DEPC. The reaction with diethylpyrocarbonate (DEPC) was performed as previously

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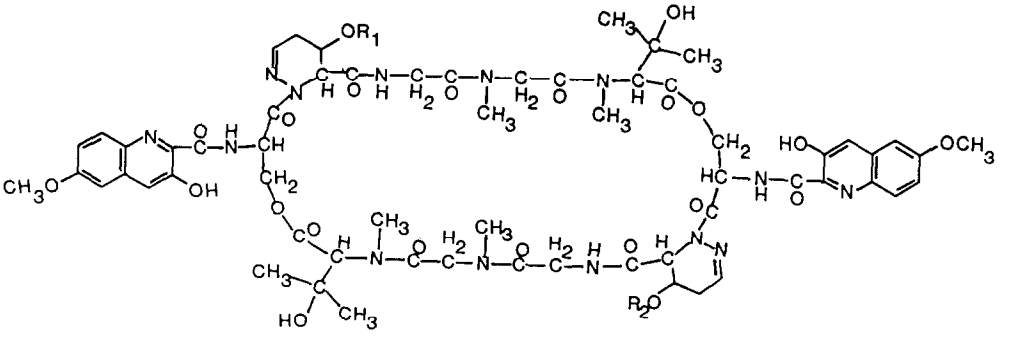

Luzopeptin A $R_1 = R_2 = COCH_3$

Luzopeptin B $R_1 = H \quad R_2 = COCH_3$

Luzopeptin C $R_1 = R_2 = H$

Fig. 1. The structures of luzopeptin.

DNA Sequences.

70-mer
3'-AATTACGCCATCAAATAGTGTCAATTTAACGATTGCGTCAGTCCGTGGCACATACTTTAGATTGTTAC
40 50 60 70 80 90

130-mer
5'-CACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAA
4270 4280 4290 4300 4310 4320

AATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTCTCATGTTTGACAGCTTATCATCGA-3'
4330 4340 4350 4360 10 20

135-mer
3'-AACCGTCCTGTTCCCGTCGAGACGTTTGACATTTTGGCCTGTTTCCGAAAGGGGACCGAATGTGCGT
10 20 30 40 50 60 70

TTTCCCTTCCCGGAAAGGACTCCTCCACTCGCCGTTGGACCTGAGCCCCTACCGCGACCTTCA-5'
70 80 90 100 110 120 130

TyrT.
3'-AAGGCCAATTGGAAATTAGGCAATGCCTACTTTTAATGCGTTGGTCAAGTAAAAAGAGTTGCATGTG
0 10 20 30 40 50 60

AAATGTCGCCGCGAGTAAACTATACTACGCGGGGCGAAGGGCTATTCCCTCGTCCGGTCATTTTTCGTAA
70 80 90 100 110 120 130

TGGGGCACCACCCCCAAGGGCT-5'
140 150

Fig. 2. Sequences of four DNA fragments used in this work. All fragments were labelled at the 3'-end with α-[^{32}P]dATP. The labelled residues are underlined. Only the strand bearing the radioactive label is shown.

described [11–15] by reacting 5 μL of a drug–DNA complex with 5 μL DEPC for 10 min at 37°. The modified DNA was precipitated with ethanol, boiled in 10% piperidine for 15 min and lyophilized. Bands in the digest were assigned by comparison with dimethylsulphate-piperidine markers specific for guanine.

