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**Involvement of DNA gyrase in the transcription of ribosomal RNA**

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**ABSTRACT**

The DNA gyrase inhibitor novobiocin specifically inhibits the transcription of ribosomal RNA *in vivo* while protein synthesis and the mRNA transcription are only partly affected. *In vitro* the novobiocin inhibition is only observed when a protein fraction, which stimulates ribosomal RNA synthesis, is present. These results indicate that DNA gyrase is involved in the transcription of ribosomal RNA, probably at an initiation step.

**INTRODUCTION**

In *E. coli* seven ribosomal RNA operons comprise about 1% of the genome (1). At high growth rates the synthesis of rRNA accounts for up to 70% of the RNA synthesis (2). Despite many studies the detailed molecular mechanism of the regulation of rRNA synthesis is still unknown (3), nor has the coordination of rRNA synthesis and that of ribosomal proteins been elucidated (4). The DNA sequences of a number of rRNA promoter regions have been determined which show common features (5,6). Comparison of the structure of these promoters with those for ribosomal proteins did not reveal any striking similarity nor are there striking differences from other promoters (7). Apparently we still miss or have overlooked important clues. The tertiary structure which is not immediately evident from the DNA sequence, may be involved.

In a bacterial cell chromosomal DNA is in a negatively supercoiled form. Seeburg et al. (8) have shown that *in vitro* the presence of negative supercoils stimulates the ability of DNA to act as template for *E. coli* RNA polymerase. Since the formation of the initiation complex is believed to require partial unwinding of the double helix (9) negative supercoiling which

facilitates unwinding of the DNA, probably enhances binding of RNA polymerase to open DNA.

The enzyme DNA gyrase introduces negative supertwists into double-stranded closed circular DNA (for a review see ref. 10). DNA gyrase is a tetramer composed of two kinds of subunits A and B. The A subunits are particularly associated with the breakage and rejoining of DNA and this activity is inhibited by nalidixic acid and oxolinic acid (11). The B subunits mediate energy transduction and are inhibited by novobiocin and coumermycin A<sub>1</sub> (12).

Several reports have appeared which indicate that DNA gyrase inhibitors cause specific inhibition of the transcription of certain genes in vivo: phage N<sub>4</sub> DNA (13); catabolite-sensitive operons of maltose, lactose and galactose (14); tryptophan operon under control of the lambda phage promoter (15). The effect of inhibitors of DNA gyrase on transcription in vitro of several bacterial and plasmid genes in a cell-free system was reported (16). The promoters of the lactose operon, the rRNA operon *rrnB* and the *col E1* gene were found to be most the sensitive ones while other genes were unaffected.

In this paper we describe the effect of novobiocin on stable RNA synthesis in vivo and in vitro. The rRNA synthesis in vitro in a system with purified DNA, carrying the rRNA operon *rrnX*, and RNA polymerase is preferentially stimulated by a protein factor (17). The stimulation of rRNA synthesis, which is due to increased initiation frequency, is inhibited by novobiocin while novobiocin has no effect on the transcription by RNA polymerase alone. In vivo, besides the DNA synthesis, the stable RNA synthesis is strongly inhibited by novobiocin while protein synthesis and (therefore) mRNA synthesis are much less depressed.

#### MATERIALS AND METHODS

Rifampicin was purchased from Schwartz/Mann, Orangeburg, N.Y., U.S.A.; novobiocin was from Sigma, St. Louis, U.S.A.. [5-<sup>3</sup>H]-Uracil (25 Ci/ mmol), [<sup>14</sup>C]amino-acid mixture (45 mCi/milliatom of carbon) and [5-6-<sup>3</sup>H] UTP were from The Radiochemical Centre, Amersham, UK.; [Methyl-<sup>3</sup>H]Thymidine (6.7 Ci/mmol) was from New England Nuclear, Dreieich, GFR.

