Sequence-specific inhibition of RNA elongation by actinomycin D

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We have developed a method to localize specific sites where RNA elongation is arrested due to DNA-bound ligands. The method was used to determine apparent binding sites for actinomycin D. We have found 14 strong RNA hindrance sites along nucleotide sequence of T7 and D111 T7 DNA of 380 nucleotides full length under low actinomycin D concentration conditions. Nucleotide sequence of all the sites is described by general formula XGCY where X ‡ G and Y ‡ C.

RNA polymerase, of E. coli *Transcription, in vitro Inhibition Actinomycin D Binding specificity RNA sequencing*

1. INTRODUCTION

Interaction of DNA with ligands capable of transcription inhibition has been thoroughly investigated (review [1]). The interaction of actinomycin D (act.D) with DNA [2] is the best studied. Nevertheless, the nucleotide sequence most preferable for act.D binding is not known. To reveal the sequence we have used the method in [3–6] for studying nucleotide sequence dependence of RNA elongation rate. We have searched for the sequences where RNA elongation is retarded due to the presence of act.D.

This report shows that act.D preferentially interrupts elongation in the vicinity of the tetranucleotide 5′ẊĠCY3′, where X ‡ G and Y ‡ C. The last nucleotide of the retarded RNA chain is either X or G, as indicated by dots above the letters. Other DNA sequences are also capable of binding act.D. However, much higher concentrations of act. D are needed to observe RNA elongation inhibition at these sites.

2. MATERIALS AND METHODS

2.1. *Preparation of ternary complex of RNA polymerase with DNA template and labeled nascent RNA*

T7 or D111 T7 DNA (0.1 g/l; 0.13 mM basepairs) was incubated at 20–22°C in 25–100 μl mixture containing: buffer A [50 mM Tris–HCl (pH 8.0), 0.1 M KCl, 5 mM $MgCl_2$], 2 μM each of CTP, GTP, UTP and [α-^{32}P]ATP (100–400 Ci/mmol), 0.1 mM CpA, 10 mg/l of *E. coli* RNA polymerase. RNA synthesis was initiated by adding the enzyme and terminated after 6 or 15 min with 50 mM EDTA. Under the conditions employed, RNA synthesis was initiated mostly from the A1 promoter and the nucleotide sequence of nascent RNA is readily followed [3,5]. The ternary complex containing nascent RNA of 60–160 nucleotides was purified from free NTPs by gel-filtration through a 14 × 0.3 cm A-1.5 m agarose gel column equilibrated with buffer A [4].

2.2. *RNA chain elongation and pyrophosphorolysis*

The ternary complex was incubated with

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1–10 μM act.D for 30 min at 20°C. The complex was then divided into 5–10 μl portions and 0.5 mM $Na_4P_2O_7$ or a mixture of 0.5 mM of each of the 4 unlabelled NTPs were added to initiate reactions. Both reactions were terminated with 50 mM EDTA.

2.3. *Nascent RNA sequencing with 3′-OCH$_3$-NTPs*

This was done as in [3,4]. To terminate RNA at a certain nucleotide, 100 μM each of the 4 3′-OCH$_3$-NTPs were added to the 4 separated 5 μl samples at 6–8 min of the [^{32}P]RNA synthesis reaction. The nucleotide sequence was in agreement with DNA sequence of T7 [7]. Electrophoresis conditions and other experimental details are described in [3,5].

