Binding of actinomycin D to DNA revealed by DNase I footprinting

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We have analyzed the specificity of the actinomycin D–DNA interaction. The 'footprint' method has been used in this investigation. It is shown that: (i) The presence of dinucleotide GC or GG is required for binding of a single drug molecule. (ii) The strong binding sites are encoded by tetranucleotide XGCY; where X ≠ G and Y ≠ C in accordance with RNA elongation hindrance sites [1]. (iii) There is a positive cooperativity in binding of actinomycin D with DNA.

Actinomycin D *Promoter* lac UV5 *DNA–ligand interaction* *Footprint* *DNase I*

1. INTRODUCTION

Actinomycin D (act D) is typical of a wide group of base specific DNA ligands. Act D is believed to be guanine specific, requiring for binding a single guanine base [2] or a dinucleotide GpC [2]. According to the currently accepted view, it occupies a 4.5 nucleotide base pair (bp)· long space in the small groove of the B DNA and excludes binding of another act D to the region.

In our study of RNA elongation inhibition by act D we have found that the inhibition efficiency is nucleotide sequence dependent. The most prominent inhibition sites are encoded by a consensus tetranucleotide 5′XGCY, where X is any nucleotide but G, and Y is any nucleotide but C. Therefore, we analyzed footprints [4] of act D·DNA complex in order to verify that the antibiotic binding sites correspond to the RNA synthesis inhibition sites. We here present data showing that the antibiotic really binds to the above-mentioned sites and that the binding is cooperative.

2. MATERIALS AND METHODS

The *lac UV5* tandem promoters of plJ-3 plasmid [5] was recloned to pBR322. It was made by Dr L.P. Savochkina in this laboratory via insertion of purified *Eco*RI flanked 285 bp *lac UV5* tandem promoters into the *Eco*RI site of pBR322. *E. coli* K12 was used as a host strain. The cloning procedure is described in [6]. The plasmid was used for preparation of *lac UV5* promoter-operator containing DNA fragment labelled at the 3′ ends as in [7].

DNase I (Worthington) digestion was performed as in [4]. DNA was incubated with act D (Reanal) at 22°C for 3 h in 100 μl of 20 mM Tris–HCl buffer (pH 8.0), 10 mM $MgCl_2$, 5 mM $CaCl_2$, 0.1 mM DTT and 10 mg/l BSA. DNA and act D concentrations are stated in the legends to fig.1,2,3. The concentration of ^{32}P-labelled *lac UV5* promoter-operator DNA fragment was 0.5 mg/l for all experiments. DNA of pBR322 plasmid was used as a carrier when higher DNA concentrations were needed. DNase I was added to 0.13 mg/l for 30 s.

Digestion was terminated by adding 100 μl of 0.2 M LiCl with 100 mM EDTA. DNA was then treated with 1 vol. of hot phenol. After alcohol precipitation the DNA preparations were electrophoresed as in [4]. DNA sequence reactions were performed as in [8].

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3. RESULTS AND DISCUSSION

3.1. *Location and length of protected regions*

Fig.1 shows a typical autoradiogram of a footprint made under different act D concentrations. Upon inspection of this and other similar electrophoregrams one can see that the specificity of act D binding under the limited ligand concentration is rather high. There are only a few sites protected against DNase I action within a 100 bp long naturally occurring DNA. Protection grade is not the same at different sites. For instance, while at 5×10^{-7} or 10^{-6} M act D the region in the proximity of the −20th nucleotide is fully protected, the regions near the −45th and near the +10th nucleotides are only partially protected.

The number of discrete regions protected against DNase action does not depend upon the increase in act D (fig.1). Instead, the protected regions enlarged with increasing ligand concentration (fig.2). But even under the highest (nearly saturated) act D concentration a large portion of GC-containing DNA sequence regions remains unprotected.

