LOCALIZATION OF *ESCHERICHIA COLI* RNA POLYMERASE INITIATION SITES IN T7 DNA EARLY PROMOTER REGION

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1. Introduction

Bordier and Dubochet [1] have mapped the *E.coli* RNA polymerase strong binding sites of T7 DNA at 1.15, 1.41 and 1.65 map units from the left DNA end. Initiation of transcription, which follows the binding of RNA polymerase, proceeds from the three different initiation points closely related to the three specific binding sites [2,3]. Recently a method has been developed in order to restrict initiation of transcription at a single initiation site [4]. In this report we describe the physical mapping of the three main initiation sites of T7 DNA early region. Data obtained demonstrate that binding and initiation sites of the early promoter region are very closely related if not identical. The term promoter used here covers the entire region at the beginning of a transcription unit which promotes and regulates the initiation of specific RNA transcripts [5].

2. Materials and methods

2.1. *Enzymes*

E.coli RNA polymerase was prepared according to Burgess [6]. RNAase III was purified as described by Darlix [7]. Both enzymes were pure proteins as seen by gel electrophoresis and free of contaminating nucleases.

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2.2. *Nucleic acids*

T7 DNA was extracted by phenol-SDS from T7 bacteriophages grown on *E.coli* B/r Thy$^-$. (rI)n was obtained from P.L. Biochemicals.

2.3. *Dinucleotides and nucleotides*

ApC, CpG, CpC and the nucleoside triphosphates were purchased from P.L. Biochemicals.

2.4. *Enzymatic assay*

The ionic conditions were: 0.04 M Tris–HCl pH 7.9, 0.01 M $MgCl_2$, 0.1 mM EDTA, 6 mM 2-mercaptoethanol and 0.05 or 0.2 M NaCl. Amounts of RNA polymerase, RNAase III, T7 DNA, (rI)n, dinucleotides and nucleotides are indicated in the individual legends of figures.

2.5. *Slab gel electrophoresis of RNA*

It was performed with polyacrylamide gels containing 3% (w/w) acrylamide and 0.5% (w/w) agarose as previously described [8].

2.6. *Specimen preparation*

Specimens for electron microscopy were prepared by adhesion on positively charged carbon films according to the method described by Dubochet et al. [9]. The binding and initiation complexes were diluted to a final DNA concentration of 0.4 to 1.5 μg/ml just before adhesion. The dilution buffer was the same as that for enzymatic assay.

Thin carbon supporting films were prepared by evaporation on mica and transferred on 400 mesh copper grid or on 200 mesh copper grid covered with a thick perforated carbon film [10]. The carbon films, treated by glow discharge in amylamine vapors, were further processed as described in [1].

2.7. *Electron microscopy*

Observations were made with a Phillips 301 or a Siemens Elmiskop 101 electron microscope. The darkfield was obtained by the tilted beam method. A thin film objective aperture of 20 μm was used. Micrographs were recorded on 65 X 90 mm Kodak films 4489 at magnification ranging from 13 000 to 40 000 times. Interesting features of the micrographs were redrawn on paper at ten times magnification using an Automega enlarger. Length measurements were made on these drawings with a map ruler.

Under our conditions length of T7 DNA was 12 μ ± 0.5 μ in agreement with the data of Bordier and Dubochet [1]. The positions on the DNA molecule are expressed in physical map units, one unit corresponding to 1% of the DNA length.

3. Results and discussion

3.1. *Selection of RNA chain initiation at a single site of T7 DNA early promoter*

The method developed to restrict the start of T7 DNA transcription by *E.coli* RNA polymerase at a single initiation site was used. This method briefly presented below, will be entirely described elsewhere [4].

Initiation of transcription takes place in the presence of an appropriate dinucleotide and one purified nucleoside triphosphate. The RNA polymerase molecules that did not initiate are definitively removed from the template by adding (rl)n and by lowering the incubation temperature to 0°C. As soon as incubation is brought back to 37°C, the other three nucleoside triphosphates are added to complete transcription.

To determine at which site chain initiation has occurred, the RNA 7600 nucleotides made [11,12] is sized by RNAase III which individualizes the transcription products of each early gene and of the early promoter region (2); these RNAs are analyzed by polyacrylamide gel electrophoresis. The interesting observation is that the RNAs from the early promoter region are separated into three species of different molecular weight which reflects the existence of three initiation points [2] (see fig.1). Accordingly the disappearance of one or two of these RNAs means that initiation of transcription did not occur at the corresponding initiation sites.

