INVOLVEMENT OF DNA GYRASE IN THE REPLICATION AND TRANSCRIPTION OF MYCOBACTERIOPHAGE I3 DNA

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1. Introduction

Supercoiling of DNA is necessary for replication and transcription [1]. DNA gyrase catalyzes the conversion of relaxed closed circular DNA into negatively supertwisted form, thereby promoting replication and transcription [2–5]. The apparent inhibition of replication by novobiocin and coumermycin A_1 is by interaction with one of the subunits of DNA gyrase [3,4,6]. Similarly, nalidixic acid and oxolonic acid block DNA synthesis by binding to the other subunit of the enzyme [3,6,7]. These antibiotics have been conveniently employed to investigate the role of gyrase on replication and gene expression in various systems [5].

During our attempts to study replication and transcription of mycobacteriophage I3, we observed complete inhibition of growth of the phage in the presence of gyrase-specific drugs. This prompted us to investigate the role of DNA gyrase in the replication and transcription of phage 13. Our results suggest that DNA gyrase is required for DNA and RNA synthesis. Most significantly the enzyme is needed throughout during transcription unlike other systems where transcription only at certain promoters is influenced by this enzyme.

2. Materials and methods

A clear plaque mutant (C5) of phage I3 [8] was used in these studies. The host strain used was *Mycobacterium smegmatis* SN 2. Novobiocin resistant *M. smegmatis* was isolated by mutagenesis with *N*-methyl, *N*-nitro, *N*-nitrosoguanidine and selective enrichment.

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Growth of phage and bacteria was carried out as in [9].

Oxolonic acid (Bristol Labs), nalidixic acid and novobiocin (Sigma) were added to the culture from a stock solution (10 mg/ml) in DMSO. Appropriate volume of DMSO was added to control cultures. ^{3}H-Labelled thymine, uracil and cytosine were from Bhabha Atomic Research Centre, Bombay.

2.1. *DNA and RNA synthesis in the presence of novobiocin and oxolonic acid*

Bacteria were grown at 37°C until exponential phase (16 h, 1×10^8 cells/ml) and infected with phage at a multiplicity of infection of 15. At the time of infection ^{3}H-labelled thymine, uracil or cytosine (2 μCi/ml) were added to the culture. At various times 0.5 ml samples were withdrawn and added to 4 ml chilled 10% trichloroacetic acid. The precipitates were processed for the determination of radioactivity.

2.2. *Kinetics of DNA and RNA synthesis*

To measure the rate of DNA and RNA synthesis, 0.5 ml samples of infected cultures were exposed to 10 min pulses of ^{3}H-labelled thymine, uracil or cytosine (2 μCi/ml) at various times after infection, until the end of the latent period (200 min). The synthesis was stopped by adding 10% trichloroacetic acid and precipitates were processed for radioactivity.

3. Results

3.1. *DNA gyrase is essential for the growth of phage I3*

Growth of *M. smegmatis* was completely inhibited at low concentrations of novobiocin (10 μg/ml) and oxolonic acid (50 μg/ml). Addition of these drugs during growth of the phage completely inhibited phage

production. These results suggested the presence of DNA gyrase in *M. smegmatis* and its possible involvement in phage I3 development.

The presence of DNA gyrase in *M. smegmatis* cell extracts was confirmed by monitoring the conversion of relaxed circular pBR 322 DNA into supercoiled form using agarose gel electrophoresis. Novobiocin (10 μg/ml) and oxolonic acid (40 μg/ml) inhibited this reaction (not shown).

3.2. *Effect of oxolonic acid and novobiocin on total DNA and RNA synthesis*

The pattern of DNA and RNA synthesis after phage infection is shown in fig.1. In presence of 100 μg oxolonic acid/ml, 60% inhibition of macromolecular synthesis was observed. Similar results were obtained when nalidixic acid was used. Inhibition of DNA and RNA synthesis was much more pronounced in presence of 100 μg novobiocin/ml, with practically no synthesis taking place. RNA isolated after phage infection was predominantly (>90%) phage specific (observations). These results support the idea that gyrase is needed for phage I3 replication and transcription.

