

## ELIMINATION OF PROMOTER FUNCTION BY BASE MODIFICATION OF DNA

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### 1. Introduction

Promoters are DNA regions at which highly selective RNA polymerase binding and RNA chain initiation take place. They are not characterized by a unique primary structure. Comparison of published promoter sequences [1–14] reveals that there is not even an absolute requirement for a particular base at any position within such a region.

In view of this situation we started to examine the dependence of promoter function on specific structural elements of the nucleobases. Thus we 'removed' the 5-methyl of deoxythymidine ( $T_d$ ) by replacement of this nucleoside by deoxyuridine ( $U_d$ ). The modification was introduced into the codogenic strand of *Escherichia coli* phage fd RF DNA. Individual promoters were separated by cleavage of the circular DNA with restriction endonuclease *HpaII* and polyacrylamide gel electrophoresis. *E. coli* RNA polymerase binding assays in the absence and presence of ribonucleoside triphosphates revealed that upon the DNA modification described the fd gene II promoter loses its affinity for the enzyme.

### 2. Materials and methods

DNA polymerase I of *E. coli* was isolated by H. Müller following the procedure in [15]. The large fragment of DNA polymerase I obtained by subtilisin treatment of the enzyme [16] was purchased from

**Abbreviations:** RF DNA, replicative form DNA of bacteriophage fd; PEG, polyethyleneglycol; DTE, ditrioinerythritol; deoxyribonucleosides are marked by  $_d$  following the nucleoside symbol

Boehringer Mannheim. *E. coli* DNA ligase was purified according to [17]. T4 DNA ligase was isolated by R. Frank and M. Wippermann as in [18]. *HpaII* restriction endonuclease from *Haemophilus parainfluenzae* was purchased from Miles Labs, Elkhart, IN. *E. coli* RNA polymerase holoenzyme was a generous gift of Professor W. Zillig, Martinsried. fd-infected *E. coli* K12 Hfr 3300 was grown and fd DNA was isolated essentially as in [19,20] with the modification that phages were purified by two successive precipitations with 3% PEG in 0.5 M NaCl [21]. fd-specific oligonucleotide primers of chain length 11–13 originating from a DNase digest of fd RF DNA were prepared and purified by D. Müller. Ribo- and deoxyribonucleoside triphosphates were from Boehringer Mannheim.  $[\alpha\text{-}^{32}\text{P}]A_d\text{TP}$  was purchased from the Radiochemical Centre, Amersham. RF DNA was synthesized in vitro in the presence of  $[\alpha\text{-}^{32}\text{P}]A_d\text{TP}$ . Synthesis and isolation were done essentially as in [22]. 100% substitution of  $T_d$  by  $U_d$  within the codogenic strand was achieved by replacement of  $T_d\text{TP}$  by  $U_d\text{TP}$  and of DNA polymerase I by its large fragment. Detailed descriptions of these procedures will be published elsewhere (in preparation). For fragmentation of fd RF, 10  $\mu\text{g}$  normal RF or 5  $\mu\text{g}$  modified RF were incubated with 15 units or 25 units, respectively, of *HpaII* at 37°C for 10 h in 0.5 ml of 30 mM Tris · HCl (pH 7.5), 10 mM  $\text{MgCl}_2$ , 1 mM DTE, 3.5% glycerol. The fragments were deproteinized by 2 phenol extractions and desalted on Sephadex G-50. RNA polymerase binding experiments were performed in 20 mM Tris · HCl (pH 8.0), 10 mM  $\text{MgCl}_2$ , 120 mM KCl, 0.1 mM DTE, 0.1 mM EDTA, 5% glycerol at 37°C [23]. To allow RNA chain initiation GTP, ATP and UTP were present in some experiments at 0.1 mM

each RF was 0.15 pmol/ml RNA polymerase. RF ratio was 20. Aliquots withdrawn from the reaction mixture were added to solutions containing denatured calf thymus DNA, 5 min later they were filtered through cellulose nitrate (MF 15 from Sartorius, Göttingen) and the filter bound material was eluted [23,24]. When RNA chain initiation was measured filters were washed with ice-cold binding buffer (see above) containing 1 M KCl [25] followed by binding buffer without KCl. DNA fragments eluted from the filters were separated on 1 mm thick 3.5% polyacrylamide/7 M urea slab gels equilibrated with 50 mM Tris-H<sub>3</sub>BO<sub>3</sub>, 1 mM EDTA (pH 8.3) [26] and run at 20 V/cm. The frozen gels were analyzed by autoradiography using Kodak X-Omat R films.