Gel electrophoresis. Non-denaturing polyacrylamide gels (40 cm, 6% w/v depending on the fragment length) were run at 800 V for 2–3 hr. Three microliters of 20% sucrose containing 10 mM EDTA was added to each sample before loading onto the gel. The products of reaction with DEPC were analysed on denaturing polyacrylamide gels containing

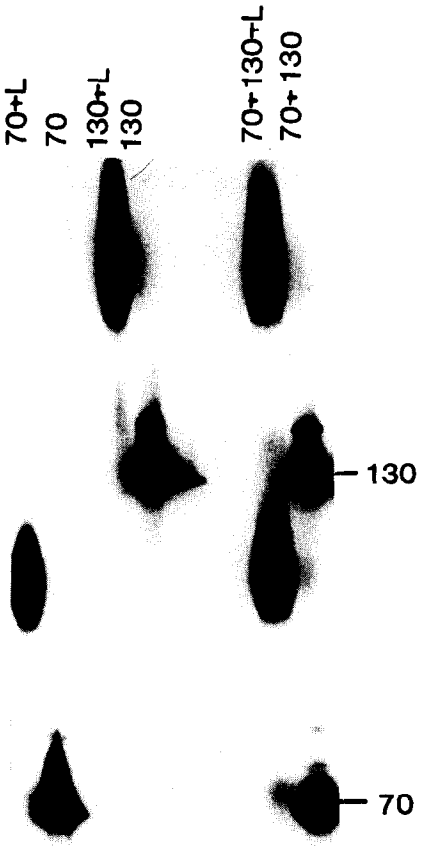

Fig. 3. Effect of 25 μM luzopeptin (L) on the mobility of the 70 and 130 base pair DNA fragments, both singly and together. The products of the reaction were separated on a 6% non-denaturing polyacrylamide gel.

8 M urea. After electrophoresis all gels were fixed in 10% acetic acid for 10 min transferred to Whatman 3MM paper, dried under vacuum at 80° and subjected to autoradiography at −70° with an intensifying screen.

RESULTS AND DISCUSSION

Nature of the reaction with DNA

We have tested the ability of luzopeptin to form interduplex cross-links by incubating the antibiotic with equal concentrations of two DNA fragments of 70 and 130 base pairs, either separately or together, and analysing the products on non-denaturing polyacrylamide gels. It can be seen from Fig. 3 that 25 μM luzopeptin decreases the mobility of both DNA fragments. If this is due to the formation of cross-linked DNA duplexes then the retarded species correspond to 70 + 70 and 130 + 130 dimers. When both species are present in equal quantities an extra band corresponding to a (70 + 130) heterodimer should be observed, running in between the 130 + 130 and 70 + 70 homodimers. No such additional band can be detected. The luzopeptin-induced changes in DNA mobility must therefore be due to drug binding to individual duplexes thereby affecting their mass and/or rigidity and length, and can not be due to any form of interduplex cross-linking.

For the drug molecule to reduce the mobility of DNA its binding must be strong (long dissociation time compared to gel running time). By contrast echinomycin even at concentrations as high as 100 μM causes no changes in the mobility of DNA fragments. Although many examples of proteins causing gel retardation can be cited we are only aware of one other instance of small molecules (diacridines) affecting DNA mobility [16]. The strength of the interaction with luzopeptin can be demonstrated by eluting the drug-retarded bands from the gel, precipitating with ethanol and rerunning on polyacrylamide gels; the DNA still retains its altered mobility. All this is suggestive of a covalent type of interaction. However, when 1 mM ethidium bromide was added to a preformed complex of 10 μM luzopeptin and DNA it caused a small but significant increase in mobility, suggesting that a portion of the drug molecules are bound reversibly. A similar result could be obtained by adding a large excess of unlabelled DNA. The interaction with DNA can be reversed using denaturing conditions (see below). We are of the opinion that the interaction is reversible but is very strong ($K_a > 10^9\ M^{-1}$).