Once the binding of RNA polymerase to T7 DNA is completed, addition of (rl)n does not remove the polymerase molecules specifically bound provided the incubation temperature is maintained at 37°C. Under these conditions the transcription process starts normally from the three initiation points after addition of the four nucleoside triphosphates (fig.1), and the three small initiator RNAs are present. On the other hand if the incubation temperature is lowered to 0°C before the initiation process, RNA synthesis is completely prevented. Three combinations of dinucleotides and nucleoside triphosphates were used to direct initiation of transcription at each site [4].

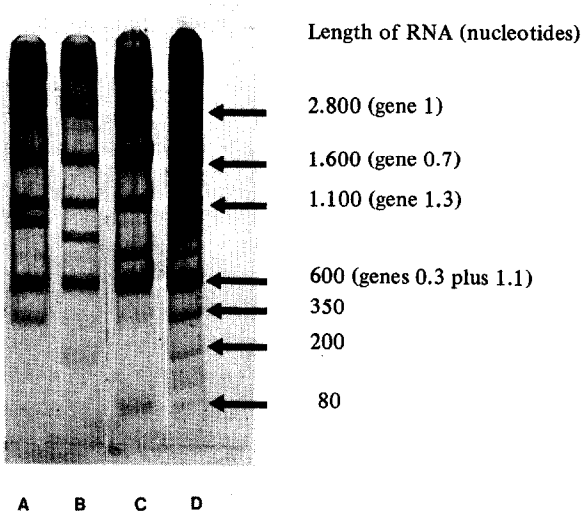
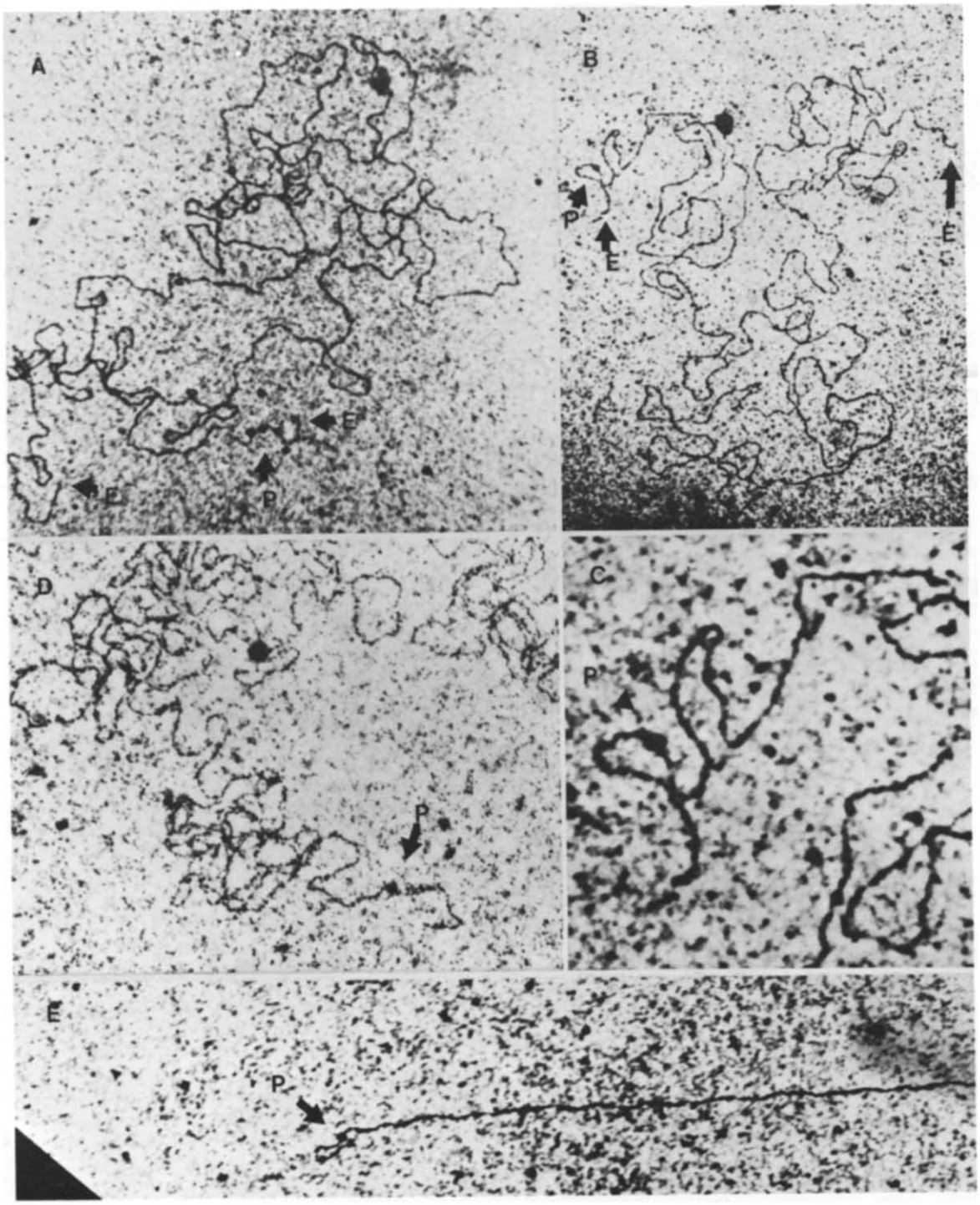