3.3. *Effect of novobiocin on the rates of DNA and RNA synthesis*

In order to study the specific role of the gyrase, the rates of DNA and RNA synthesis were measured in the presence of 100 μg novobiocin/ml. Both DNA and RNA synthesis were completely inhibited by the drug (fig.2). The effect of varying concentrations of novobiocin on DNA and RNA synthesis after infec-

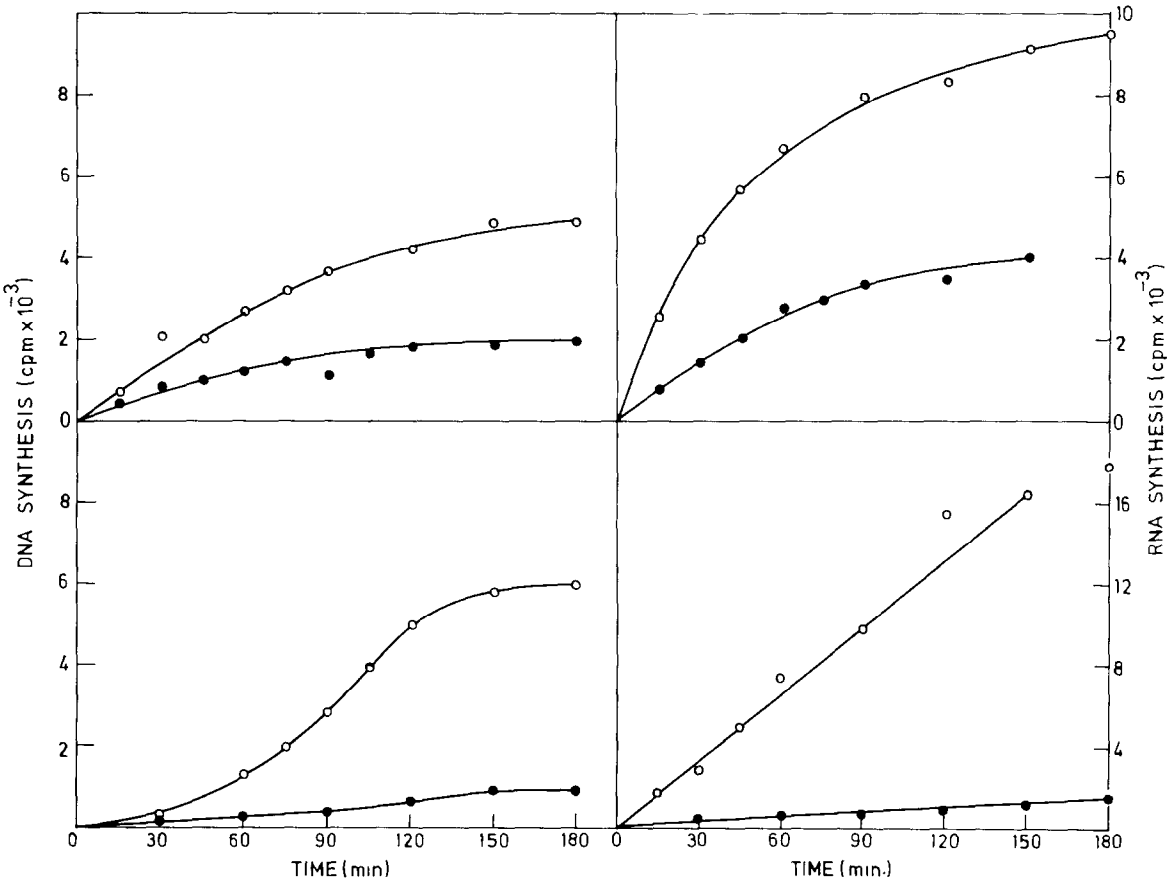

Fig.1. Effect of oxolonic acid (top panel) and novobiocin (bottom panel) on DNA and RNA synthesis: (○) control (no drug); (●) in the presence of the drug. Since the GC content of phage I3 DNA is 68%, the incorporation of [^{3}H]cytosine to DNA or RNA gave a much higher value compared to thymine or uracil. When [^{3}H]cytosine was used, the trichloroacetic acid precipitates were treated with alkali or pure preparation of RNase A. The difference in radioactivity before and after digestion gave the incorporation into DNA. The patterns obtained with [^{3}H]thymine of [^{3}H]uracil were similar.

