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Differential inhibition of abortive transcription initiation at different promoters catalysed by *E. coli* RNA polymerase

Effect of rifampicin on purine or pyramidine-initiated phosphodiester synthesis

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The action of rifampicin on the RNA chain initiation catalysed by *E. coli* RNA polymerase over different templates has been studied. The steady-state formation of dinucleoside tetraphosphate under the condition of abortive initiation reaction was assayed. It was observed that rifampicin shows a spectrum of inhibitory effects on transcription initiation at different promoters. At two different promoters with a pyrimidine nucleotide at the 5′-initiation site, e.g. *rrn*B P2 having CTP and *lac* P2 having UTP, the effect of rifampicin on the abortive synthesis of the first phosphodiester bond was found to be total, even at low concentrations of the antibiotic. On the other hand, in most cases the effect of rifampicin on the abortive synthesis with a purine nucleotide at the 5′-initiation site was found to be only partial, with the exception of the T7A2 promoter, where rifampicin stimulates the abortive synthesis of pppGpC. It was also noticed that if there was a purine nucleotide at the second position of a dinucleotide which had already been synthesised by the enzyme, then further addition of the third nucleotide was not blocked in the presence of rifampicin. It appeared that a purine nucleotide at the initiation site or at the product terminus site of a translocated dinucleotide behaved similary towards rifampicin. In the same way, if this position was occupied by a pyrimidine, rifampicin would inhibit further phosphodiester synthesis, even at a very low concentration. The stimulatory effect of rifampicin at the T7A2 promoter was presumably because here a ternary complex containing the promoter, enzyme and the abortive transcript pppGpC was initially stable, but dissociated upon addition of rifampicin, resulting in the rapid turn-over of the product.

E. coli; RNA polymerase; Rifampicin; Inhibition; Promoter

1. INTRODUCTION

The rifampicin class of antibiotics have been extensively studied ever since the observation of Sippel and Hartmann that they inhibit the initiation of RNA synthesis in *E. coli* [1]. A number of studies carried out in the past showed that RNA polymerase mutations conferring resistance to rifampicin are located exclusively in the β subunit [2–5]. Since RNA polymerase from *E. coli* is a large, multi-subunit enzyme ($\alpha_2\beta\beta'\sigma$,$M_r$ 450,000), its nature of binding with rifampicin has been a matter of interest for sometime [6,7]. It appears that a proper juxtaposition of four segments of the β subunit is necessary for the creation of the rifampicin binding site [5], which requires the assembly of core RNA polymerase ($\alpha_2\beta\beta'$). This probably explains why the isolated β subunit [8] and subassembly, $\alpha_2\beta$ [9], show, respectively, no or little binding with rifampicin.

On the mechanism of action of rifampicin, two experimental observations are noteworthy. Firstly, 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 [10]. It has also been proposed that rifampicin destabilizes the ternary complex by interfering with the binding of the oligonucleotides to the active enzyme–DNA complex [11]. However, Kessler and Hartmann [12] showed that rifampicin strongly stimulated dinucleotide synthesis at 25°C and inhibited it, as expected, at 37°C. The reason behind this behaviour remains unexplained to date. It was also observed that the formation of pppApApC at the $\lambda P_{R'}$ promoter in the abortive initiation reaction was not inhibited by rifampicin [10]. The question remains of whether the action of rifampicin on the transcription initiation is promoter dependent. Thus, despite exhaustive and generalised studies in this area there remain a number of points which need to be addressed.

In the present study, we have tried to answer these questions by selectively studying the effect of rifampicin on transcription initiated at T7 early promoters which are known to be strongest *in vitro* [13], as well as rRNA promoters. Availability of the promoters as separate transcription units cloned in pBR322 [14] greatly aided the use of these promoters as templates for the transcription reaction studied here.

