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Inhibition of the first phosphodiester bond formation catalyzed by *Escherichia coli* RNA polymerase in the presence of bovine seminal plasmin: promoter dependency

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Inhibition of the abortive initiation of transcription catalyzed by *E. coli* RNA polymerase has been studied here in the presence of bovine seminal plasmin. Seminal plasmin, which is known to be a stronger inhibitor than rifampicin binds at the same site as rifampicin to RNA polymerase. However, unlike rifampicin, seminal plasmin showed the inhibition of the formation of both the first and second phosphodiester bonds. We observed, in vitro, that the degree of inhibition of transcription was different at different promoters. Thus, the percent of inhibition of transcription initiation by seminal plasmin was much less at r-RNA promoters in comparison to that at the early promoters of bacteriophage T7.

Transcription; Inhibition; Seminal plasmin; Promoter; (*Escherichia coli*)

1. INTRODUCTION

Seminal plasmin, a 6 kDa protein from bovine seminal plasma, had been shown to be an inhibitor of transcription in *Escherichia coli* and it does so in vitro by binding strongly to RNA polymerase [1,2]. It was further reported by us that no binding domain for rifampicin was available on RNA polymerase–seminal plasmin complex, indicating that both the inhibitors share a common binding site on the enzyme [3]. It is known that rifampicin binds to the β-subunit of RNA polymerase [4]. McClure and Cech [5] reported that the major effect of rifampicin was found to be a total block of the translocation step that would ordinarily follow the formation of the first phosphodiester bond. It was mentioned here that rifampicin is a partial inhibitor of the formation of the first phosphodiester bond in RNA synthesis having an effect only on the binding of the first two triphosphates to the enzyme.

Thus, with an analogy to rifampicin, seminal plasmin is also expected to bind to the β-subunit of RNA polymerase. However, one would expect the binding domain of seminal plasmin to the enzyme would encompass a larger area than a comparatively smaller molecule like rifampicin (MW 832). This would mean that the inhibitory effect of seminal plasmin on the initiation of transcription would be more pronounced and probably one would be able to look at the effect of a transcription inhibitor at every step like promoter recognition, DNA unwinding, and formation of the first few phosphodiester bonds, which leads to overall initiation of transcription. In this report we have studied the effect of seminal plasmin on the abortive initiation of transcription at different promoters individually cloned in a plasmid vector.

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2. EXPERIMENTAL PROCEDURE

All the chemicals and buffers used here are of purest grade available. Radio nucleotides used for the transcription assay were obtained from Bhabha Atomic Research Centre, Bombay, India. *E. coli* RNA polymerase was purified and assayed according to the method already developed in this laboratory [6]. Derivatives of *E. coli* strain HMS174, each containing plasmid pAR 1435 (T7A1), pAR1539 (T7A2) were kindly provided by Dr F.W. Studier of Brookhaven National Laboratory, N.Y. The above plasmids have a single early promoter of T7 DNA as mentioned in the parentheses [7]. Plasmid pKK3535 (*rrn*B operon containing both P1 and P2 promoters) was a kind gift from Dr David Schlessiger's laboratory at Washington Univerity, St. Louis. Natural bovine seminal plasmin constituting 47 amino acids [8] has been routinely used in the past after purifying the protein from bovine seminal plasma. However, we noticed before that this preparation may have at times a minor contamination of RNase, which makes the transcription assay difficult. Recently, Dr R. Nagaraj from this laboratory has successfully synthesised this protein by solid-phase synthetic methods at Imperial Cancer Research Founds, London, U.K. using an Applied Biosystem Machine. Details of such synthesis along with the purification protocol will be published elsewhere. Synthetic seminal plasmin was found to be as biologically active as the natural one in various assays and was used in this study.

Abortive initiation of transcription was carried out at T7A1 and T7A2 promoters as well as at the r-RNA promoter by supplying only two nucleotides corresponding to the 5′-terminus of the RNA message. Dinucleotide tetraphosphates were turned over by RNA polymerase in the absence of other nucleotides according to the protocol already described by Johnston and McClure [9]. In certain cases

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we also added the first 3 nucleotides corresponding to the RNA start site, to generate a tri-nucleotide product at equilibrium. Thus the abortive product at T7A1 was pppApU or pppApUpC, at T7A2 and *rrn*B P2 promoters were pppGpC and pppCpCpC respectively [7,10]. The products were subsequently analysed over a 25% acrylamide (20:1, acrylamide:bis acrylamide) gel run with tris-borate-EDTA buffer (pH 8.3). The intensity of the bands on the autoradiogram were quantitated with the help of a Biomed densitometer equipped with a soft laser source.