Effects on DNA mobility

Figure 4 shows the effects of luzopeptin (1 nM–100 μM) on the mobility of tyrT DNA (160 base pairs) and the 135-base pair DNA fragment. In each case the DNA mobility decreases in a concentration dependent fashion up to a maximum value. At the highest drug concentrations the DNA is smeared, though at lower values discrete bands can be seen. We assume that each band represents the addition of a further drug molecule to the DNA helix. By measuring the distance between the bands at the lower concentrations, and assuming a linear relationship between mobility and the number of drug molecules bound, we estimate that the 160- and 135-base pair fragments can bind 34 and 28 drug molecules, respectively, i.e. 4.7 base pairs per drug. This analysis is likely to underestimate the number of ligands bound. Once about six luzopeptin molecules have been bound discrete bands are no longer observed, presumably because of either the degenerate number of ways of arranging the ligands on the DNA helix or because some slow dissociation is occurring. At the highest ligand concentration when the DNA should be saturated, a single species is still not seen. This must reflect the overlap of potential binding sites so that there is no unique way of saturating the DNA. For example if drug binding occludes four base pairs and the gap between adjacent ligands is only three bases then the helix will not be able to accommodate any further drug molecules and the apparent ligand size will be seven base pairs [17]. Therefore for a ligand occupying n base pairs binding to a lattice of N bases, when the DNA is fully saturated bands from N/n to $N/(2n-1)$ drug molecules bound should be evident, though not in equal proportions.

In order to clarify the effect of luzopeptin on DNA mobility we have studied its interaction with short DNA fragments (15–40 base pairs). Figure 5 shows