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2. MATERIALS AND METHODS

2.1. *Materials*

All the chemicals and buffers used in this study were of the purest grade available. The nucleotides were purchased from Boehringer and rifampicin from Sigma. Radionucleotides were obtained from Amersham or Bhabha Atomic Research Centre, India.

For any kind of abortive initiation assay, it is essential to check the quality of the triphosphates as well as to characterise the product formed. All the triphosphates were routinely chromatographed on a polyethylene amine (PEI) plate against ammonium carbonate buffer [15] and their identity confirmed under UV light by either their R_f value or by comparison with a known standard.

E. coli RNA polymerase was purified from mid-log phase cells of a RNase I⁻ strain (MRE 600) following essentially the method of Burgess and Jendrisak [16] with a modification developed in this laboratory [17]. The enzyme was found to have a specific activity of 2200 U/mg consisting of 60% active molecules. The enzyme purified in this way was routinely stored in 40 mM Tris-Cl (pH 7.9), 50% glycerol, 0.2 M KCl, 10 mM $MgCl_2$, 0.2 M EDTA and 0.1 mM dithiothreitol (DTT) at –20°C. However, we also observed that this enzyme is very stable in its solid form upon lyophilization. The *E. coli* RNA polymerase was dialysed against a glycerol free buffer containing 10 mM Tris-Cl (pH 7.9), 0.1 mM EDTA and 0.2 mM DTT overnight prior to lyophilization. Subsequently the product was kept at 4°C and reconstituted when necessary with a buffer containing 10 mM Tris-Cl (pH 7.9), 5% glycerol and 0.2 mM DTT. The protein concentration was determined by UV absorption using an extinction coefficient (1% $A_{280\ nm}$) of 6.2 [18]. The rifampicin concentration was fixed using the molar extinction coefficient 28,000 at 334 nm [19].

Derivatives of *E. coli* strain HMS 174, each containing plasmid pAR 1435 (T7A1), pAR 1539 (T7A2) or pAR 1354 (T7A3), were kindly provided by Dr. A.H. Rosenberg and Dr. F.W. Studier of Brookhaven National Laboratory, NY. Each of these plasmids has a single T7 early promoter as denoted in parenthesis [14]. Plasmid DNA pKK 3535 with the whole *rrn*B operon, containing both P1 and P2 promoters, was a kind gift from Dr. David Schlessinger's laboratory at Washington University, St. Louis. pUC9 plasmid was used as a template when the synthesis of the abortive product over the *lac* promoter was followed.

2.2. *Methods*

The plasmids were purified by the alkaline-lysis method, followed by CsCl gradient to isolate supercoiled DNA [20]. The promoter-containing inserts in the *Bam*HI site of pBR322 were checked by the run-off transcription of *Sal*I-digested plasmid over 4% denaturing polyacrylamide gel. In all cases the length of the transcript indicated that the initiation was taking place at the desired position. It should be mentioned at this point that the vector promoters are much weaker in comparison to the inserts containing T7 early promoters in vitro and therefore, even in the presence of a molar excess of RNA polymerase, transcription almost exclusively initiates at the inserts. In all the cases mentioned below plasmids linearized with *Cla*I were used as templates. In the case of pKK3535, a *Bam*HI–*Hin*dIII fragment containing both P1 and P2 promoters were used as template. Syntheses of the dinucleoside tetraphosphates at different promoters were carried out following the protocol described before [10,21] for the abortive initiation reaction in the transcription buffer containing 40 mM Tris-Cl (pH 7.9), 5 mM $MgCl_2$, 50 mM KCl and 1 mM DTT. To ensure that the abortive transcripts were initiated at the correct position, we checked the abortive transcription with various combinations of nucleotides at the vectors alone, as well as those containing the promoter inserts, and the products of such reactions were analysed in the following way: firstly, the R_f values of the different products were monitored and checked with standard values from literature wherever available. The products of the abortive initiation assay were also confirmed by treating the di- or trinucleotides, as appropriate, with alkaline phosphatase and then co-migrating them over a PEI plate with commercial or synthetic oligonucleotides. The length of the transcripts was also checked by running them over a 25% polyacrylamide gel [22]. Gel mobility shift assay of the complex was carried out according to the method of Fried and Crothers [23]. Urea-polyacrylamide gel electrophoresis of the abortive transcription products were performed essentially following the standard protocol and also with the help of a recently developed modification [24].