3. RESULTS

It was observed earlier in our institute (Kumar and Chatterji, personal communication) that in the presence of strong promoters like T7A1 or T7A2 [11] or r-RNA promoters in a vector like pBR322, the productive run-off transcript as well as abortive synthesis of dinucleotides exclusively take place at these promoters and the promoters of the vector alone showed no significant RNA synthesis.

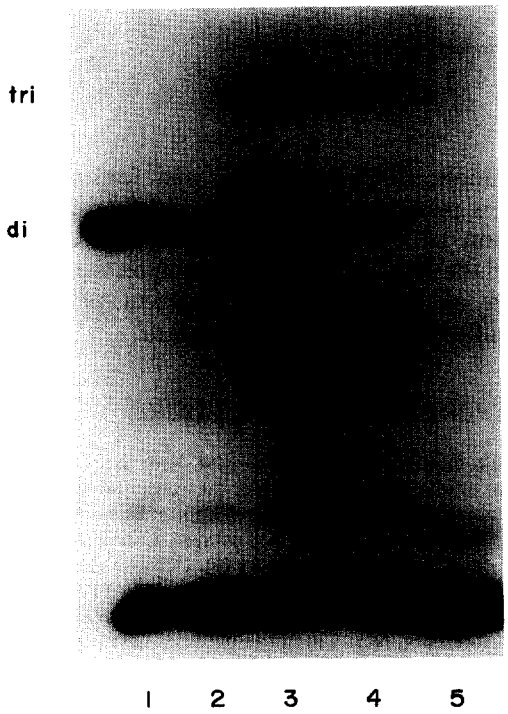

Fig.1. Analysis of abortive synthesis of the dinucleotide and trinucleotide products catalyzed by *E. coli* RNA polymerase at T7A1 promoter over a 25% acrylamide gel. Synthesis of the dinucleotide tetraphosphate was carried out in 25 μl of cocktail A containing 80 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 50 mM KCl, 2 mM dithiothreitol, 0.15 μM RNA polymerase and 0.4 mM DNA-phosphorus. Enzyme and DNA were preincubated at 37°C for 5 min and the abortive initiation of transcription was triggered by the addition of an equal volume of cocktail B containing 200 μM ATP, 6 μM α-^{32}P-UTP (1 μCi/nmol) and further incubated for 10 min at 37°C (Lane 1). Lane 2 shows the effect of seminal plasmin on the synthesis of pppApU. In this case, RNA polymerase was preincubated with seminal plasmin for 15 min at 37°C in a molar ratio of 1:40 (enzyme:seminal plasmin) before its addition to cocktail A. Lane 3 shows the synthesis of both pppApUpC and pppApU in which case 6 μM CTP was also added to cocktail B. Lane 4 shows the effect seminal plasmin (enzyme: seminal plasmin, 1:40) on the synthesis of trinucleotide. Lane 5 is the incubation mixture with RNA polymerase, but no DNA. The gel was subsequently autoradiographed.

Fig.1 shows the inhibition of the abortive initiation of transcription carried out at the strongest promoter of *E. coli* in vitro, i.e. T7A1. We have specifically attempted the abortive initiation reaction to follow the action of seminal plasmin as this assay mimics the events leading to the formation of the first or second phosphodiester bond only in the transcript with no elongation of the RNA chain [5,9]. It can be seen from fig.1 that on the addition of only two nucleotides, corresponding to the 5′-initiation site of the transcript, RNA polymerase turns over pppApU at T7A1 promoter which can then be resolved in the gel. In the presence of the inhibitor, bovine seminal plasmin, the formation of the dinucleotide product was inhibited (lane 2). Similarly when all the three nucleotides, ATP, UTP and CTP were supplied a trinucleotide product was also accumulated in the absence of seminal plasmin (lane 3) along with the dinucleotide. It has been reported that the enzyme usually releases the dinucleotide abortive product even when the productive RNA synthesis is going on in the presence of all the four