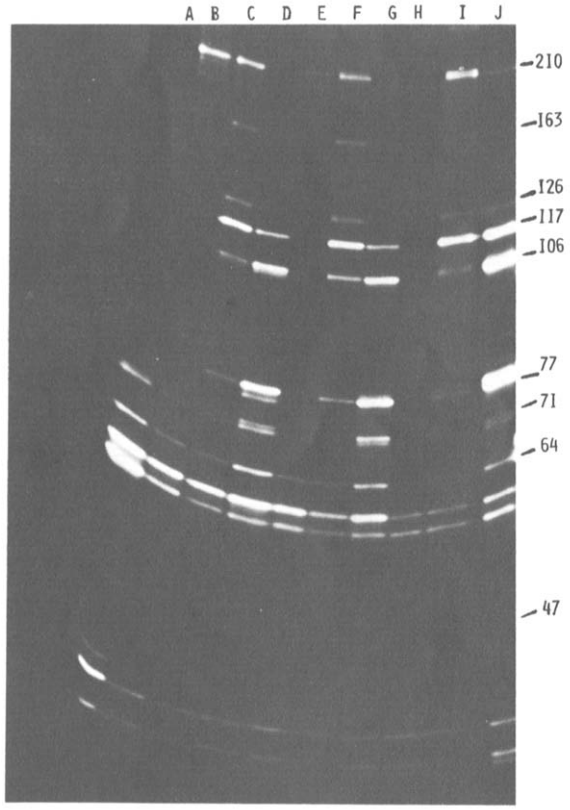

Fig.1. Inhibition of DIII T7 RNA elongation by act.D. Autoradiography of [^{32}P]RNA after electrophoresis in 20% polyacrylamide gel: (A) original ternary complex synthesized for 6 min from DIII T7 DNA; (B,E,H) the ternary complex the same as in (A) incubated with 0.5 mM of unlabelled NTPs for 10 s, 30 s and 30 min, respectively; (C,F,I) the same as (B,E,H), but before NTP addition the ternary complex was incubated with 1 μM act.D for 30 min; (D,G,J) the same as (C,F,I), but at 10 μM act.D. Nucleotides are numbered starting from the initiating dinucleotide CpA. Indicated numbers correspond to the 3′-terminal nucleotide residues of retarded RNAs.

3. RESULTS

3.1. *Location of RNA chain elongation hindrance sites*

Distribution of the nascent RNA length in the original ternary complexes is shown in fig.1A, 2A and 3H. The complexes were used as starting

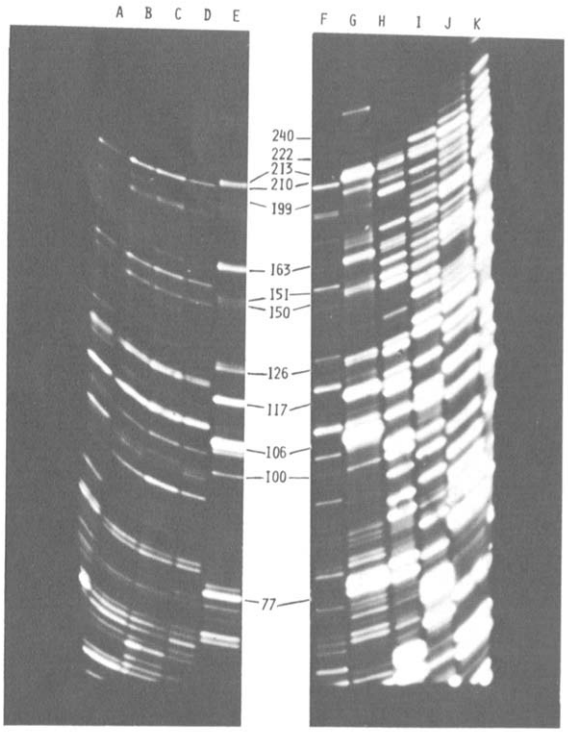

Fig.2. Influence of act.D on DIII T7 RNA elongation and pyrophosphorolysis. Autoradiography of [^{32}P]RNA after electrophoresis in 15% polyacrylamide gel: (A) initial ternary complex synthesized for 15 min; (B,C) RNA pyrophosphorolysis for 1 and 20 min, respectively; (D,F) as (B,C) but in the presence of 10 μM act.D for 1 and 20 min, respectively; (E,G) RNA elongation at 10 μM act.D for 1 and 20 min, respectively; (H–K) RNA sequencing with 3′-OCH$_3$-CTP, 3′-OCH$_3$-GTP, 3′-OCH$_3$-ATP and 3′-OCH$_3$-UTP, respectively.