3. RESULTS

3.1. *Rifampicin-induced inhibition of abortive synthesis*

During the course of our investigation with T7 early promoters, we obtained a clone of T7A2 promoter where the sequence in the –10 region was altered, resulting in reduced strength of the promoter [25]. The order of the base was 'TAGCGA' instead of TAGAAT, and we named this promoter T7A2*. The initiation sequence under the control of T7A2*, however, remained unaltered.

Table I lists all the promoters that we studied here and the effect on them of rifampicin. The percentage of inhibition was calculated from the changes in the total area of the peaks in the paper chromatograph corresponding to the oligonucleotides. It is now well accepted that rifampicin does not inhibit the formation of the first phosphodiester bond [10], but interferes with the translocation event during the formation of the second phosphodiester bond. As expected, rifampicin did not show any appreciable effect on the abortive synthesis at T7A1, or at T7A3 (Table I). However, at *lac* P1 even the formation of a trinucleotide product, pppGpApA, was only minimally abrogated by the antobiotic. Abortive synthesis at T7A2 showed enhanced accumulation of the product when rifampicin was added to the medium (Fig. 1a). AT T7A2*, the effect of rifampicin was found to be minimal (Table I). However, in both these cases as well as with poly (dG-dC), when the reaction was initiated with GDP instead of GTP, rifampicin showed a partial inhibitory effect on the abortive synthesis. It should be noted at this point that at the poly (dG-dC) template the use of GDP instead of GTP ensured the synthesis of only the dinucleotide product, ppGpC.

It should also be mentioned at this point that the abortive initiation assay at each of these promoters was carried out for different time-spans so that sufficient amounts of product were accumulated in each case and percent inhibition of dinucleotide synthesis in the presence of rifampicin could be followed with sufficient ease. Under such assay conditions only the differential strength of these promoters is reflected. However, gel analysis and chromatographic runs ensured that no other products were formed other than those expected. Thus Table I lists a comparative study of the rifampicin-induced inhibition of the synthesis of the first few phosphodiesters under steady-state conditions.

Surprisingly, it was observed that rifampicin, even at a concentration level as low as 12 μM, totally inhibited abortive synthesis both at the *rrn*B P2 and *lac* P2 promoters (see Table I and Fig. 1). Both of these inhibitons