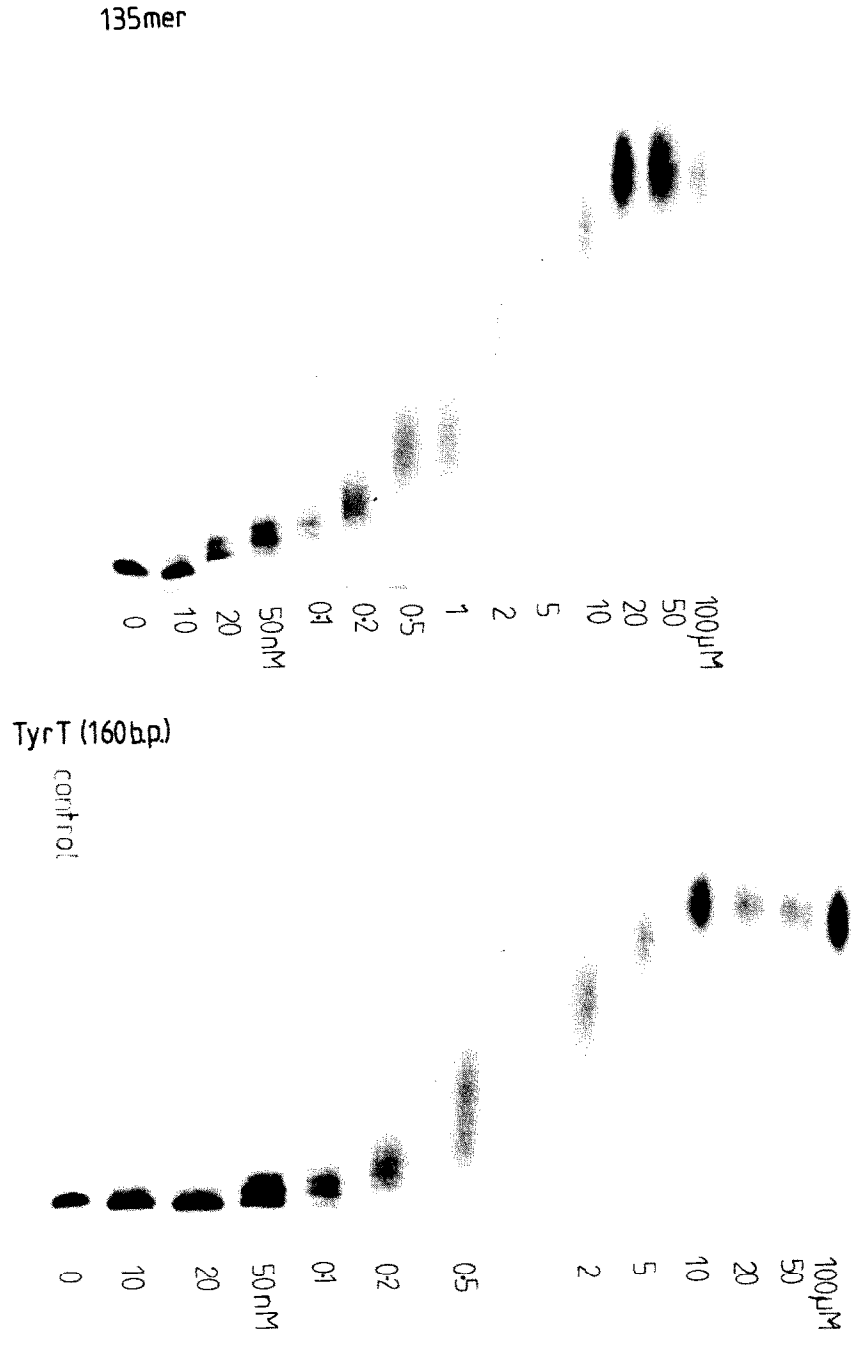

Fig. 4. Effect of various concentrations of luzopeptin on the mobility of tyrT DNA (160 base pairs) and the 135 base pair DNA fragment. Products were resolved on 5% non-denaturing polyacrylamide gels.

the effect of luzopeptin (1 nM–100 μM) on the mobility of the 24, 29 and 34 base pair DNA fragments. In each case the DNA is retarded by luzopeptin and clear bands can be seen at all drug concentrations. The maximum number of bands seen with each DNA is presented in Table 1. The data are consistent with one drug binding site for every four base pairs and suggests that luzopeptin can interact with all the available sequences. With each DNA more than one band is visible at the highest drug concentration due to the overlap in potential binding sites and the degenerate ways of saturating the DNA helix, as noted above.

It is worth noting that in several instances, especially with the shorter fragments, the amount of radioactivity detected is greater in the drug-treated than the control lanes, even though identical quantities of radiolabelled DNA was used in all lanes. This is probably because luzopeptin protects the DNA from denaturation.