The sequence is shown in fig.3 which presents in schematic form the results of the experiments shown in fig.1,2. Fig.3 demonstrates that act D protects roughly the same regions of the opposite DNA strands, though these regions are slightly shifted to the 3′ ends of the corresponding strands. It is apparent from fig.3 that the length of the protected regions increases stepwise with increase in act D concentration. The lengths are equal to 7–8, 11–12, 14–16 and 18–20 bp. Each step of the series seems to be about 4 bp in agreement with the 'anticooperative length' of the antibiotic [2]. The shortest protected region, however, is 7–8 bp. This could mean either that the minimal size of region protected against DNase by a single act D molecule is 7–8 bp or that the binding of at least 2 molecules of act D is needed to protect DNA. We have insufficient data to make an unambiguous choice, but we prefer the first explanation.

3.2. *Cooperativity of act D binding*

Anticooperativity of act D binding is well known [2]. The binding region widening upon the increase of act D concentration found in this study is strictly in favor of positive cooperativity of dye–DNA interaction. This interaction is also supported by the ability of act D to occupy $\overset{5'\text{CTCATTA}\,3'}{3'\text{GAGTAAT}\,5'}$ (near

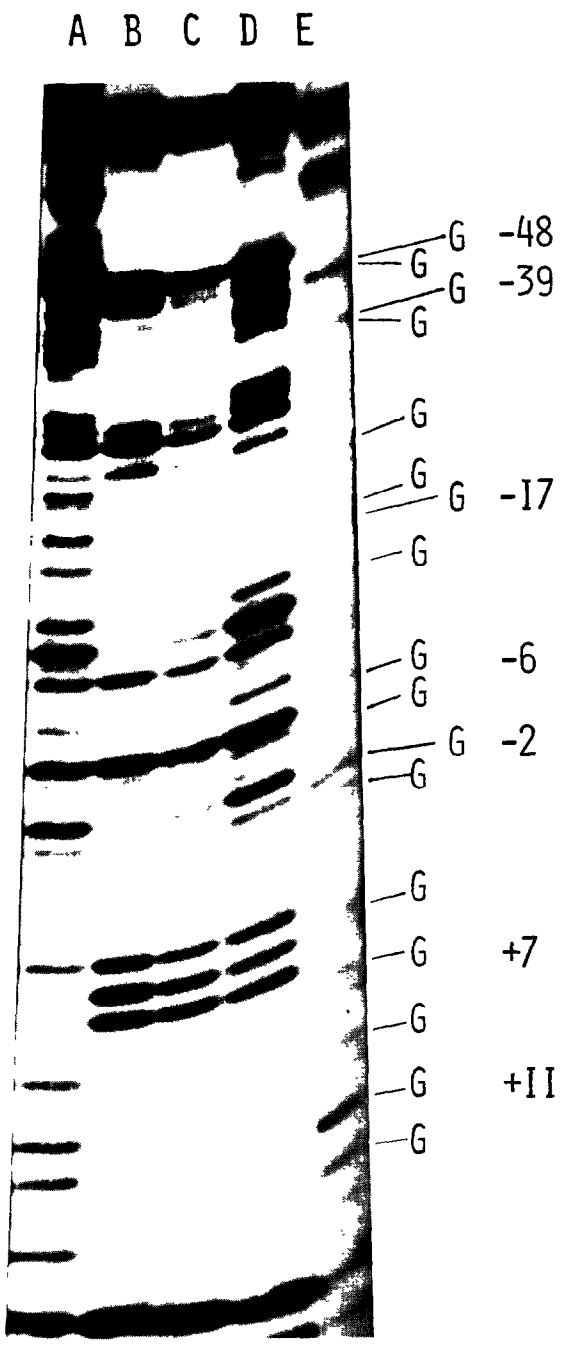

Fig.1. Footprint of act D–*lac UV5* promoter-operator complex. DNA concentration 2.3 mg/l (4 μM nucleotide bp). Upper strain is labelled. Polyacrylamide concentration was 20%. Concentration of act D: (A) 0 μM, (B) 8 μM, (C) 2 μM, (D) 0.5 μM, (E) A + G dimethyl sulfate reaction. DNA G nucleotides are numbered as in fig.3.