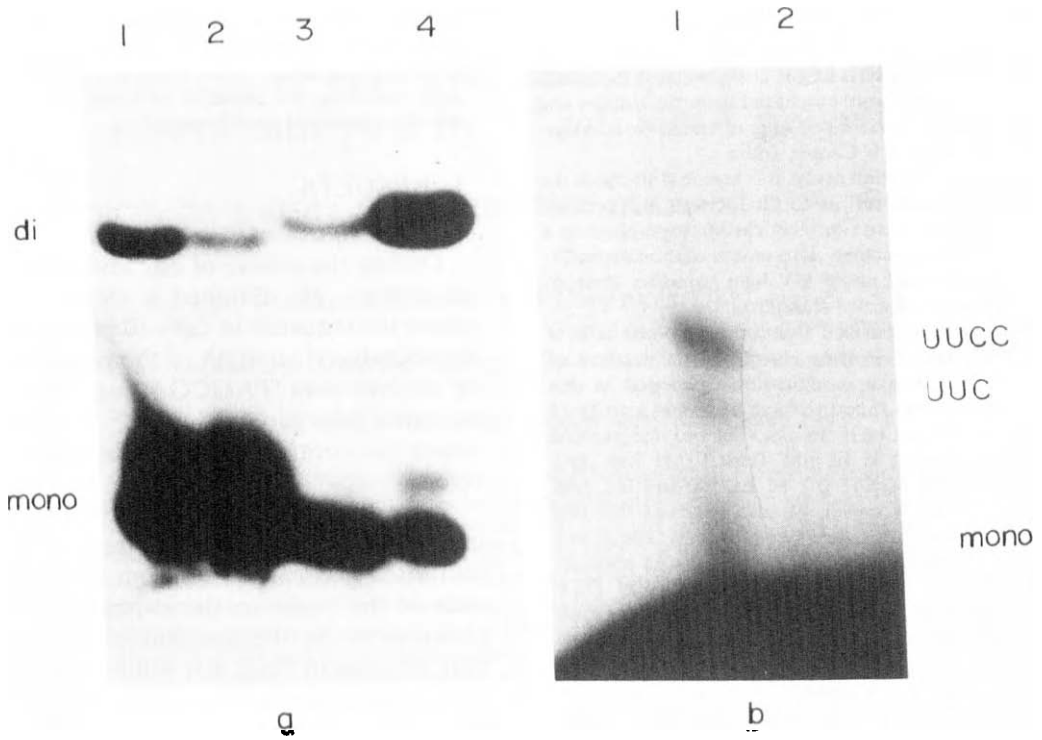

Fig. 1. (a) Polyacrylamide gel (25%) electrophoresis of the abortive transcripts in the presence and absence of rifampicin. Lanes 1 and 2 represent transcription over the template pAR1435 (T7A1) in the absence and presence, respectively, of rifampicin (50 μg/ml). The substrates were 800 μM ATP and 6 μM[α-^{32}P]-UTP (2 μCi/nmol). Lanes 3 and 4 represent transcription over the template pAR1539 (T7A2) in the absence and presence of rifampicin, respectively. GTP was 800 μM and [α-^{32}P]-CTP was 6 μM (2 μCi/nmol). The concentration of DNA was 0,4 mM DNA-phosphorus, and of RNA polymerase 0.16 μM. (b) A similar transcription reaction was carried out at the *lac* P2 promoter using UTP and [α-^{32}P]-CTP following the same protocol as given in [24]. Lane 1, without rifampicin; lane 2 with rifampicin (50 μ gm/ml).

were studied by paper chromatographic as well as gel electrophoretic analysis (Fig. 1b). On examination of Table 1 it was revealed that both of these promoters have a pyrimidine nucleotide at the 5′ initiation site. Generally two abortive products were obtained at *lac* P2, UUC and UUCC (Fig. 1b), upon the addition of UTP and CTP, as observed before by Gartenberg and Crothers [24]. In both cases, accumulation of a dinucleotide was also not observed in the presence of rifampicin, either by gel or by chromatographic separation, indicating that rifampicin totally blocked even the fromation of the first phosphodiester bond. (At *rrn*B P1, [α-^{32}P]ATP was used as tracer instead of CTP to avoid signals from *rrn*B P2.)

Table 1

Effect of rifampicin (60 μM) on the abortive initiation of transcription at different promoters

Promoter	Initial transcribed sequence	Nucleotides provided in the abortive initiation reaction	Expected products	R_f value	Percentage of inhibition[a]
T7A1	AUCGAGAGGGACAC	ATP,[α-^{32}P]UTP	pppApU	0.44	40
T7A2	GCUAGGTAACACTA	GTP,[α-^{32}P]CTP	pppGpC	0.56	Nil (several-fold increase)[b]
T7A2	GCUAGGTAACACTA	GDP,[α-^{32}P]CTP	ppGpC	0.49	44
T7A2*	GCUAGGTAACACTA	GTP,[α-^{32}P]CTP	pppGpC	0.56	0 (slight increase)
T7A2*	GCUAGGTAACACTA	GDP,[α-^{32}P]CTP	ppGpC	0.49	58
poly d(G-C)	GCGCGCGCGCGCGC	GDP,[α-^{32}P]CTP	ppGpC	0.49	65
T7A3	AUGAAACGACAG	ATP,[α-^{32}P]UTP	pppApU	0.44	20
*rrn*B P1	ACUGACACGG	[α-^{32}P]ATP, CTP	pppApC	0.38	0
*rrn*B P2	CCCGCGCCGC	[α-^{32}P]CTP	pppCpCpC		100[c]
lac P1	GAAUUGTGAGC	[α-^{32}P]GTP, ATP	pppGpApA	–	7
lac P2	UUCCGGGCTCG	[α-^{32}P]CTP, UTP	pppUpUpC and pppUpUpCpC	–	100[c]