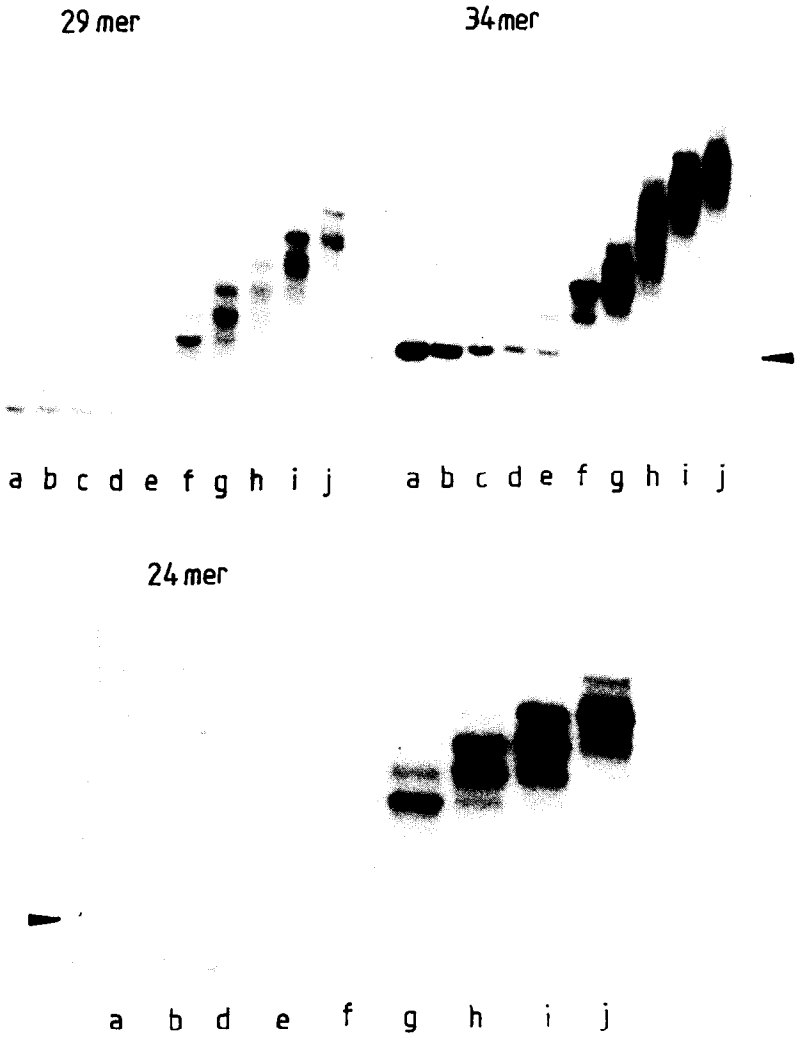

Fig. 5. Effect of various concentrations of luzopeptin on the mobility of the 24, 29 and 34 base pair DNA fragments. The concentrations of luzopeptin were: (a) 0; (b) 3 nM; (c) 10 nM; (d) 30 nM; (e) 100 nM; (f) 300 nM; (g) 1 μM; (h) 3 μM; (i) 10 μM; (j) 30 μM. The arrows mark the position of the unmodified DNAs.

Table 1. The maximum number of additional bands seen in polyacrylamide gels in the presence of luzopeptin

Fragment length	Number of bands
16	3
18	4
24	6
29	7
31	8
34	9

Effects of luzopeptin on DNA structure

Diethylpyrocarbonate (DEPC) has recently been used to assess the effects of drug binding on DNA structure [11–15]. Although it was originally intended as a probe for Hoogsteen base pairs [11, 12] it appears to detect DNA unwinding and is sensitive to the accessibility of the purine N7 atom. Figure 6 shows the results of DEPC reaction with four DNA fragments in the presence and absence of luzopeptin and echinomycin. In each case reaction in the control is weak and occurs at purines. The pattern produced by echinomycin on tyrT DNA is similar to that previously reported [11, 13] with strong enhancements at adenines at positions 32, 44, 79, 82 and 83. The pattern in the presence of luzopeptin is very different; the enhancements at positions 44 and 79 are missing and new bands can be seen at positions 51 and 67. The best luzopeptin binding sites on this fragment are between positions 55 and 70, so there is no direct correlation between the location of the binding site and DEPC-reactivity. Similar differences in reactivity of the other three DNAs to DEPC in the presence of luzopeptin and echinomycin are readily apparent. With echinomycin