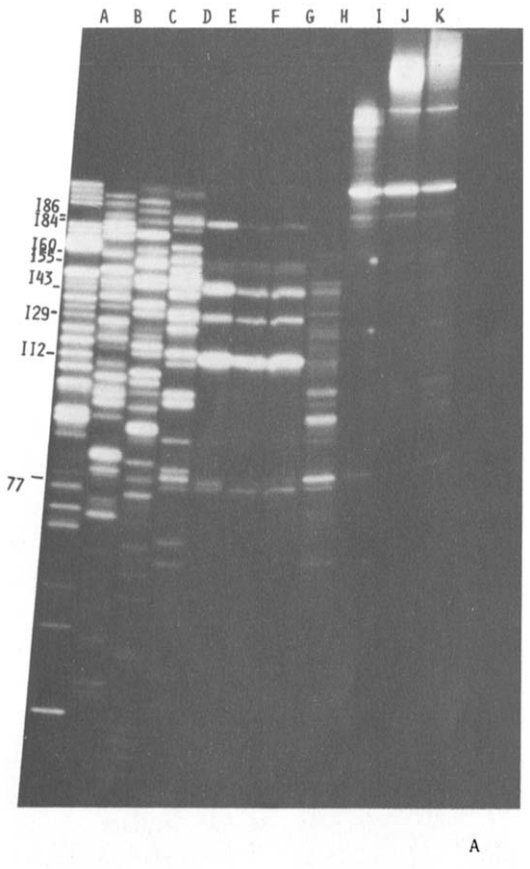

Fig.3. Inhibition of T7 RNA elongation by act.D. Autoradiography of [^{32}P]RNA after electrophoresis in 20% polyacrylamide gel: (H) initial ternary complex synthesized for 6 min; (I–K) RNA was elongated with 0.5 mM of unlabelled NTP without act.D for 30 s, 2 min and 30 min, respectively; (E–G) RNA was elongated with 0.5 mM of unlabelled NTP in the presence of 10 μM act.D for 30 min, 2 min and 30 s, respectively; (A–D) RNA sequencing with 3′-OCH_3-UTP, 3′-OCH_3-ATP, 3′-OCH_3-GTP and 3′-OCH_3-CTP, respectively.

material for these act.D experiments. The features of the distribution of RNA lengths in the ternary complexes have been discussed [5,6].

Addition of concentrated unlabelled NTPs to the original ternary complex results in fast RNA elongation and consequently in redistribution of the radioactivity to the upper part of the gel, as one can see comparing fig.1B,E,H or fig.3I–K with fig.1A and fig.3H, respectively. In the presence of act.D new bands (fig.1C,D,F,G,I,J; fig.3E–G) corresponding to RNA lengths from nucleotides 40–200 appear. Most of the new bands differ from those present in the original complexes and ternary complexes elongated in the absence of act.D

A

CCACACGUCCAACGGGGCAACCGUAUGUACACCUGAUGGGUUCGCAAUGAAACAACGAAU
40 50 60 70 80 90

CGAACGCCUUAAGCGUGAACUCCGCAUUAACCGCAAGAUUAACAAGAUAGGUUCCGGCUA
100 110 120 130 140 150

UGACAGAACGCACUGAUGGCUUAAAGAAAGGUUAUAUGCCCAAUGGCACACUAUACGCUG
160 170 180 190 200 210

CAAAUCGGCGAAUAGUGAGAACUUGGCG
220 230 240

B

CGACACCGGGGUCAACCGGAUAAGUAGACAGCCUGAUAAGUCGCACGAAAAACAGGUAUU
40 50 60 70 80 90

GACAACAUGAAGUAACAUGCAGUAAGAUACAAAUCGCUAGGUAACACUAGCAGCGUCAAC
100 110 120 130 140 150

CGGGCGCACAGUGCCUUCUAGGUGACUUAAGCGCACCACGGC
160 170 180 190

Fig.4. RNA elongation hindrance sites along primary structure of DIII T7 RNA (A) and T7 RNA (B). Bold lines above letters denote the location of 3′-ends of RNAs retarded at 1 μM of act.D and 30 s elongation time. Consensus sequences are underlined. Dots above letters denote the 3′-ends of RNAs retarded at >10 μM act.D.

(fig.1A and B,E,H; fig.3H and I–K). These new bands correspond to the RNA retarded by act.D and therefore, point to the sites occupied by act.D molecules. Positions of these bands are not altered when RNA elongation time varies from 10 s–30 min or [act.D] varies from 1–10 μM (fig.1,3). Location of the sites of elongation hindrance are determined via sequencing the nascent RNA (fig.2H–K; fig.3A–D). Fig.4 shows the positions of the sites along nucleotide sequences of the RNAs analyzed. When [act.D] is >10 μM ([DNA] being 130 μM nucleotide basepairs), a number of additional bands appear. Some of them (positions 47, 64, 70, 71 in fig.1D,G; and 100, 151, 152, 222, 240 in fig.2G) are already visible as weak bands at 10 μM act.D. At higher [act.D] their intensities increase. At 100 μM act.D they become as intense as the strong bands at < 10 μM act.D. Positions of all the additional bands are pointed out in fig.4.

3.2. *Pyrophosphorolysis of RNA in the presence of act.D*

Results shown in fig.2D,F compared with those in fig.2B,C clearly indicate that act.D is not able to inhibit the pyrophosphorolysis reaction [4]. It is worth noting that the antibiotic olivomycin shows the ability to specifically inhibit both RNA elongation and the pyrophosphorolysis reaction (submitted).