[a] This is an average value obtained from various chromatographic and electrophoretic assays
[b] No inhibition was observed with concentrations of rifampicin up to 240 μM
[c] Total inhibition was observed with concentrations of rifampicin as low as 12 μM

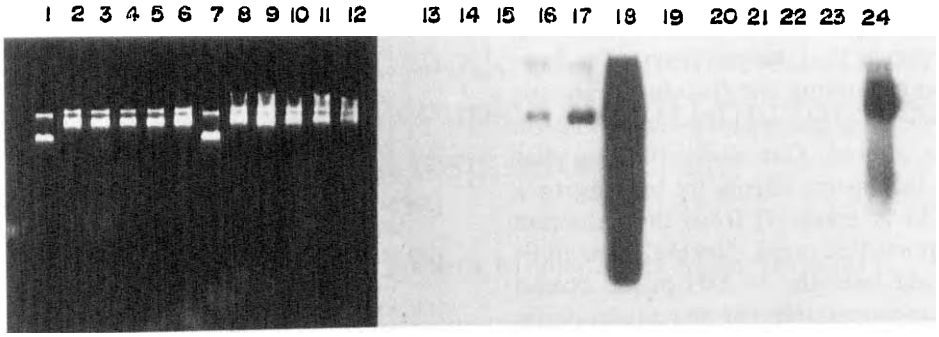

Fig. 2. Gel mobility retardation and ternary complex analysis of pAR1539 (T7A2) with RNA polymerase and NTPs. The control plasmid DNA (20 nM, lanes 1 and 7) was treated at 37°C for 10 min with *E. coli* RNA polymerase (200 nM) in the absence of any NTP (lane 2), in the presence of (lane 3) GTP alone, (lane 4) GTP and CTP, (lane 5) GTP, CTP and UTP, and (lane 6) all four NTPs. Lanes 8–12 are the same as lanes 2–6, respectively, except that these were incubated with 100 μM rifampicin at 37°C for 5 min prior to loading. The concentration of GTP was 200 μM, while the other NTPs were used at 10 μM: [α-^{32}P]-CTP was at 2 μCi/nmol. Samples were analyzed over a 1% agarose gel in Tris-borate-EDTA buffer (pH 8.3) at 1.5 V/cm for 6 h. Lanes 13–24 represent the same samples as lane 1–12, respectively, when dried and autoradiographed.

Upon examination of Table I, two observations appear to be noteworthy: (i) there is a difference between the purine or pyrimidine at 5′ initiation site towards rifampicin-induced inhibition of abortive synthesis, and (ii) both T7A2 and T7A2* show stimulation of abortive transcription in the presence of rifampicin.