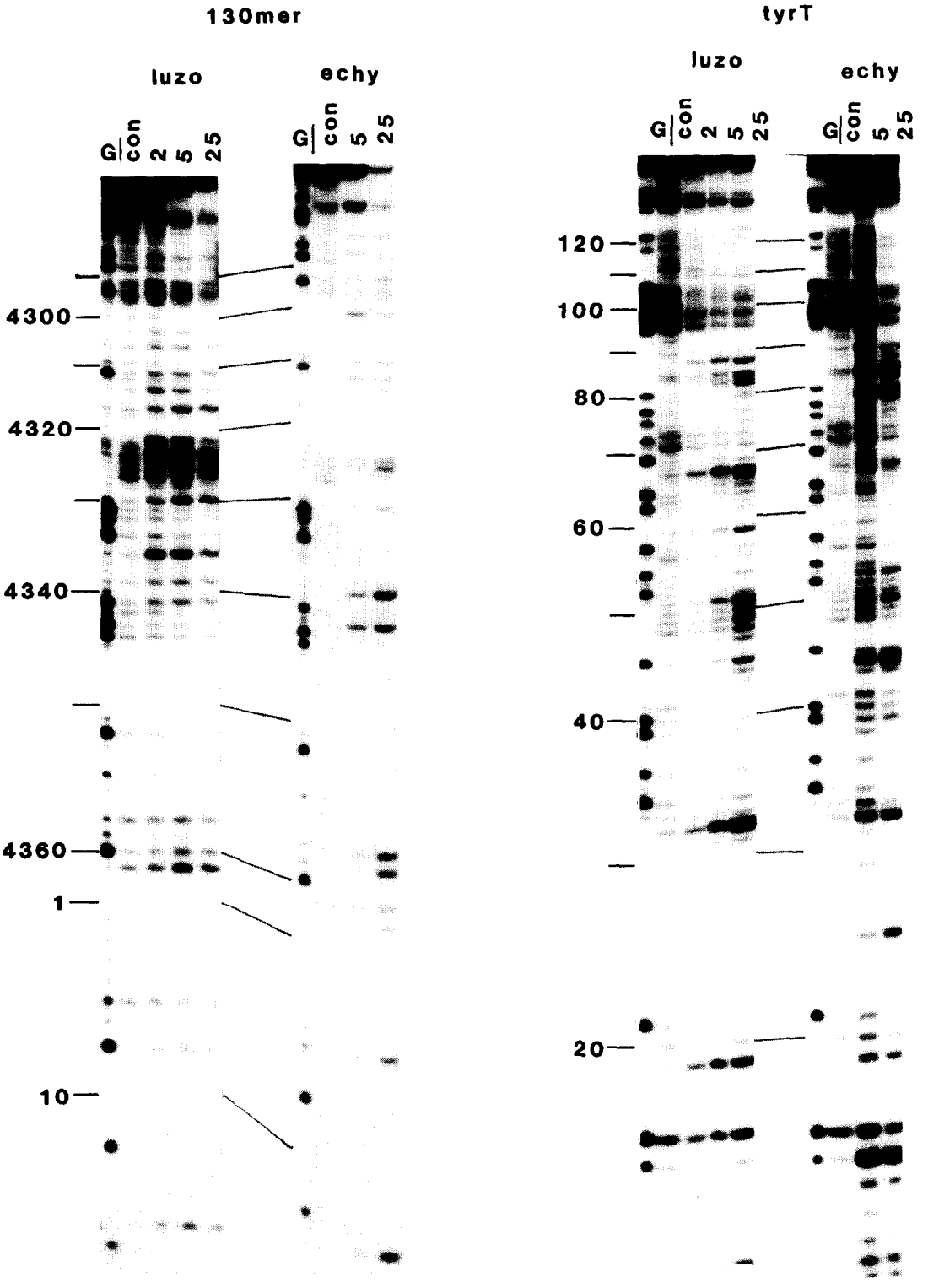

Fig. 6. Patterns of DEPC mediated DNA strand cleavage in the absence (C) and presence of luzopeptin (L) and echinomycin (E) on the four DNA fragments. The concentration of each ligand as micromolar is given at the top of each gel lane. For the 135-mer each ligand was at 15 μM. Tracks labelled "G" are dimethylsulphate-piperidine markers specific for guanine.