Fig.1. Slab gel electrophoresis of T7 RNA made in the presence of a dinucleotide. Each incubation contained: 13 μg T7 DNA, 2.5 μg RNA polymerase, ionic conditions as described in method (NaCl concentration was 0.2 M). When added dinucleotides (ApC, CpG and CpC) and nucleotides (ATP and CTP) were present at 2.10^{-4} M. Final volume 0.04 ml. After 5 min at 37°C (rl)n was added (0.1 mg/ml) and incubation temperature lowered to 0°C. (In the control without dinucleotide, the four nucleotides were added together with poly I and RNAase III and incubation was for 20 min at 37°C). 10 min later incubation was brought back to 37°C and the nucleotides lacking were added (2.10^{-4} M) together with [^{14}C]UTP (0.5 mCi/μmole) and RNAase III (0.01 μg). Incubation was for 20 min at 37°C. 130 000 counts/min of radioactive RNA were put in each well of the slab gel. Rates of RNA synthesis were:

Control	100%	D
ApC plus ATP	36%	C
CpG plus CTP	29%	B
CPC plus ATP	36.5%	A


A
E
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When initiation takes place in the presence of the dinucleotide ApC with ATP and under the conditions mentioned above, only one transcript of T7 DNA early promoter is synthesized, that of about 80 nucleotides long. With the dinucleotides CpG plus CTP and CpC plus ATP, again only one small RNA is made, those 150 and 300 nucleotides, respectively (fig.1). As expected the RNA of genes 0.3, 0.7, 1, 1.1 and 1.3 are also produced and, in either case, the final amount of RNA made is a third of what it is in the control (legend of fig.1).

3.2. *Localization of E.coli RNA polymerase initiation sites in the early promoter region of T7 DNA*

Complexes of T7 DNA and RNA polymerase have been studied by darkfield observation of specimens prepared by adhesion on positively charged carbon films.

As a control we have located the binding sites of *E.coli* RNA polymerase on the physical map of T7 DNA at a molar ratio of RNA polymerase to T7 DNA equal to ten and in the presence of (rI)n (fig.2a). As expected three enzymes were found bound on the DNA near the left end at 1.10, 1.40 and 1.65 map units (200 recorded bound enzyme molecules; result not shown), in complete agreement with the results of Bordier and Dubochet [1].

The initiation complexes T7 DNA–RNA polymerase–oligoribonucleotide were formed as described in the first section, and were adsorbed on the films at 0°C after the addition of (rI)n. With either dinucleotide ApC, CpG or CpC a large fraction, 65–80%, of the T7 DNA molecules bear one or more RNA polymerase molecules. In most cases (90%) only one enzyme molecule is present on the DNA, within two map units of one DNA end (figs.2b,c and d). Figs. 2b,c and d show initiation complexes made in the presence of ApC plus ATP, CpG plus CTP and CpC