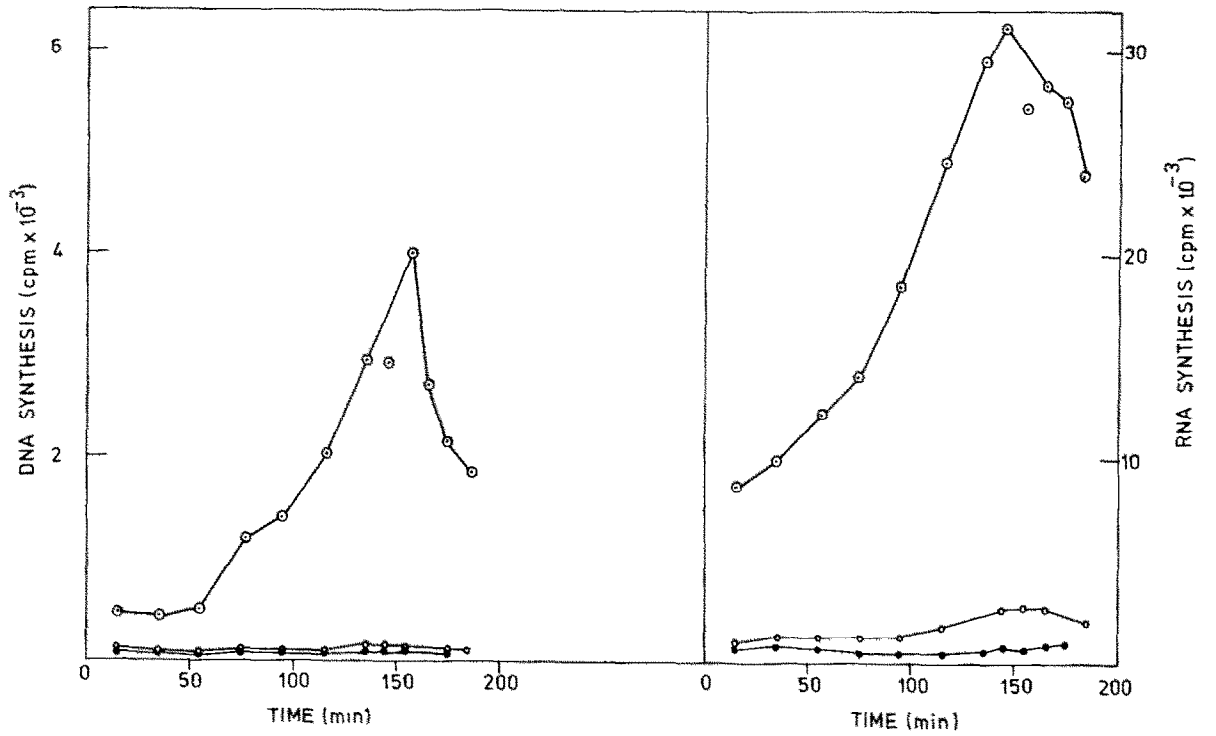

Fig.2. Kinetics of DNA and RNA synthesis: for details, see text. Novobiocin was added at the time of infection. In control, the appropriate quantity of DMSO was added. The points shown are the mid points of the individual pulses: (◉) control (no drug); (○) 10 μg novobiocin/ml, (●) 25,50, 100 μg novobiocin/ml.

tion was also investigated. At all the concentrations (10,25,50,100 μg/ml) of the antibiotic tested, both replication and transcription were completely inhibited (fig.2). This is in contrast to the results with phage T7 where transcription was only partially inhibited even at high concentrations of the antibiotic [10].

To demonstrate that the inhibition of DNA and RNA synthesis by novobiocin results from inhibition of host DNA gyrase, we determined the effect of this antibiotic on phage DNA and RNA synthesis in novobiocin resistant cells (fig.3). In these cells synthesis proceeds normally, though at a reduced rate in presence of the drug.

These results strongly suggest the requirement for DNA gyrase in phage I3 replication as well as transcription. This requirement is not transient and is seen throughout during the synthesis. Apparently, there is no preferential action on the expression of certain genes.

3.4. *Is DNA gyrase needed only for initiation of replication?*

Selective action of novobiocin on initiation of DNA synthesis has been observed in *Bacillus subtilis* [11]. On the other hand complete inhibition of replication is reported in *Escherichia coli* when gyrase specific antibiotics were used [12]. To ascertain whether gyrase is needed only during initiation or during the entire span of chain growth, we did the following experiment. DNA synthesis was measured at 150 min (the time at which maximal synthesis occurred) in phage-infected cells which were exposed to novobiocin for different periods. If gyrase is acting only at the initiation of DNA replication there should be no inhibition when the drug is added at a stage later than at the initiation process. Alternatively, if gyrase is needed throughout during replication, there would be total inhibition at any time. The results are presented in fig.4A. There is almost complete inhibition irrespective of the time of drug addition establishing the involvement of the enzyme during the entire span of replication. Inhibition at >70% was observed even when the drug and label were added together. The rapid inhibition pattern is clearly suggestive of an effect in the elongation phase. Gyrase involvement during the entire course of transcription is also evident from this experiment (fig.4B).