3.2. *Stability of the transcription complex at T7A2 or T7A2* in the presence of rifampicin*

Schultz and Zillig [11] reported earlier that rifampicin destabilises the ternary complex of DNA, enzyme and short transcripts. Such effects can best be seen at the promoters, where a stimulatory effect of rifampicin on abortive transcription was observed. Therefore, we decided to check the stability of the ternary transcription complex in the presence of rifampicin at the T7A2 promoter. When the second nucleotide, CTP (see Table I), was kept radio-labelled, and the transcription was carried out over a *Cla*I-linearised plasmid, pAR 1539, that contains the T7A2 promoter, a stable ternary complex was formed upon the addition of the first two or three nucleotides, producing, respectively, pppGpC and pppGpCpU. This was confirmed by autoradiography where the retarded DNA–enzyme complex band was detected (Fig. 2). We did not try to quantitate the percentage of the DNA–RNA polymerase complex that retained the short transcripts. However, these complexes were stable to heparin but dissociated completely in the presence of rifampicin, as observed by following the disappearance of radioactivity in the gel (see Fig. 2). Thus, it appears that rifampicin stimulated the turnover of the abortive transcripts synthesized at T7A2 by destabilizing the ternary transcription complex.

DISCUSSION

If the differential inhibition by rifampicin of abortive initiation at different promoters could be explained in terms of a mechanism, then it should also reflect some aspects of the initiation of transcription. Analysis of the results in Table I indicate that there is a correlation between the differential effect of rifampicin and the nature of the abortive initiation products at the various promoters. When a pyrimidine nucleotide was occupying the 5′ initiation site, as in the case of *rrn*B P2 or *lac* P2, contrary to the observation made by McClure and Cech [10], even the first phosphodiester synthesis was totally inhibited by rifampicin. As most of the RNA message in *E. coli* initiates either with A or G, this observation has eluded us for a long time. Interestingly, we obzerved that in the cases where the second nucleotide or the third nucleotide were also purines, rifampicin-induced inhibition of the second or third phosphodiester formation was minimal. It can be expected that after the synthesis of the first phosphosdiester bond, the dinucleotide translocates in such a way that the second nucleotide of the translocated dimer occupies the position which was hitherto occupied by the first nucleotide. Thus the product terminus site is the same as the initiation site before product formation. From out results in Table I, we would like to propose that if there is a pyrimidine nucleotide at the initiation site or product terminus site, then the rifampicin effect would be total for the next phosphodiester synthesis. On the other hand, if this site is occupied by a purine nucleotide, rifampicin-induced inhibition for the next phosphodiester would be much less. It was noticed earlier [26] that the synthesis of UpApU from UpA and UTP over poly d(A-T) was only partially inhibited by rifampicin.

The assumption that the rifampicin-bound RNA polymerase distinguishes the substrates at the initiation (or product terminus) site for its inhibitory effects becomes further evident when GDP is used instead of GTP in the abortive initiation reactions. Rifampicin exerted a partial inhibition on the synthesis of ppGpC with GDP and CTP as substrates at the T7A2 and T7A2* promoters, as well as poly d(G-C). One plausible

explanation for all these results is that the binding of rifampicin to polymerase induces a conformational change in the enzyme such that the geometry of at least the initiation nucleotide binding site (product terminus site, so called after the first phosphodiester formation and translocation) is altered. Our earlier finding that rifampicin exerts its inhibitory effects by binding to a site on the enzyme 30 Å away [7] from the substrate binding site also supports this point. Normally the initiation nucleotide should hold the 3′ -OH of the bound nucleotide oriented appropriately for the nucleophilic attack on the NTP bound at the elongation nucleotide site, resulting in phosphodiester formation. Thus, in the rifampicin-bound enzyme, the differential inhibition obtained with different nucleotide substrates may be because of their altered orientation in the binding site, with the pyrimidines fitting least well.

The fact that a transcription complex at the T7A2 promoter retains a dinucleotide transcript, pppGpC, explains the very low rate of abortive initiation at this promoter. The stability of this dinucleotide product in the complex is reduced by the binding of rifampicin to the enzyme, thus resulting in an enhanced rate of turnover of the dinucleotide. This also explains a lone observation made earlier [12] that rifampicin activates abortive synthesis at 25°C. It is expected that the turn-over of the abortive product at this temperature is rate-limiting but is enhanced in the presence of rifampicin.

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