### Strains

Phage  $\lambda$ d51lv (carrying the rRNA operon *rrnX*) was a gift from Dr. P. Jørgensen (18). *E. coli* NF537 (*leu*<sup>-</sup>, *valS*<sup>ts</sup>, *relA1*), which is derived from AS19, a rifampicin-sensitive strain of *E. coli* B, and NF87 were obtained from Dr. N.P. Fiil.

### Growth and labelling

NF537 was grown at 30°C in medium K, containing E salts (19), 0.1% Bacto tryptone, 0.05% yeast extract and 0.2% glucose. The doubling time was about 45 min. When an A<sub>450</sub> of 0.45 was reached novobiocin was added at a concentration of 25 µg/ml. [<sup>3</sup>H]Uracil, [<sup>14</sup>C]amino-acid mixture or [<sup>3</sup>H]thymidine were added as indicated in the legends of the figures. Samples of 0.1 ml were taken and mixed immediately with 2.0 ml ice-cold 6% trichloroacetic acid. The samples for <sup>14</sup>C-amino acids incorporation were heated for 15 min at 95°C to solubilize labelled amino acids bound to tRNA. The precipitates were collected and washed on glass-fiber filters (Whatmann GF/C).

### Preparation of $\lambda$ d51lv DNA

$\lambda$ d51lv was grown by thermal induction of the lysogen and separated from helper phage according to Miller (20). Phage DNA was stored in 10 mM Tris-HCl, pH 7.5 (25°C), 0.1 mM EDTA at 4°C.

### RNA synthesis in vitro

The standard reaction mixture contained 40 mM Tris-HCl, pH 7.9 (25°C); 110 mM KCl; 0.4 mM potassium phosphate; 10 mM MgCl<sub>2</sub>; 5% (v/v) glycerol; 0.3 mM each of CTP and GTP; 0.5 mM ATP; 0.1 mM <sup>3</sup>H-UTP (0.8 Ci/mmol); 0.1 mM EDTA; 0.1 mM dithiothreitol; 0.2 mg bovine serum albumin per ml; 0.1 mM EGTA (ethyleneglycol-bis- $\beta$ -aminoethyl ether) N,N'-tetraacetic acid); 10 µg/ml  $\lambda$ d51lv DNA and, when present, 3 µg/ml novobiocin.

The mixture was preincubated for 10 min at 37°C; then RNA polymerase (26 µg/ml) and, where indicated, the protein fraction which precipitates between 30 and 47% ammonium sulfate saturation were added (see for its preparation Oostra et al. (17)). The reaction was stopped after 30 min by addition of an equal volume of 4xSSC. Phenol extraction and measurement of total RNA and rRNA were carried out as described earlier (21).

Pulse-labeling and rRNA hybridization

To 0.5 ml of an exponential growing culture of NF537 ( $A_{450}=0.5$ ) novobiocin was added to a final concentration of 30  $\mu\text{l/ml}$ . At 8 min, 40  $\mu\text{Ci}$  [ $5\text{-}^3\text{H}$ ]-Uracil (specific activity 25 Ci/mmol) were added and shaken for 30 sec. The cells were transferred to a tube at  $90^\circ\text{C}$ , containing 0.5 ml SDS-lysis mixture (0.01 M Tris-HCl, pH 7.5 ( $25^\circ\text{C}$ ); 0.1 M NaCl; 0.002 M EDTA and 1% SDS). After 1 min at  $90^\circ\text{C}$  the tubes were cooled at  $0^\circ\text{C}$ . The lysed cells were twice extracted with one volume of phenol saturated with 2xSSC. To the water layer 2.5 volumes of 96% ethanol were added; the mixture was held for 10 min at  $-70^\circ\text{C}$ . The precipitate formed was collected by centrifugation. The pellet was dissolved in 0.25 ml 2xSSC containing 0.05% SDS and aurin tricarboxylic acid (50  $\mu\text{g/ml}$ ).