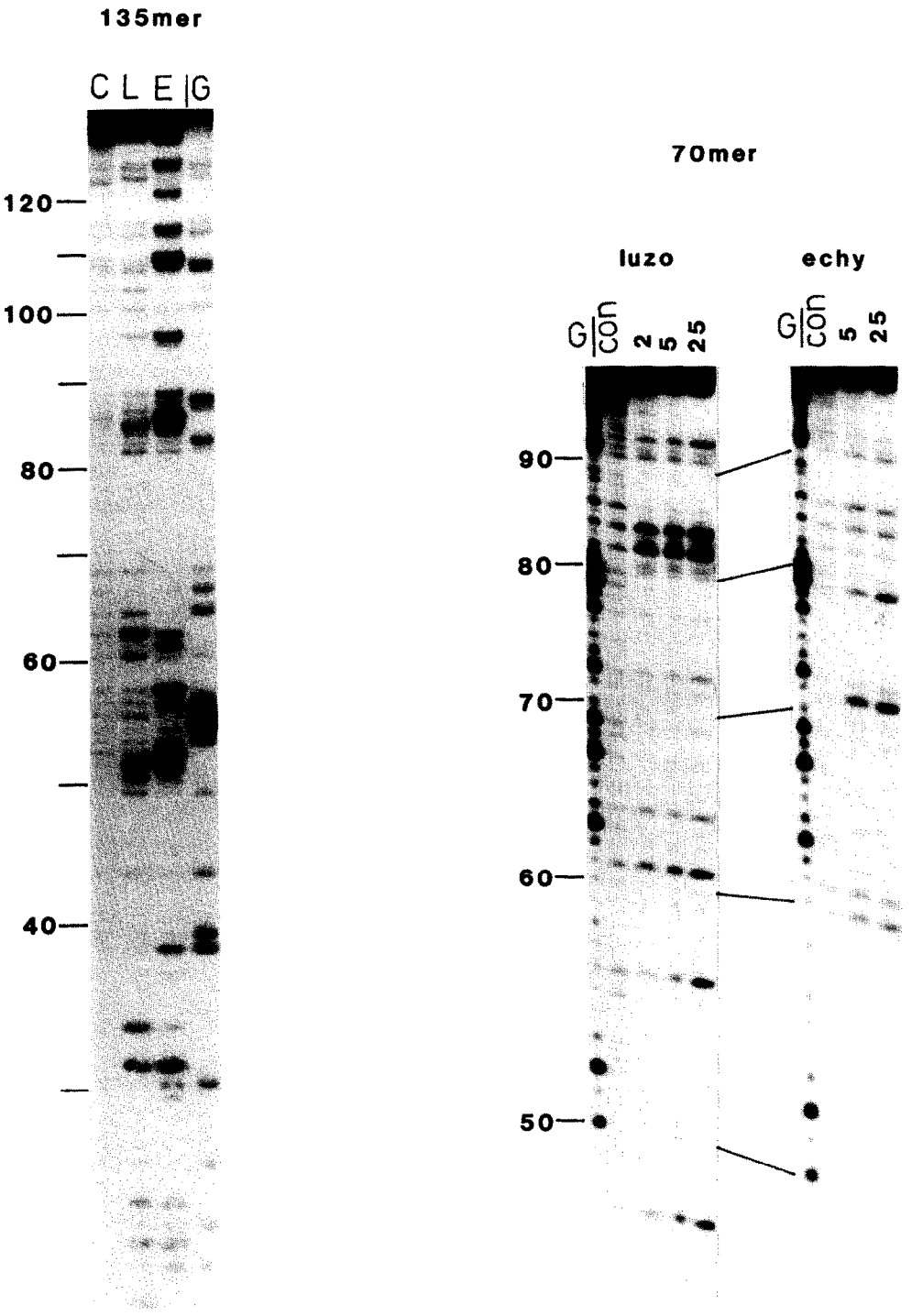

Fig. 6. *Continued.*

hyperreactive sites often appear at adenines adjacent to the putative CpG binding sites, for example around positions 4270 and 4340 on the 130-mer. The 135 and 70 base pair fragments do not possess many CpG steps flanked by adenines. Many of the DEPC reactive sites observed with luzopeptin occur at runs of adenines, for example around positions 50, 67, and 83 on tyrT DNA, 4325 on the 130-mer, and 51 on the 135-mer. Although it is not possible to explain why certain bands become hypersensitive to DEPC in the presence of each of the antibiotics, it is clear that the two ligands have different effects on DNA structure.