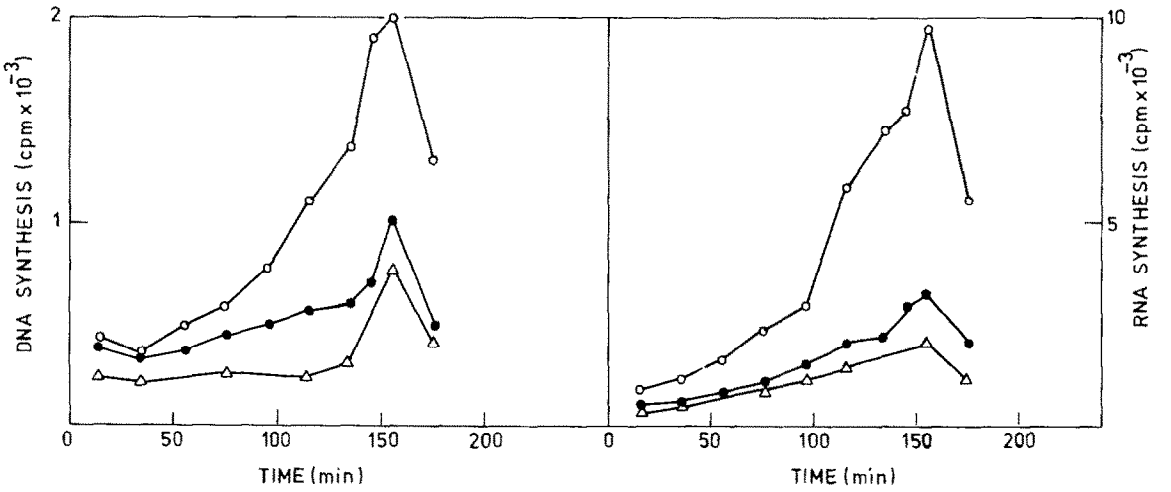

Fig.3 Kinetics of DNA and RNA synthesis in novobiocin resistant *M. smegmatis*. (○) control (no drug); (●) 25 μg novobiocin/ml; (△) 50 μg novobiocin/ml.

The effect of oxolonic acid and novobiocin on DNA and RNA polymerase activities in phage infected cells was also investigated. There was no inhibition of [^{3}H]TMP or [^{3}H]UMP incorporation in the presence of different concentrations (10–500 μg/ml) of novobiocin under the standard assay conditions (table 1). Oxolonic acid also did not inhibit RNA polymerase activity. It is therefore evident that the inhibition of in vivo DNA- and RNA-synthesis by these antibiotics is not due to direct inhibition of DNA- and RNA-polymerases.

4. Discussion

Our results with mycobacteriophage I3 implicate a role for DNA gyrase in phage replication and transcription. During the initiation of replication, main-