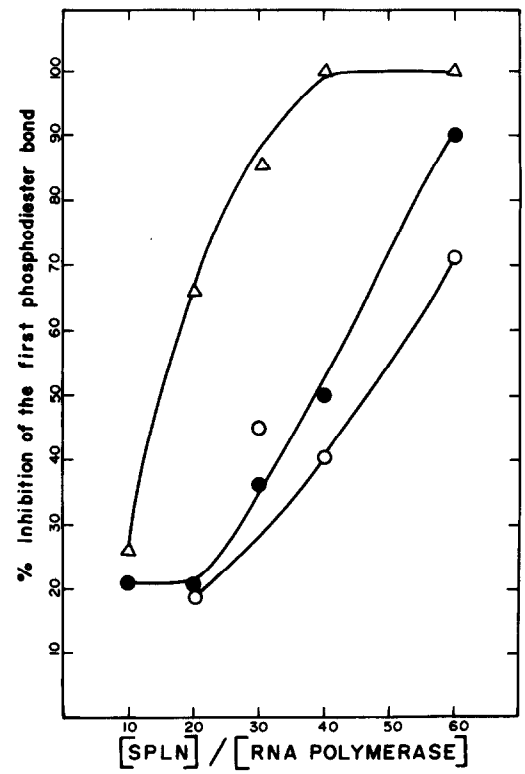

Fig.2. Effect of varying concentration of bovine seminal plasmin on the abortive initiation of transcription reaction carried out at T7A2 (—△—), T7A1 (—●—) and *rrn*B P2 (—○—) promoters. In the case of T7A2 the product was pppGpC and pppApU or pppCpCpC at T7A1 and *rrn*B P2 promoters respectively. The conditions of the reaction were the same as in the legend to fig. 1. As both the r-RNA promoters and T7A2 promoters are weaker than T7A1, the final incubation was carried out for 60 min to accumulate detectable amounts of the products. Quantitation of the product formed was carried out by scanning the autoradiogram of the acrylamide gel.

nucleotides [12]. Thus, the appearance of both di- and trinucleotides in lane 3 is expected.

However, formation of both these products was inhibited in the presence of seminal plasmin (lane 4). We could observe similar relationship between the presence of seminal plasmin and the degree of inhibition of the transcription initiation at all the promoters that we studied. Fig. 2 shows such a relationship. It can be noted from fig. 2 that the pattern of inhibition at varying molar concentrations of seminal plasmin was more or less the same in all the cases, although r-RNA promoter showed significantly less effect at all the concentrations of the inhibitor.

4. DISCUSSION

We have mentioned earlier in this paper that although seminal plasmin and rifampicin compete for the same binding site on RNA polymerase [3] their sizes are quite different from each other. With the help of a series of abortive initiation assays McClure and Cech have shown [5] that rifampicin is not a good inhibitor of the formation of the first phosphodiester bond; rather, it inhibits the translocation step. Our results show that seminal plasmin inhibits the synthesis of the first phosphodiester bond in the transcript as well. We have shown earlier that seminal plasmin, like rifampicin, is not capable of inhibiting the elongation of the pre-initiated RNA chain [13]. Thus, both these bacterial RNA polymerase inhibitors have various properties in common. However, it seems that seminal plasmin, being a larger molecule, encompasses a larger domain of the enzyme which is responsible for all the catalytic functions like first phosphodiester bond formation, translocation etc., leading to overall initiation of transcription.

Interestingly, it was observed that the concentration of seminal plasmin required for the 50% inhibition of transcription initiation was different at different promoters studied here (see fig. 2). Although it was reported earlier [2] that seminal plasmin inhibits ribosomal RNA synthesis in vivo maximally, we observed that the r-RNA promoter directed abortive initiation of transcription in vitro was least blocked in the presence of seminal plasmin. However, a recent report has postulated that r-RNA promoters are very weak promoters in vitro due to certain sequence characteristics [14]. Nevertheless this sharp effect of the nature of promoters on the degree of inhibition of initiation reaction suggest that this inhibitor probably interferes directly with the promoter binding ability of RNA polymerase. No such promoter dependent inhibition of transcription has been shown so far with rifampicin. If RNA polymerase has the intrinsic ability to recognise different promoters in different ways then one may be able to visualise this spectrum of inhibitory effect of seminal plasmin on transcription initiation. Some time ago it was shown that the stoichiometry of the reaction between DNA and RNA polymerase differ with different types of promoters [15]. Our experiments reported here suggest that seminal plasmin can be used to monitor this varying mode of promoter recognition by *E. coli* RNA polymerase.

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