4. DISCUSSION

4.1. *Consensus sequence for strong hindrance sites*

Every prominent elongation hindrance site for low act.D concentrations is situated within a generalized sequence 5′XGCY3′ (fig.4). For short incubation times with high NTP concentrations (fig.1C,D) RNA is predominantly terminated with X. For longer incubation times (fig.1I,J) one GMP residue is slowly added and the last RNA nucleotide becomes either X or G. It is of interest that the rate of the GMP incorporation is by several orders of magnitude lower than that of free RNA elongation under the conditions used.

Table 1 summarizes all elongation hindrance sites visible at < 5 μM act.D with their location in the RNA sequences *vs* the associated tetranucleotide. Frequencies of occurrence of the tetranucleotide and the hindrance sites are also indicated. One can see that any time RNA polymerase meets XGCY, if X‡G and Y‡C, RNA synthesis is interrupted by act.D. There is only one exception: AGCG situated between nucleotides 146–149 of T7 RNA seems to be unable to stop RNA elongation (fig.4). Here, however, the site is overlapped with AGCA located 3 nucleotides upstream.

Table 1

A list of RNA elongation hindrance sites

5′XGCY3′	*N*	*n*	Position of elongation hindrance sites
CGCA	7	7	77–80, 117–120, 126–129, 163–166 of DIII T7 RNA 77–80, 159–162, 186–189 of T7 RNA
UGCG	0	0	
UGCA	2	2	213–216 of DIII T7 RNA, 112–115 of T7 RNA
CGCU	2	2	210–213 of DIII T7 RNA, 129–132 of T7 RNA
AGCG	3	2	106–109 of DIII T7 RNA, 146–149, 184–187 of T7 RNA
AGCA	1	1	143–145 of T7 RNA
UGCU	0	0	
CGCC	1	0	99–102 of DIII T7 RNA
GGCG	2	0	239–242 of DIII T7 RNA, 157–160 of T7 RNA
UGCC	2	0	191–194 of DIII T7 RNA, 166–169 of T7 RNA
GGCA	2	0	50–53, 199–202 of DIII T7 RNA
AGCC	1	0	64–67 of T7 RNA
GGCU	2	0	150–153, 172–175 of DIII T7 RNA
CGCG	0	0	
GGCC	0	0	
AGCU	0	0	

Complementary tetranucleotides are combined in pairs: *N*, occurrence frequency of the tetranucleotides in RNA fragment in fig.4; *n*, frequency of hindrance sites associated with tetranucleotide

Some of the probable hindrance sites UGCG, UGCU, AGCU and CGCG did not happen to occur in the T7 RNAs analyzed (table 1). Tetranucleotides UGCG and UGCU are found in transcripts of pBRS 2950 [8] initiated from cloned T7 A3 promoter. These two tetranucleotides are found to be the sites of strong inhibition of RNA elongation by act.D (not shown).

The probability of occurrence of the concensus sequence (if correct) in random DNA sequence with 50% GC content is equal to 0.036, which pretty well agrees with the value found in the experiment: 14 sites/380 nucleotides = 0.037 (fig.4).

4.2. *Other hindrance sites*

When the sites described in the previous section are saturated when the act.D concentration is increased, other RNA elongation hindrance sites become visible. As far as nucleotide sequence specificity is concerned, the sites definitely include all the XGCY with X = G or Y = C excluded from the strong hindrance sites. In addition some unique hindrance sites appear in the same range of [act.D]: ŮGGG, ĊGGG, ĊACC and ĠAGG (fig.4). No general formula for these sites can be drawn from the data obtained, but it seems that a single G or a dinucleotide CG is not sufficient to induce act.D binding. When transcription of DIII T7 DNA is inhibited with 100 μM act.D rather wide guanine-containing regions are still apparently free of act.D: these are for instance regions from nucleotide 80–99 and 127–143 of DIII RNA (fig.4).

This study and similar experiments with methidium–propyl-EDTA, DNA polymerase I and DNase I produce to some extent inconsistent results [9–11]. The discrepancy seems to come from conjoint influence of dye–DNA and enzyme–DNA interaction modes on the experimental results.

In this connection experiments described in this paper gave an amusing result that RNA elongation is arrested just in the middle of the apparent act.D binding site. Taken together with the well-known concept that RNA polymerase opens one turn of the double-stranded DNA helix at the RNA growing point and the inability of act.D to interact with both RNA·DNA hybrid and single-stranded DNA, the finding raises a number of questions on the mechanism of RNA synthesis. It is of interest that another powerful inhibitor of RNA elongation, olivomycin, interrupts elongation 3–4 nucleotides upstream the apparent binding sequence which is any of trinucleotides GGG, CGG or GGC (submitted).

We have shown in this paper that act.D binding constant depends on DNA nucleotide sequence and therefore, interpretation of binding isoterms [2] needs reevaluation.

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