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The 5′-CA DNA-sequence preference of daunomycin

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The DNA-sequence specificity of daunomycin was investigated by DNase I footprinting and an *E. coli* RNA polymerase transcription-inhibition assay. The 5′-CA sequence was identified as being the highest affinity binding site, although other modest affinity (5′-GC, CG, CT, TC, AC) and poor affinity sites (5′-AA, AT, TA) were also observed. The preference of daunomycin for 5′-CA nucleotide sequence suggests that its biological activity may arise from association with the 5′-CA-containing sequences thought to be associated with genetic regulatory elements in eukaryotes.

Daunomycin; DNA; Footprinting; Sequence selectivity; Transcription inhibition

1. INTRODUCTION

In the light of a growing awareness of the importance of DNA-sequence recognition by a number of antibiotics [1–3], there have been several attempts to ascertain the DNA-sequence specificity of anthracycline anti-cancer drugs, such as daunomycin and adriamycin (fig.1). However, there has been no consensus from either the experimental evidence [3–5] or theoretical computations [6–8] concerning the nature of DNA-sequence discrimination by daunomycin.

Although footprinting techniques have emerged as powerful tools for analyzing drug/DNA association phenomena [1,2], they have been unsuccessful in providing a clear indication of the DNA-sequence specificity of daunomycin [9,10]. As these techniques rely on a competitive equilibrium between a drug and a destructive probe for the DNA substrate, this failure suggests a reexamination at subphysiological temperatures, where the kinetics of drug-DNA dissociation would be slower [10].

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Here, we present the first experimental evidence of the DNA-sequence specificity of daunomycin using DNase I footprinting and an RNA polymerase transcription-inhibition assay at reduced temperatures.

2. EXPERIMENTAL

2.1. *Reagents*

Daunomycin was a gift from Farmitalia (Carlo Erba, Milan). The 203-bp *Eco*RI fragment of *lac* DNA containing the L8-UV5 double mutant was kindly supplied by Professor D.M. Crothers (Yale University).

2.2. *DNase I footprinting*

DNase I footprinting was performed as in [11] using a

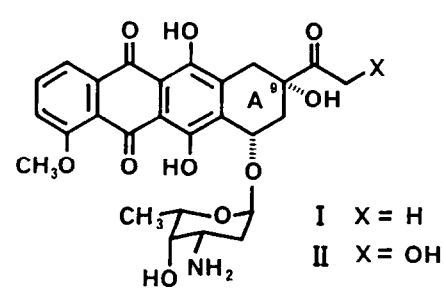

Fig.1. Structure of daunomycin (I) and adriamycin (II).

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*Hin*dIII/*Pvu*II restriction fragment from pSP65, labelled at the 3′-terminus of the *Hin*dIII restriction site using [α-^{32}P]dATP and AMV reverse transcriptase. The nucleotide sequence of the restriction fragment was established by G-specific cleavage [12] with reference to the known sequence of the plasmid [13].

2.3. *Transcription inhibition*

Transcription with *E. coli* RNA polymerase in vitro [14] utilised a 497-bp *Pvu*II/*Sal*I restriction fragment of a modified pBR322 vector lacking the *tet* and P1 promoters and containing the 203-bp *Eco*RI *lac* DNA fragment ligated into the unique *Eco*RI site [16]. Nucleotide sequencing of the RNA transcript was performed using 3′-*O*-methyl-CTP and 3′-*O*-methyl-ATP [17].

2.4. *Electrophoresis and autoradiography*

Electrophoresis, autoradiography and densitometric analysis were performed using standard techniques [18].

3. RESULTS

3.1. *DNase I footprinting*

The equilibrium between the drug and DNA was probed by DNase I at several temperatures and a range of drug/DNA ratios. It was apparent from the autoradiograms (not shown) that the inhibition of enzymatic hydrolysis was dependent on both the DNA sequence and the drug/DNA ratio, ν. This effect was much more pronounced at 5°C than at 25°C, and only the data taken at 5°C are presented here. Below ν = 0.03, the apparent sequence-dependent inhibition of DNase I became independent of ν. Thus, to ensure that the highest affinity binding sites of daunomycin were being probed, densitometric analysis of the autoradiograms was confined to very low drug/DNA ratios.

In fig.2, the ordinate represents the percentage ratio of the areas under complementary bands in a control lane (ν = 0) to that of a suitable digest (ν = 0.025). Domains of cleavage inhibition (ordinate values less than 100%) correspond to the drug-binding sites. A comparison of the number of times a dinucleotide sequence is implicated as a binding site (n) to the sum of all such sites in the visualized DNA fragment (N) demonstrates that the highest affinity binding site is (5′ or 3′)-CA (n and n/N% of 20 and 91%, respectively) followed by lower affinity sites (5′ or 3′)-CG (5, 55%), (5′ or 3′)-CT (7, 50%), (5′ or 3′)-GG (2, 33%), (5′ or 3′)-AT (2, 20%) and (5′ or 3′)-AA (3, 21%).