For the determination of total RNA synthesized 10  $\mu\text{l}$  was treated by TCA as described earlier (21). For the estimation of rRNA, 25  $\mu\text{l}$  was hybridized in a total volume of 250  $\mu\text{l}$  as described earlier (21) except that the hybridization time was 16h. The hybridization efficiency was 35 to 40%.

RESULTSEffect of novobiocin on rRNA synthesis in vitro

The influence of novobiocin on rRNA synthesis in vitro in a system with purified  $\lambda\text{d511v}$  DNA and RNA polymerase is shown in Table 1. As can be seen there is no effect on the RNA synthesis in this system. The ribosomal RNA is preferentially stimulated by a protein factor (17). When novobiocin is added together with

Table 1. Effect of novobiocin on rRNA synthesis in vitro

Extract	Novobiocin	$^3\text{H-UMP inc. (dpm} \times 10^{-3})$		rRNA (%)
		non-rRNA	rRNA	
-	-	76.9	20.2	24.7
+	-	55.2	53.7	54.9
-	+	84.9	18.9	21.8
+	+	63.5	19.5	27.8

RNA was synthesized under standard reaction conditions as described in Materials and Methods

this factor the rRNA synthesis is drastically lowered (Table 1). Apparently, the DNA gyrase inhibitor novobiocin in this system acts only when the stimulator is present but is without effect on RNA polymerase proper. Whether this inhibition is real or an artifact we tested the effect of novobiocin on rRNA synthesis in vivo.

#### Effect of novobiocin on RNA accumulation

The effect of novobiocin on RNA accumulation is shown in Fig. 1. Apparently, novobiocin has a dramatic effect. The rapid attainment of a plateau of incorporated label shows a complete inhibition of stable-RNA synthesis. Whether labile RNA is still being synthesized at an appreciable rate can be decided by the use of rifampicin which blocks initiation of all RNA synthesis (22). Since elongation is not blocked by this drug all labile RNA in production will be completely terminated and within a short time broken down. A drop in the plateau level should thus occur since the steady-state concentration of labile RNA

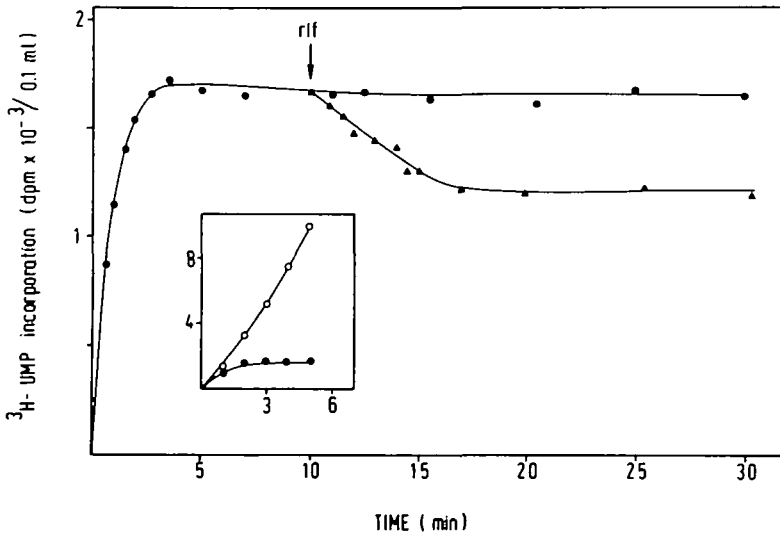


Fig.1. Influence of novobiocin on RNA synthesis

To an exponentially growing culture of NF537 novobiocin (25  $\mu\text{g/ml}$ ) and  $^3\text{H}$ -uracil (5  $\mu\text{Ci/ml}$ , 20  $\text{mCi/mg}$ ) were added at  $t=0$ . At the times indicated 100  $\mu\text{l}$  aliquots were removed. At  $t=10$  to a part of the culture rifampicin (50  $\mu\text{g/ml}$ ) was added.  $\circ$ — $\circ$  control;  $\bullet$ — $\bullet$  novobiocin;  $\blacktriangle$ — $\blacktriangle$  novobiocin and rifampicin.

contributes to it. Fig. 1 shows that there is still synthesis of labile RNA occurring when stable RNA synthesis has already ceased. Thus, the synthesis of ribosomal RNA which is for all practical purposes stable (23) is much stronger inhibited by novobiocin. Whether mRNA is affected at all cannot be concluded from this experiment.