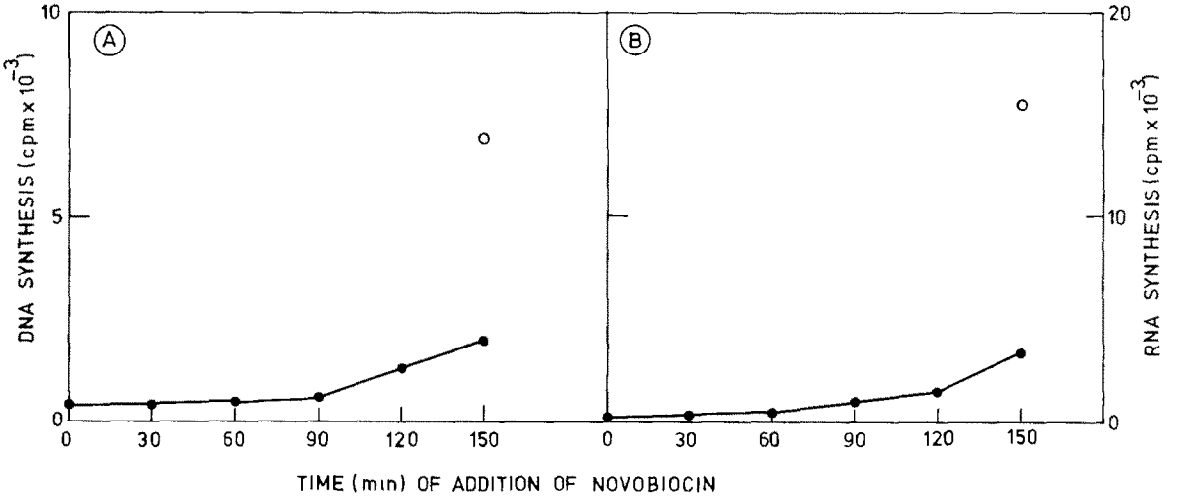

Fig.4. Requirement for DNA gyrase throughout DNA and RNA synthesis. At various times after phage infection, aliquots were exposed to 100 μg novobiocin/ml. After 140 min, [^{3}H]cytosine (2 μCi/ml) was added to all the samples. After 20 min, the labelling was terminated and radioactivity was determins: (●) time of novobiocin addition; (○) level of synthesis in the absence of the drug, at 150 min.

Table 1
RNA polymerase and DNA polymerase reactions in vitro

	pmol [^{3}H]UMP incorp. 5 min^{-1} . mg protein^{-1}		pmol [^{3}H]TMP incorp. 15 min^{-1} . mg protein^{-1}
	Phage I3 DNA template	Calf thymus DNA template	Calf thymus DNA template
Control	3484	3341	3681
Novobiocin			
(10 μg/ml)	3564	n.d.	n.d.
(50 μg/ml)	3429	3436	n.d.
(100 μg/ml)	3223	3568	3548
(500 μg/ml)	3520	3177	3986

The enzyme used was the S-100 preparation from phage I3-infected *M. smegmatis* cells. The RNA polymerase assay system contained in 125 μl, 20 mM Tris–HCl (pH 7.9), 1 mM Mg-acetate, 0.1 mM EDTA, 0.1 mM dithiothreitol, 100 mM KCl, 20 μg bovine serum albumin/ml, 1% glycerol, 0.4 mM CTP, GTP and ATP, 0.05 mM [^{3}H]UTP (200 μCi/μmol), 5 μg phage I3 or calf thymus DNA and the enzyme. After incubation at 37°C for 5 min, the samples were processed for acid precipitable radioactivity

The reaction mixture for DNA polymerase contained in 125 μl final vol., 50 mM Tris–HCl (pH 9.0), 6 mM $MgCl_2$, 0.5 mM β-mercaptoethanol, 40 μM each of dGTP, dATP, dCTP, and 10 μM d[^{3}H]TTP (100 μCi/μmol), 50 μg bovine serum albumin, 10 μg activated calf thymus DNA and the enzyme. After 15 min at 37°C, samples were processed for acid-precipitable radioactivity

In all cases, the drug was added prior to the addition of substates. The appropriate quantity of DMSO was added to the controls; n.d., not determined

tenance of DNA in an underwound state (i.e., negatively supercoiled state) would aid in the necessary unwinding at the growing replication fork.

Our most significant observation, however, is the requirement for gyrase throughout during transcription unlike the other systems examined so far, where transcription of different operons are influenced to different extents by gyrase [10,13,14]. In phage T7 the expression of early genes at the extreme left of the genome is insensitive to gyrase inhibitors; expression in the middle of genome exhibit intermediate levels of inhibition whereas late gene expression is completely inhibited [10]. Further, expression of a particular gene is varied when it is transcribed from different promoters, in the presence of the gyrase inhibitors [13,14]. Our results imply that gyrase is required for gene expression from the entire genome. The double-stranded DNA of phage I3 has extensive single strandedness on either side of the genome (S. Karnik, K. P. G., unpublished). Thus one would expect the DNA to circularize immediately after infection, resulting in supertwisted form. Consistent with this argument is the observation that inhibitors of DNA gyrase block the supercoiling of the infecting phage λ DNA [2,3]. Further, DNA isolated from phage I3 forms highly concatenated structures in vitro. Formation and resolution of DNA catenates by DNA gyrase has been established [15].

Novobiocin and oxolonic acid had no effect on in vitro [^{3}H]TMP and [^{3}H]UMP incorporation into DNA and RNA, respectively. This may be due to the fact that DNA isolated from the phage is linear in vitro and hence gyrase may not have any specific effect. In vivo certain topological constraint is imposed on DNA, making it mandatory for the gyrase to act on it.

Acknowledgements

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