Fig.2. Cooperativity of act D–DNA binding. DNA concentration was 0.5 mg/l (0.8 μM nucleotide bp). Lower strain of DNA is labelled. Polyacrylamide concentration was 20%. (A) A + G dimethyl sulfate reaction; act D concentration: (B) 1 μM, (C) 10 μM, (D) 20 μM.

position −50) and $^{5'\text{CACTTTA}\,3'}_{3'\text{GTGAAAT}\,5'}$ (near position −30). These AT-rich sequences are situated close to strong act D binding sites located near the −45th and −25th nucleotides (fig.3). Under the same conditions a similar sequence $^{5'\text{CACATTA}\,3'}_{3'\text{GTGTAAT}\,5'}$ is not occupied by act D. This sequence is situated near the −5th nucleotide which is not in close proximity to any strong binding site. The positive cooperativity mode of the act D–[d(G–C)] interaction was also found by an independent method in [9].

3.3. *Nucleotide sequence preferable for act D binding*

At low act D concentration (10^{-6}–10^{-7} M), cooperativity of interaction is negligible as far as each binding site is occupied by a single dye molecule. In this case the minimal requirement for the nucleotide sequence of DNA to induce binding seems to be the presence of dinucleotide GC or GG in the tetranucleotide occupied by act D. Dinucleotides CG, TG, AG, GT and GA are unable to induce act D binding. The most prominent sites of protection (fig.3) seem to be associated with tetranucleotide described by the formula XGCY where $X \neq G$ and $Y \neq C$ in agreement with the most prominent RNA elongation hindrance sites [1]. The same tetranucleotides are located at the most prominent nick-translation hindrance sites situated between the 135th and 142th and between the 188th and 191st nucleotide of a pBR313 fragment [10]. A single site protected by act D (act 3×10^{-6} M) against digestion with methidium–propyl–EDTA–Fe(II) complex [11] is also associated with oligonucleotide described by the above-mentioned formula. It is CGCGCA located between the 4255th and 4261st nucleotides of pBR322. Thus, the act D recognition sequence for the most prominent binding sites exhibited by a variety of different methods seems to be in concord. It looks probable that act D binds to XGCY or XGGY symmetrically to the phosphate following the 5′G. RNA elongation slows down or stops entirely when the last incorporated RNA nucleotide is X or G, respectively [1]. So, the inhibition occurs within the act D binding region. While DNA chain elongation seems to be interrupted just upstream of X, if 5′–3′ exonuclease precedes polymerisation, it might also be inhibited within the apparent act D binding site. As far as the DNA synthesis is a partially distributive process, it seems logical to assume that reinitiation of DNA elongation is a probable second target of inhibition for act D. If so, the last incorporated nucleotide of the arrested