Protein synthesis in the presence of novobiocin

In agreement with the maintenance of (at least some level of) labile RNA synthesis after addition of novobiocin, Fig. 2 shows that 60% of protein synthesis is unaffected by novobiocin. In view of the half-life of the mRNA which is 1.5 to 2 min (2) there must be mRNA synthesis; thus only part of mRNA synthesis is inhibited by the drug. Which genes are most affected cannot be concluded. Some mRNA genes have been reported to be quite sensitive (14,15,24,25).

Differential effect of novobiocin on RNA synthesis

The ratio of unstable to stable RNA, a measure of the

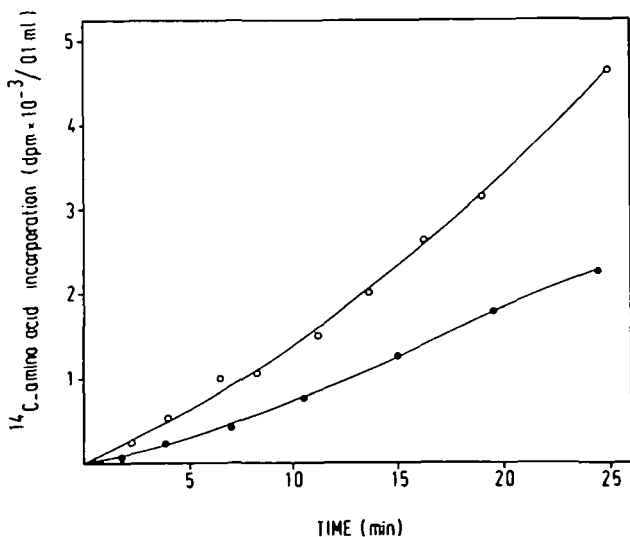


Fig. 2. Effect of novobiocin on protein synthesis

An exponentially growing culture of NF537 was labelled with  $^{14}\text{C}$ -amino acid mixture (1 $\mu\text{Ci}/\text{ml}$ ). Samples of 100  $\mu\text{l}$  were taken in the absence (○—○), or in the presence of 25  $\mu\text{g}/\text{ml}$  novobiocin (●—●).

mRNA/rRNA synthesis ratio, was determined in an experiment analogous to the experiments described by Pato and von Meyenburg (2). The incorporation of  $^3\text{H}$ -uracil was followed after simultaneous addition of label and rifampicin. As stated above rifampicin blocks initiation, but does not affect elongation and termination of RNA chains. The label incorporated in RNA reaches a maximum when nearly all chains are completed; the subsequent decay of a part of the labeled material is due to breakdown of unstable RNA. In this way the ratio labile/stable RNA synthesis at the time of rifampicin addition is determined.

Fig. 3 shows the labeling pattern. In the control experiment the ratio labile/stable RNA synthesis is about 0.60 which is in agreement with the ratio expected of a doubling time of about 45 min (2). If novobiocin is added simultaneously with label and rifampicin the same curve is obtained (data not shown). The observed inhibitory effect of novobiocin on RNA synthesis (Fig. 1)

