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Fast abortive initiation of uvrA promoter in a supercoiled plasmid studied by stopped-flow techniques

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In order to follow the fast kinetics of abortive initiation (lag time from 1 ms to 10 s), we have built a stopped-flow apparatus equipped for fluorescence detection. The small volume used for each assay (35 μl), and the short dead time (~0.5 ms) are the essential advantages of this apparatus. Supercoiling of DNA affects considerably the initiation of transcription from the uvrA promoter. It decreases the lag time due to the isomerisation process 3-fold. Nevertheless, it does not change significantly the product $K_B k_2$, which is indicative of promoter strength and shows that uvrA is an 'association-limited' promoter. The presence of the LexA repressor increases the lag time considerably. At least for small RNA polymerase concentrations this increase is stronger for supercoiled than for linearized DNA.

Regulation; Transcription initiation; RNA polymerase; Supercoiling; Fluorescence; Stopped-flow

1. INTRODUCTION

The regulation of transcription initiation from bacterial promoters by the *E. coli* RNA polymerase can occur in many different ways. At every moment the cell must be able to adapt its machinery to any environmental change, to satisfy all the needs with the lowest loss of energy.

The promoter sequence has been assumed so far to be associated with an intrinsic 'promoter strength'. Indeed, correlations were found between the values of transcription parameters and deviations from the so-called consensus sequences [1,2].

(i) This strength can be modulated by several external actions: perturbations of the medium (temperature, ionic strength, pH), which can occur in the cell and which have been largely studied in vitro [3–5].

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(ii) Three-dimensional variations of DNA structure, like DNA supercoiling [3,4].

(iii) Effector (repressors or activators) proteins which are, in some cases, responsible for a major part of the transcription regulation [6–9].

The uvrA gene belongs to the SOS system, formed by at least 15 genes, all negatively regulated by the LexA protein. Activation of these genes is obtained during the induction of the SOS system by the cleavage of LexA in the presence of another protein (the activated form of RecA) (reviews [10,11]).

Recently, we have followed the initiation of transcription of the uvrA gene, on a linear plasmid, by abortive initiation with a fluorescence assay, as well as its regulation by the LexA repressor [6].

In the absence of any repressor molecule, and on linear DNA the uvrA promoter appears to be an 'association-limited' promoter, with lag times in the range of 50–100 s. With the same uvrA promoter on supercoiled DNA these lag times are considerably lowered, so that these measurements

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were not possible without a rapid mixing system. A new stopped-flow apparatus was used for this study, and is described here. We show by these stopped-flow fluorescence measurements, that on supercoiled DNA lag times are lowered by a factor of four to five without really affecting the product $K_B k_2$. The effect of LexA on this transcription was also followed.

2. MATERIALS AND METHODS

2.1. *Materials*

Plasmid pA_7galk containing the uvrA promoter-operator region was a generous gift from Backendorf et al. [12].

The RNA polymerase was extracted from a K12 strain of *E. coli*, according to Burgess and Jendrisak [13]. The specific activity on calf thymus DNA was 899 U/mg, where 1 unit is equivalent to 1 nmol UTP incorporated in 10 min.

LexA repressor was purified according to Schnarr et al. [14].

Unlabeled nucleoside triphosphates and dinucleotide monophosphates were purchased from Sigma and P.L. Chemicals, respectively.

ANS (1-naphthylamine-5-sulfonic acid) was from Fluka. UTPγANS was prepared as described by Yarbrough et al. [15].

2.2. *Abortive initiation assays*

As reported previously [3,4,6,16], this technique relies on the production of short oligonucleotides. In our case, using the dinucleotide GpU as starting nucleotide and UTPγANS for elongation, leads to the tetranucleotide GpUpUpU for the uvrA promoter. Standard 'transcription buffer' was: 50 mM Hepes (pH 7.9), 0.4 mM potassium phosphate buffer, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, 100 μg/ml bovine serum albumin (BSA), 0.5 mM GpU and 0.1 mM UTPγANS as elongating nucleotide. The DNA template, in standard buffer, was 2.6 nM in plasmids and RNA polymerase concentration varied from 50 to 300 nM, in the same buffer. After equilibration at 37°C, the RNA polymerase was directly mixed with DNA in the stopped-flow fluorescence cell.