3.2. *Inhibition of RNA polymerase transcription activity*

Elongation of the RNA transcript was allowed to proceed at 10°C in the presence and absence of daunomycin (ν ~0.03) for various time intervals (not shown). A densitometric analysis of the 30 s lane on the autoradiogram is presented in fig.3. Sites where drug induced pausing corresponding to natural pausing have been ignored, except where a clear drug-induced enhancement is apparent on the corresponding control lane. Of the nine drug-induced block sites, seven involve transcription up to a 5′-CA site, with the other two being 5′-CG sites. The transcription was observed up to the nucleotide on the upstream (5′) side of the drug site, or one nucleotide further upstream. The transcription-inhibition method appears insensitive to the lower affinity binding sites detected by DNase I footprinting, and we have taken the sequences at which transcription-inhibition occurs as betraying the highest affinity receptor sites for daunomycin.

The unambiguous conclusion drawn from analysis of the transcription data is that daunomycin associates preferentially with 5′-CA sequences and that 5′-CG sequences form a lower affinity binding site.

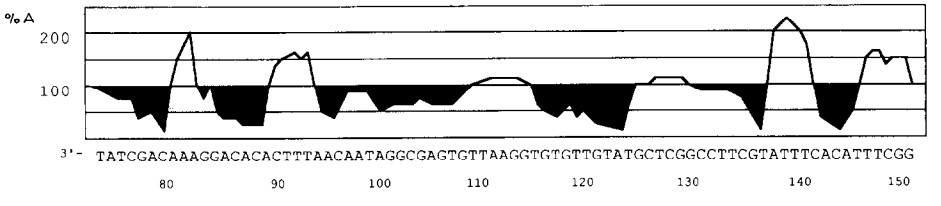

Fig.2. Densitometric analysis of the DNase I footprinting data. The ordinate represents the fractional (%) enzymatic cleavage (ν = 0.025 compared to ν = 0) plotted as a function of the nucleotide sequence visualized on the autoradiogram (not shown). The darkened areas correspond to regions of diminished enzymatic cleavage and indicate drug-binding sites.

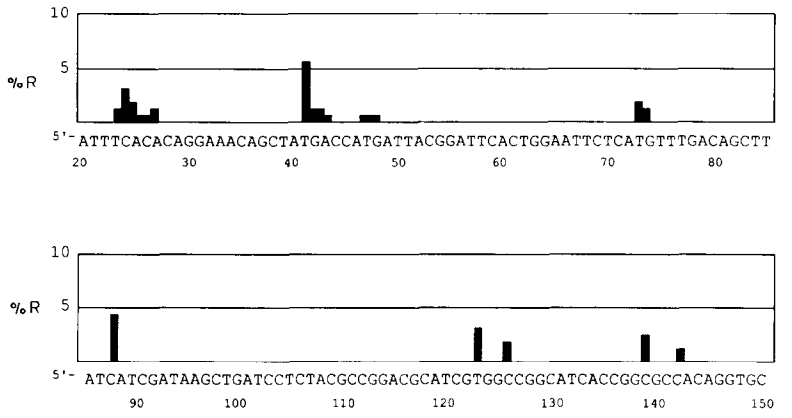

Fig.3. Transcription-detected drug block sites. The data were obtained from densitometric analysis of the 30 s elongation track on the autoradiogram (not shown). The transcribed sequence is shown from the nucleotides +20 to +150 downstream of the GpA-initiated UV5 promoter. The ordinate represents the percentage blockage normalized against the total radioactivity incorporated into transcripts beyond 10-mers. The effects of natural pausing were subtracted from the apparent drug-induced blockage. Drug-induced block sites were ignored if they did not exceed natural pausing by 15%.

4. DISCUSSION

In consideration of both the footprinting and transcriptional data, the overall sequence specificity of daunomycin appears very similar to adriamycin and bis-adriamycin [15,16], being manifested as a distribution between high affinity (5′-CA), modest affinity (5′-AC, CG, GC, TC, CT, CC) and low affinity (5′-TA, AT, AA) sites. The observed inhibition of *HpaI* by daunomycin [19] is consistent with our observations. The two restriction sites corresponding to the low (IC_{50}, 12.4 μM) and high affinity (IC_{50}, 6.7 μM) drug-binding sites contained one and two 5′-CA flanking sequences, respectively. DNase I footprinting studies on the related anthracycline nogalomycin, have also implicated (5′ or 3′)-CA sequences as the highest affinity binding sites [20]. Moreover, eight of the twelve reported adriamycin-induced DNA polymerase-inhibition sites are associated with neighbouring CA sequences [21].

In accord with the 5′-pyrimidine-purine-3′ sequence preferences of intercalators [22] and the importance of the OH (9) moiety in conferring biological activity [23] and stabilizing the drug/DNA interaction [24], several theoretical computations suggest the 5′-CA sequence as a high affinity binding site [13,14]. Both our results [15,16] and those of others [20,21] on related anthracyclines indicate that some 5′-CA sites remain unoccupied. Although it is certain that flanking sequences modify the intrinsic sequence preference of daunomycin, the nature of this relationship is presently obscure.

Given the prevalence of alternating CA tracts in eukaryotes [25] and the postulated role of their B←→Z conversion [26] in the activation of domains of DNA [27], the documented ability of daunomycin to reverse the B←→Z transition [28] suggests that its biological activity may arise from the preferential interaction with the 5′-CA genetic regulatory elements in DNA [29].

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