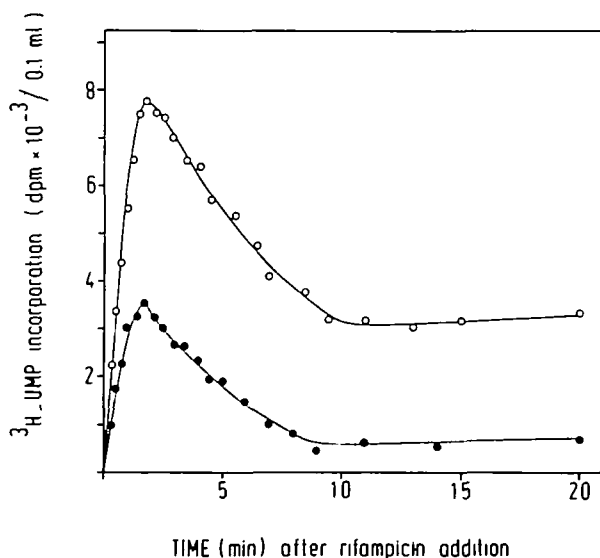


Fig. 3. RNA synthesis after inhibition of initiation

To an exponentially growing culture of NF537 rifampicin (50  $\mu\text{g}/\text{ml}$ ) and  $^3\text{H}$ -uracil (5  $\mu\text{Ci}/\text{ml}$ , 29  $\text{mCi}/\text{mg}$ ) were added. Samples of 100  $\mu\text{l}$  were taken.  $\circ$ — $\circ$  control; nalidixic acid to prevent incorporation of label into DNA was present in a concentration (10  $\mu\text{g}/\text{ml}$ ) which does not affect RNA synthesis (2,26).  $\bullet$ — $\bullet$  novobiocin (25  $\mu\text{g}/\text{ml}$ ) was added 8 min prior to addition of label.

is thus most probably due to a decrease in the frequency of initiation of RNA synthesis, and not an effect on elongation, a premature random arrest or termination as could already be expected from Fig. 1.

If label and rifampicin were added 8 min after addition of novobiocin, when accumulation of RNA has ceased, there is a drastic change in the ratio labile/stable RNA synthesis (Fig. 3). Most of the labeled material decays: at least 85% of the RNA made is unstable. Comparing the mRNA synthesis in the absence and presence of novobiocin we see that the mRNA synthesis is reduced by about 35% which is in agreement with the measured reduction of the protein synthesis (Fig. 2).

The maximum of the label in RNA in the presence of only novobiocin is reached later than in the presence of rifampicin. Thus novobiocin acts more slowly, or only indirectly, on rRNA synthesis. This apparent lag is also evident from the ratio labile/stable RNA in Fig. 1. It is not caused by a low permeability of the drug as can be seen in Fig. 4. DNA synthesis is inhibited by

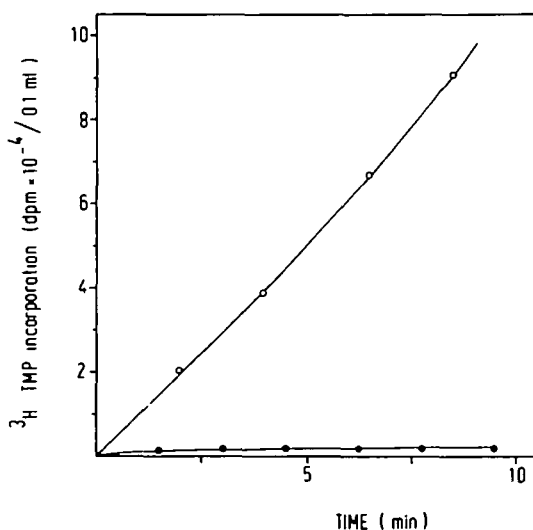


Fig. 4. Effect of novobiocin on DNA synthesis

An exponentially growing culture of NF537 was labeled with  $^3\text{H}$ -thymidine (5  $\mu\text{Ci}/\text{ml}$ , 5  $\text{mCi}/\text{mg}$ ). Samples of 100  $\mu\text{l}$  were taken in the absence (○—○), or in the presence of 25  $\mu\text{g}/\text{ml}$  novobiocin (●—●).



novobiocin within a few seconds. We must conclude that either the effect of the drug on the rRNA synthesis is indirect or a higher concentration is necessary for the inhibition of the rRNA synthesis.