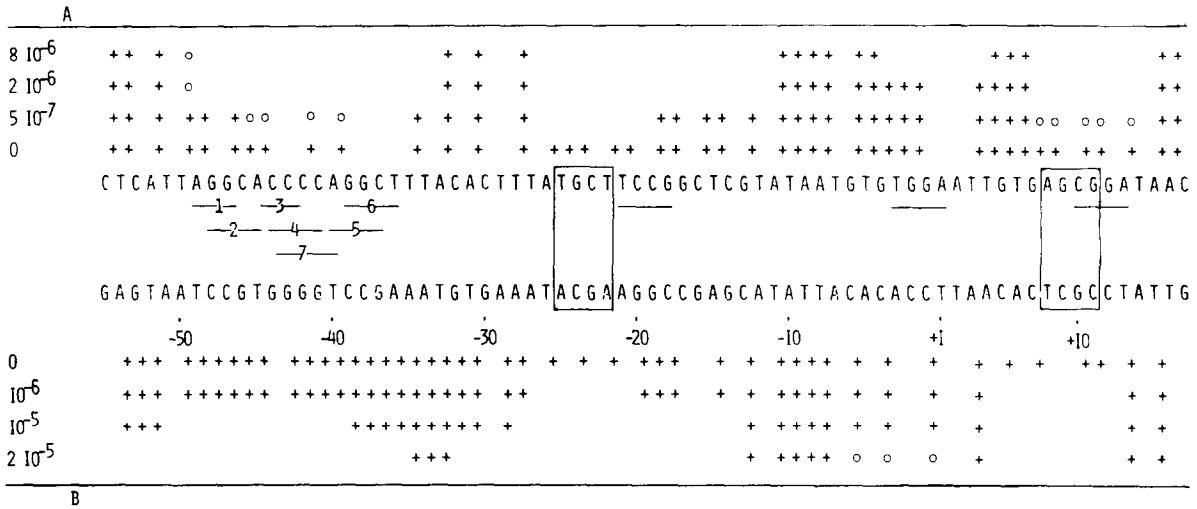

Fig.3. Schematic representation of act D binding sites along the *lac UV5* promoter-operator region. Asterisks indicate sites cut down by DNase I. Open circles denote sites where the digestion was partially inhibited by act D. Concentrations of act D are shown to the left. (A) Upper chain is labelled; DNA concentration 4 μM bp. (B) Lower chain is labelled; DNA concentration 0.8 μM bp. Concensus tetranucleotide – XCGY, where X $\neq$ G and Y $\neq$ C are boxed. Putative binding sites of act D molecules are underlined. Inserted numbers designate alternative putative binding sites discussed in the text.

DNA chain might be Y or a nucleotide immediately following Y. Inhibition at both sites of the putative act D strong binding sites is described in [10].

As far as DNase I hydrolysis is concerned it seems that the enzyme can freely cut DNA 3 nucleotides to the 5′ side and 5 nucleotides to the 3′ side from the center of the binding sequence

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↓↓↓          ↓
5′NNNNGCNNNN3′
3′NNNNCGNNNN5′
 ↑           ↑↑↑
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This generalized formula is derived from the superposition of protection patterns of all the separately situated act D binding sites and describes the protection pattern of idealized 'unspecific DNase'. These are sites situated at the −2 to +1st, +8 to +11th and −26 to −22nd nucleotides in fig.3. The idealized protection pattern is taken to be symmetrical with respect to both DNA strains. No real protection site corresponds to the formula due to clear nucleotide sequence specificity of pancreatic DNase I, but the formula does not contradict any of the actually observed sites. The formula is most closely illustrated by a site situated near the +1st nucleotide which is occupied by a single act D molecule. Here, the upper chain digestion pattern is organized such that both 3′ and 5′ protection boundaries are localized unequivocally.

The analysis of binding at weaker sites, especially at overlapping binding sites, is much more complicated. An example of such a region is AGGCACCCCAGGCT located between the −50th and −36th nucleotides in fig.3. Here there are 7 overlapping putative binding sites (underlined); the shielding (but not binding) apparently starts with ACCC (fig.1). The protection of the site is not complete, so either there is a competitive binding to adjacent overlapping sites (probably 3, 4 or 7, see fig.3) or the act D binding constant is not high enough to yield full protection. Upon act D concentration increase 3 non-overlapping sites out of a putative 7 sites become occupied (1, 3 and 5; 2, 4 and 5; 2, 4 and 6; or 2, 7 and 6) creating full protection of the region from the −50th to the −36th nucleotide. Binding to the regions located in the vicinity of the −20th and the +10th nucleotides can be analyzed in a similar way. But in this case the first binding site is certainly the XGCY. We have no criteria to decide which of the weak sites will be occupied first. Probably, a

number of factors including local DNA structure may influence the binding.

It seems important to note that incomplete protection (as well as DNA hydrolysis activation) of central regions can be induced by an indirect influence of act D bound to some distant sites due to change of the local DNA structure. The resulting redistribution of the DNA digestion pattern may be misinterpreted as evidence of ligand binding. The methods used in this and other studies of nucleotide sequence specificity of ligand–DNA binding are indirect, so the conformity of apparent main sites revealed by all the methods gives certainty that the results really reflect the feature of act D–DNA binding, and do not specify the DNA–enzyme interaction mode.

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