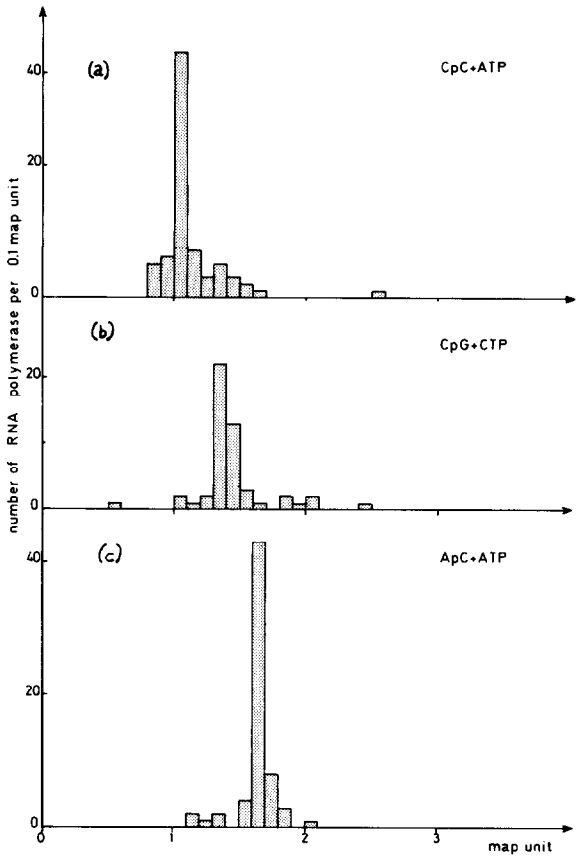

Fig.3. Position of the initiation sites. The specimens were prepared and analyzed as described in method. (a) Histogram of the position of 70 RNA polymerase molecules that have initiated transcription with CpC plus ATP. 56 T7 DNA molecules have one enzyme molecule and 7 have 2 enzyme molecules bound. (b) Histogram of the position of 51 RNA polymerase molecules that have initiated transcription with CpG plus CTP. 43 T7 DNA molecules have one enzyme molecule and 4 have 2 enzyme molecules bound. (c) Histogram of the position of 64 RNA polymerase molecules that have initiated transcription with ApC plus ATP. 52 T7 DNA molecules have one enzyme molecule and 6 have 2 enzyme molecules bound.

Fig.2. *E.coli* RNA polymerase-T7 DNA complexes. Incubation conditions were the same as in figure 1 except that final concentration of (rI)n was 0.05 mg/ml. After addition of (rI)n the samples were diluted with the same buffer containing NaCl at 0.05 M, and specimen preparation was carried out as described in method. (A) RNA polymerase – T7 DNA binding complexes in the presence of (rI)n. One entire DNA molecule is spread in the field. RNA polymerase molecules are visible either bound near one DNA end or in the background of the film. Magnification: 83 000 ×. DNA end: E; RNA polymerase: P. (B) RNA polymerase–ApCpA–T7 DNA initiation complex. One entire DNA molecule is visible in the field with only one RNA polymerase near one end. Magnification: 83 000 ×. (C) Same as B but magnification 250 000 ×. (D) RNApolymerase–CpGpC–T7 DNA initiation complex. Magnification: 133 000 ×. (E) RNApolymerase–CpCpA–T7 DNA initiation complex. Magnification: 100 000 ×.

plus ATP, respectively; the corresponding histograms of the position on the DNA of 185 recorded enzyme molecules that have initiated transcription are reported in figs.3a,b and c, respectively. Thus initiation points corresponding to the three major binding sites are located at 1.05 ± 0.1, 1.40 ± 0.1 and 1.65 ± 0.1 map units.

It was shown [4] that under appropriate conditions T7 DNA transcription by *E.coli* RNA polymerase starts from a single initiation site. Selection of the sites depends on the dinucleotide used and several dinucleotide can induce the selection of the same initiation site. In the work presented here the initiation complexes were vizualised by electron microscopy and the three main initiation sites of T7 DNA early region were mapped at 1.05 ± 0.1, 1.40 ± 0.1 and 1.65 ± 0.1 map units. The very similar localization of the strong binding sites of T7 DNA early region at 1.15 ± 0.05, 1.41 ± 0.05 and 1.65 ± 0.08 map units (Bordier and Dubochet [1]) or at 1.17 ± 0.1, 1.52 ± 0.07 and 1.83 ± 0.04 (Portmann et al. [13]) is a direct proof of a very close physical relation between the binding and the initiation sites of T7 DNA early promoter region.

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