#### Characterization of RNA synthesized

We have assumed in the foregoing part that stable RNA is practically equivalent to rRNA. To show that this is indeed so, and that novobiocin did not stabilize an otherwise unstable RNA fraction we studied the novobiocin effect on pulse-labelling by using the hybridization-competition method with which 16S and 23S rRNA are determined (21). Table 2 shows the result and clearly demonstrates that novobiocin has a differential effect on rRNA and non-rRNA synthesis, rRNA synthesis being inhibited for at least 95% whereas non-rRNA is only reduced by half. The results are in good agreement with those in Figs 2 and 3 in which protein synthesis, and stable/unstable RNA synthesis are depicted.

#### DISCUSSION

Our results clearly show a specific inhibition of rRNA transcription in vivo by the DNA gyrase inhibitor novobiocin. The results indicate that the inhibitory action is exerted at an initiation step, i.e. prior to transcription proper. Although it cannot be considered proven the effect of novobiocin is most likely due to its action on DNA gyrase, a DNA topoisomerase changing the linkage number (10,12), and thus the helicity of circular double-stranded DNA (27). Protein synthesis and overall mRNA transcription are much less inhibited by novobiocin. Our results agree with those found by others for specified operons and their different sensitivity to DNA gyrase inhibitors. The question arises how the continual action of DNA gyrase is

Table 2. Effect of novobiocin on the relative synthesis of rRNA

novobiocin	<sup>3</sup> H-UMP inc. (dpm x 10 <sup>-3</sup> /25 $\mu$ l)			rRNA (%)
	total RNA	rRNA	non-rRNA	
-	175.8	66.8	109.0	43
+	59.0	3.5	55.5	7

Pulse-labelling and competition hybridization were as described in Materials and Methods.

necessary, or at least favourable, for the transcription of certain genes, in our case rRNA transcription.

The bacterial chromosome probably contains supercoiled domains (28) and is condensed in beaded substructures (29). The conformation of the bacterial DNA is determined and maintained by topoisomerases, like DNA gyrase, and some other proteins, e.g. those binding to specific conformations only (30). DNA gyrase might facilitate transcription from certain operons by making them more accessible to RNA polymerase, e.g. by local (partial) unwinding; in vitro supercoiling enhances transcription of formation of initiation complexes (8,31).

Interestingly the data of the novobiocin sensitivity of the tryptophan promoter seems at first sight conflicting (14-16,24). The flanking DNA into which the tryptophan promoter is inserted or in which it is present is different which might explain the differences in sensitivity. We have to conclude that DNA gyrase can induce an effect on DNA which can be propagated along the DNA at least over a certain distance. Supercoiling might be such a phenomenon.

Our results in vitro show that novobiocin does not inhibit purified RNA polymerase as is also found for the related drug coumermycin A<sub>1</sub> (32). The transcription of rRNA is only novobiocin sensitive if our stimulating factor is present. The easiest explanation is the presence of a DNA gyrase or DNA gyrase-like topoisomerase in the protein fraction, and its inhibition by novobiocin. The DNA gyrase would, in this view, stimulate rRNA initiation by acting on the DNA which is not closed circular DNA. We have thus to assume an action of DNA gyrase on linear DNA, or the presence of ligase in our preparation. Several times the action of DNA gyrase (or its sister enzyme from T4 phage) on linear DNA has been postulated (16,33); and its involvement in linear DNA replication has been established (34). At this stage, we cannot exclude the presence of ligase in our fraction, so the matter is still open.

However that may be, our results show that in vivo as well to some extent in vitro rRNA transcription is critically dependent upon the conformation of DNA in the vicinity of the rRNA promoter. DNA gyrase is one but probably not the only factor influen-

cing this conformation. It is not clear how DNA gyrase assists in the promoter function at binding RNA polymerase or in initiation; variation of the degree of superhelicity or induction of superhelicity within local domains could be the basis of some regulation. The DNA conformation may directly or indirectly depend upon metabolic and growth parameters and thus be a transducer of the control of rRNA synthesis by the cell metabolic state.

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