2.3. *Stopped-flow apparatus*

A stopped-flow apparatus equipped with fluorescence detection has been developed in our laboratory. In order to restrict the quantities of biological product necessary and to minimise the dead time of the measuremets, a Giken (Union Giken) type mixing system was used [17]. The two solutions, subjected to appropriate nitrogen pressure, are introduced together in the cell through a classical injector system, and the flow of solution is controlled by an opposite nitrogen pressure on an electro-pneumatic valve at the exit of the cell.

(i) Before mixing the two solutions are stocked in two identical 5 ml compartments. The two solutions are isolated from each other by two conical flood-gates in order to avoid contamination after mixing, during the acquisition. The whole system is thermostatted by water circulation.

(ii) The electro-pneumatic valve acts like an electromagnetic loudspeaker. The magnet is wrapped in an enclosure closed with an undulating membrane. A moving coil is fixed on one side of the membrane and the valve itself on the other. The time required for opening and closing of the valve is less than 1 ms.

(ii) As shown in fig.1, the measurement cell is constituted of a hollow silica cylinder, which has a length of 0.6 cm and an external diameter of 0.8 cm. The central hole has a diameter of 0.2 cm

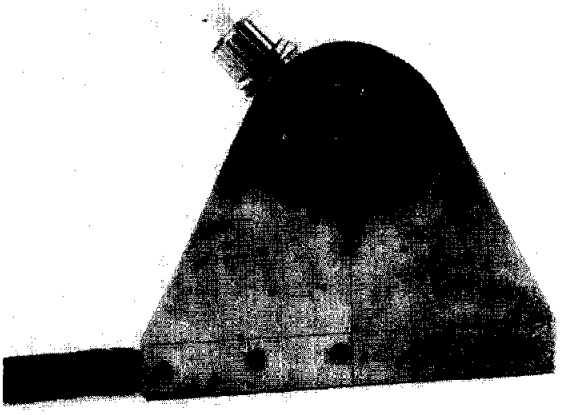

Fig.1. The measurement head. The three araldite cylinders are in place. At the center, one can see the axial hole, through which excitation light will pass. The place for optical filters as well as the exit of the evacuation channel is also shown. The big circular hole is generally closed by the optical filter holder.

and one of its ends is lighted to the exit of the mixing system. The total volume necessary for one assay is about 35 μl (20 μl cell volume + 15 μl dead volume).

The measurement head is made of three araldite cylinders which are fitted together like Russian dolls.

The cylindrical cell is embedded in the first one (internal one). Eight collimating slits (0.05 × 0.6 cm), cut parallel to the cylinder length and whose radial directions are 0 and π, $\pi/6$ and $5\pi/6$, $\pi/3$ and $2\pi/3$, $\pi/2$ and $3\pi/2$, define four observation channels.

The intermediate araldite cylinder presents eight corresponding slits which can accommodate optic filters.

On the last araldite cylinder (outside one) are fixed eight photodiode assemblies (Hamamatsu type 1227/16BQ). Each one offers a 0.11 × 0.59 cm sensitive surface and is characterized by a wide UV response.

The three coaxial cylindrical araldite blocks are moulded in aluminium alloy matrices with araldite M (Araldite resin R.T.M., Ciba Geigy), filled by 5% (w/w) of carbon black (carbon black vulcan 3 was a gift from Cabot France).

The draining of the solution is made through an inox needle, incorporated in the araldite so that the change of solution is total at each shot. The signal can be analysed either for each of the four channels, or as a summation of the signal over the four channels. The excitation light is given by an Xe lamp, lighting an HRS monochromator (Jobin Yvon). An optical feedback loop stabilizes the intensity of the excitation light.

The data are recorded with a Data 6000 digital oscillograph, which determines the sampling period and executes the analog-to-digital conversion.

A host computer assumes the full 'menu' program of the Data 6000, the transfer of the data via an IEEE-488 bus, and the processing of the data.

3. RESULTS AND DISCUSSION

3.1. *Preliminary checkings*

As a preliminary control, we have checked that neither the rapid mixing by shearing nor exposure of the solution to a light beam by photolysis alters the material in any significant way.

(i) UTPγANS is not hydrolysed, in the absence of any transcriptional activity during the mixing time, and after 2 h of exposure to the light beam.

(ii) As another control of the integrity of the material after mixing, we have tested abortive initiation of a linearized pA$_7$galk plasmid, and the measured lag times were the same as those previously described using a hand-mixing device [6].

3.2. *Stopped-flow study of abortive initiation of the uvrA promoter*

Fig.2 shows the time course of abortive initiation of transcription from the uvrA promoter in a supercoiled plasmid as measured by the fluorescence increase of the ANS marker, due to the hydrolysis of UTPγANS during the synthesis of the oligonucleotide GpUpUpU.