### 3 Results and discussion

Starting from viral single-stranded fd DNA a modified RF DNA in which all T<sub>d</sub>-moieties of the codogenic strand were replaced by U<sub>d</sub> was synthesized in an oligonucleotide-primed 'repair' synthesis [22,27] catalyzed by the large fragment of *E. coli* DNA polymerase I lacking the 5'-exonuclease function [16]. Open circular RF II species were converted to covalently closed RF I by *E. coli* or T4 DNA ligase.

The modified DNA was cleaved by restriction endonuclease *Hpa*II from *Haemophilus parainfluenza*. This allows discrimination between the interactions of different fd promoters with *E. coli* RNA polymerase [23].

The purified *Hpa*II fragments were incubated with RNA polymerase holoenzyme at 37°C and 120 mM KCl. These conditions were shown to allow only specific binding of the enzyme to promoter regions [23]. After 20 s, 50 s, 2 min and 5 min, aliquots were withdrawn and examined by a filter binding assay which traps RNA polymerase and DNA complexed by the enzyme [23,24]. *Hpa*II fragments eluted from the filters were separated by polyacrylamide gel electrophoresis. Assays with unmodified DNA were run as control.

Figure 1 shows the fragments of normal and U<sub>d</sub>-substituted fd RF DNA bound by RNA polymerase after a 5 min incubation at an enzyme:RF ratio of ~20. Both fragment patterns are qualitatively identical with the exception of fragment H carrying the fd

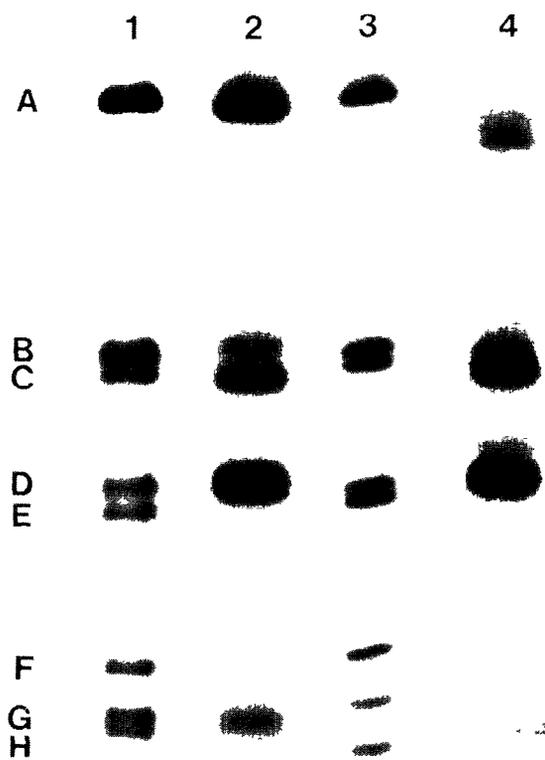


Fig 1 *Hpa*II fragments of normal and deoxyuridine-containing fd RF DNA bound by RNA polymerase. <sup>32</sup>P-labeled fd RF DNAs (37.5 pmol) pre-fragmented by *Hpa*II were incubated with 750 pmol RNA polymerase holoenzyme in 250 µl for 5 min at 37°C. The reaction was stopped by addition of excess denatured calf thymus DNA. Fragments bound by RNA polymerase were trapped on cellulose nitrate filters, separated on polyacrylamide gels and visualized by autoradiography. For details see section 2. Results of such analyses are shown in slots 2 (normal DNA) and 4 (U<sub>d</sub>-containing DNA). Slots 1 and 3 show complete sets of *Hpa*II fragments A-H containing T<sub>d</sub> or U<sub>d</sub>, respectively.

gene II promoter as the only RNA polymerase binding site.

Obviously in the case of U<sub>d</sub>-substitution this fragment does not form a complex with the enzyme. This result also holds for the samples withdrawn earlier from the reaction mixture. As can be expected promoter binding is a rather fast reaction. Half-maximal binding of the fastest fd promoters (of genes X, II and VIII) requires 15–60 s under the conditions applied, and there is no promoter-containing fragment that is not at least partially bound after only 20 s.



positions are situated within the Pribnow sequence [29] as promoters exhibiting identical modifications of this region like the gene X and gene VII promoters (see fig 2) show no or only a small decrease of promoter strength (our unpublished results)

For a precise localization of the effect described here studies with partially modified gene II promoter regions which can be obtained by use of unique primer molecules for DNA polymerase I should be quite useful

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