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REFERENCES

1. Huang C-H, Mong S and Crooke ST, Interactions of a new antitumour antibiotic BBM-928A with deoxyribonucleic acid. Bifunctional intercalative binding studies by fluorimetry and viscometry. *Biochemistry* **19**: 5537–5542, 1980.
2. Huang C-H, Prestayko AW and Crooke ST, Bifunctional intercalation of antitumor antibiotics BBM-928A and echinomycin with deoxyribunucleic acid. Effects of intercalation on deoxyribonucleic acid degradative activity of bleomycin and phleomycin. *Biochemistry* **21**: 3704–3710, 1982.
3. Arnold E and Clardy J, Crystal and molecular structure of BBM-928A, a novel antitumor antibiotic from actinomadura luzonensis. *J Amer Chem Soc* **103**: 1243–1244, 1981.
4. Searle MS, Hall JG and Wakelin LPG, ^{1}H and ^{13}C-n.m.r. studies of the antitumor antibiotic luzopeptin. Resonance assignments, conformation and flexibility in solution. *Biochem J* **256**: 271–278, 1988.
5. Fox KR, Davies H, Adams GR, Portugal J and Waring MJ, Sequence-specific binding of luzopeptin to DNA. *Nucl Acids Res* **16**: 2489–2507, 1988.
6. Huang C-H, Mirabelli CK, Mong S and Crooke ST, Intermolecular cross-linking of DNA through bifunctional intercalation of an antitumour antibiotic, luzopeptin A (BBM-928A). *Cancer Res* **43**: 2718–2724, 1983.
7. Low CML, Drew HR and Waring MJ, Sequence-specific binding of echinomycin to DNA: evidence for conformational changes affecting flanking sequences. *Nucl Acids Res* **12**: 4865–4879, 1984.
8. Van Dyke MW and Dervan PB, Echinomycin binding sites on DNA. *Science* **225**: 1122–1127, 1984.
9. Searle MS, Hall JG, Denny WA and Wakelin LPG, Interaction of the antitumour antibiotic luzopeptin with the hexanucleotide duplex $d(5'GCATGC)_2$; one and two-dimensional NMR studies. *Biochem J* **259**: 433–441, 1989.
10. Fox KR, Footprinting studies on the interactions of nogalamycin, arugomycin, decilorubicin and viriplanin with DNA. *Anti-Cancer Drug Design* **3**: 157–168, 1988.
11. Portugal J, Fox KR, Mclean MJ, Richenberg JL and Waring MJ, Diethyl pyrocarbonate can detect a modified DNA structure induced by the binding of quinoxaline antibiotics. *Nucl Acids Res* **16**: 3655–3670, 1988.
12. Mendel D and Dervan PB, Hoogsteen base pairs proximal and distal to echinomycin binding sites on DNA. *Proc Natl Acad Sci USA* **84**: 910–914, 1987.
13. McLean MJ and Waring MJ, Chemical probes reveal no evidence of Hoogsteen base pairing in complexes formed between echinomycin and DNA in solution. *J Mol Recog* **1**: 138–151, 1988.
14. Jeppesen C and Nielsen PE, Detection of intercalation-induced changes in DNA structure by reaction with diethylpyrocarbonate or potassium permanganate. Evidence against the induction of Hoogsteen base pairing by echinomycin. *FEBS Lett* **231**: 172–176, 1988.
15. Fox KR and Grigg GW, Diethylpyrocarbonate and permanganate provide evidence for an unusual DNA conformation induced by binding of the antitumour antibiotics bleomycin and phleomycin. *Nucl Acids Res* **16**: 2063–2075, 1988.
16. Nielsen PE, Zhen W, Hendriksen U and Buchardt O, Sequence-influence interactions of oligoacridines with DNA detected by retarded gel electrophoretic migrations. *Biochemistry* **27**: 67–73, 1988.
17. McGhee JD and Von Hipple JD, Theoretical aspects of DNA-protein interactions: cooperative and non-cooperative binding of large ligands to a one dimensional homogeneous lattice. *J Mol Biol* **86**: 469–489, 1974.