One can observe (fig.3) that, in comparison with many other promoters, the lag times observed on a supercoiled uvrA promoter are rather short (10–45 s). On the other hand, the formation of the open complex is systematically 4–5-times faster for the supercoiled than for the linearised uvrA promoter [6].

Using the classical treatment of abortive initia-

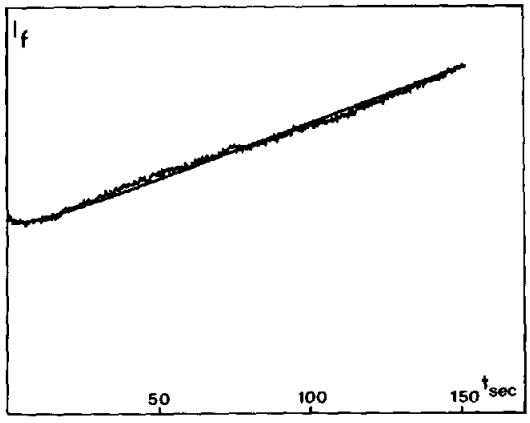

Fig.2. Kinetics of RP_o formation on the supercoiled uvrA promoter, followed with the stopped-flow apparatus. The product is GpUpUpU. The pA$_7$galk DNA was 2.6 nM, and RNA polymerase 300 nM, in the standard transcription buffer. This recording was obtained with a sampling time of 300 ms, and corresponds to the accumulation of 4 kinetics. The pressure applied on the solutions was 7 bar. The best fit of data was obtained with a lag time of 12 s.

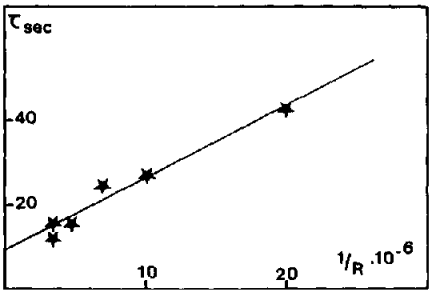

Fig.3. Plot of τ (s) obtained for the supercoiled uvrA promoter. The lag times observed for GpUpUpU synthesis are plotted vs the reciprocal of the RNA polymerase concentration.

tion, proposed by McClure [16], which is based on the two-step mechanism:

$$R + P \overset{K_B}{\rightleftharpoons} RP_c \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RP_o$$

where R represents the RNA polymerase, P the promoter, RP_c the closed complex and RP_o the open complex. The binding constant K_B as well as the isomerization rate k_2 between RP_c and RP_o, can be determined from the relation:

$$\tau = \frac{1}{k_2} + \frac{1}{k_2 K_B [R]}$$

The lag time considered here is in fact the one observed at the beginning of the abortive initiation kinetics (see fig.2).

Table 1 shows the parameters determined from plots of τ vs 1/[R]. If supercoiling increases considerably the rate of isomerisation k_2 of the uvrA promoter, it decreases in a comparable manner the association constant K_B. As in the case of the bla (ACAA) promoter and of the RNA 1 promoter [19], the two parameters K_B and k_2 are apparently linked. These important but opposite effects of negative superhelicity observed lead to a relative constancy of the product $K_B k_2$. If one considers the correlation between this product $K_B k_2$ and the promoter strength as discussed by Mulligan et al. [1], supercoiling has only a small effect on the transcription initiation of such promoters.

It should be stressed that the analysis of the uvrA promoter on a supercoiled plasmid might be complicated by the presence of a divergent promoter of the gene coding for the single-strand binding protein (ssb). The activity of this promoter has been determined in vivo by S_1 nuclease mapping [18] whereas so far no transcriptional activity has been detected in vitro on linearized templates. This ssb promoter lies very close to the uvrA promoter at a distance of 7 base pairs between the two (−35) regions. The occupancy of the two promoters by RNA polymerase may thus be mutually exclusive.

Table 1

Kinetic parameters

	K_B (M^{-1})	k_2 (s^{-1})	$k_2 K_B$ ($M^{-1} \cdot s^{-1}$)
uvrA(l)	1.5×10^7	2.7×10^{-2}	4×10^5
uvrA(s)	5.7×10^6	10.5×10^{-2}	5.98×10^5

Kinetic parameters of transcription initiation at the uvrA promoter in standard transcription buffer (uvrA(l) and uvrA(s) are respectively uvrA promoter on linear (from [6]) or supercoiled DNA)

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