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A Possible Role for RNA Polymerase in the Initiation of M13 DNA Synthesis

(DNA replication/chloramphenicol/replicative form/rifampicin/single-stranded DNA)

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Contributed by Arthur Kornberg, September 16, 1971

ABSTRACT The conversion of single-stranded DNA of bacteriophage M13 to the double-stranded replicative form in *Escherichia coli* is blocked by rifampicin, an antibiotic that specifically inhibits the host-cell RNA polymerase. Chloramphenicol, an inhibitor of protein synthesis, does not block this conversion. The next stage in phage DNA replication, multiplication of the double-stranded forms, is also inhibited by rifampicin; chloramphenicol, although inhibitory, has a much smaller effect. An *E. coli* mutant whose RNA polymerase is resistant to rifampicin action does not show inhibition of M13 DNA replication by rifampicin. These findings indicate that a specific rifampicin–RNA polymerase interaction is responsible for blocking new DNA synthesis. It now seems plausible that RNA polymerase has some direct role in the initiation of DNA replication, perhaps by forming a primer RNA that serves for covalent attachment of the deoxyribonucleotide that starts the new DNA chain.

DNA polymerases from *Escherichia coli* and phage-infected cells extend DNA chains, but as yet they have not been shown to initiate a chain (1, 2). With single-stranded circles of M13 or ϕX174 phage DNA as templates for *E. coli* DNA polymerase, it appeared that chain initiation did take place (3). Subsequently however, the presence and participation of small, linear DNA fragments as primers to initiate synthesis was recognized (1, 4).

How then is new DNA synthesis initiated? One possibility is that all DNA synthesis takes place by covalent extension of preexisting DNA chains. Thus, oligonucleotide fragments of a DNA chain or ends produced by endonucleolytic scissions of a chain might serve as primers. Experience with available enzymes favors this scheme. An alternative to this possibility is that a new enzyme initiates chains by itself, or in conjunction with one of the known DNA polymerases. Studies with intact cells favor this suggestion, but no enzyme has yet been found to do this job.

Another alternative occurred to us. Since RNA polymerase starts new RNA chains, and DNA polymerase is known to covalently extend a ribonucleotide terminus during DNA synthesis (5), a brief transcriptional operation by RNA polymerase might provide an RNA primer for DNA synthesis. This priming piece of RNA could later be recognized and excised by nuclease action. Thus, a *de novo* initiation event catalyzed by RNA polymerase would be an essential step for the start of DNA synthesis. The synthesis of the double-stranded replicative forms of M13 DNA provided an excellent system in which to test this possibility. The first step in M13 DNA synthesis involves the conversion of the phage single-stranded DNA (SS) to a double-stranded replicative form (RF) (6). This step relies entirely on host-cell enzymes; it does not require the expression of any known viral gene or any new protein synthesis. The next stage, in which the replicative forms multiply (RF→RF), requires, among other things, the product of a viral gene (6).

In this report we show that both of these DNA replication events (SS→RF, RF→RF) are strongly and immediately inhibited by the antibiotic rifampicin, a specific inhibitor of the RNA polymerase initiation step. This rifampicin action is not due to inhibition of protein synthesis because chloramphenicol, a direct inhibitor of protein synthesis, does not prevent the SS→RF conversion and has only a small effect on RF multiplication. Rifampicin does not affect M13 DNA synthesis in an *E. coli* mutant with an RNA polymerase resistant to rifampicin.

Abbreviations: SS, (phage) single-stranded DNA; RF, (phage) double-stranded replicative form.

MATERIALS AND METHODS

Materials were as follows: rifampicin and chloramphenicol from Calbiochem; unlabeled ribonucleotides from P-L Biochemicals; [^{3}H]CTP (3.2 Ci/mmol) and [^{3}H]uridine (27Ci/mmol) from Schwarz BioResearch Inc.; [^{3}H]thymidine (6.7 Ci/mmol) and [^{3}H]leucine (55 Ci/mmol) from New England Nuclear Corp.

Growth of cells and phage, and [^{3}H]thymidine-labeling of *E. coli* 5274 (F^+, T^-) and the wild-type M13 strains have been described (7, 8).

Rifampicin-resistant mutants of *E. coli* 5274 were isolated by plating 10^8 cells on agar that contained tryptone, as well as 50 μg/ml of rifampicin. The resistance to rifampicin of each of five independently isolated mutants was demonstrated to be due to an altered RNA polymerase by an *in vitro* assay of the polymerase activity in extracts of the bacteria. Both the resistant and sensitive strains were grown to late-log phase in 10 ml of Hershey broth, collected by centrifugation at 5000 $\times$ g for 5 min, resuspended in 1 ml of buffer A (see below), disrupted with a 100-W M.S.E. sonicator, and centrifuged at 10,000 $\times$ g for 10 min. 0.2 Volume of 1% protamine sulfate was added to the supernatant. The resulting precipitate was collected by centrifugation at 10,000 $\times$ g for 10 min, and suspended in 1 ml of buffer A. This fraction (50 μl) was used in an RNA polymerase assay (9) with [^{3}H]CTP (14,000 cpm/nmol) as the labeled nucleotide and calf-thymus DNA (125 μg/ml) as the template. Potassium phosphate (20 mM,

pH 7.4) was present to inhibit polynucleotide phosphorylase. The enzyme fractions were incubated with rifampicin (5 μg/ml) at 25°C for 5 min before the assay. The rifampicin-sensitive strain incorporated 320 cpm in 10 min without rifampicin, but only 5 cpm with rifampicin present (corrected for incorporation in the absence of the three unlabeled triphosphates). The rifampicin-resistant strain incorporated 184 cpm without, and 121 cpm with, rifampicin.

In most experiments, the cells were grown on a glucose–casamino acids medium supplemented with 2 μg/ml of thymine (7). When protein synthesis was measured by the incorporation of [^{3}H]leucine, cells were grown on this medium with the casamino acids replaced by 19 amino acids (excluding leucine) at 5 μg/ml.

[^{3}H]Thymidine-labeled M13 phage was prepared as described (8). The titer was 1.9×10^{12} phage/ml, with a specific activity of 5.8×10^6 cpm/ml.

Intracellular M13 DNA was extracted by a modification of the procedure of Hirt (10). Cells treated with lysozyme were lysed by the addition of sodium dodecyl sulfate to 0.5% and incubated at 37°C for 10 min. A concentrated solution of NaCl was added to a concentration of 1 M, and mixed with the lysate by slow inversion and rolling of the tube. The lysate was chilled to 0°C for 2 hr, centrifuged at $20,000 \times g$ for 15 min, and the supernatant, containing most of the M13 DNA, was removed. In some cases the DNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 6.5) and 2 volumes of cold ethanol and storing the mixture at −20°C overnight; the precipitate was collected by centrifugation at $10,000 \times g$ for 10 min and dissolved in 0.01 M Tris·HCl (pH 8.0)–1 mM EDTA.

Buffer A contains 0.05 M Tris·HCl (pH 8.0)–0.01 M $MgCl_2$–0.01 M 2-mercaptoethanol–0.05 mM EDTA. NET buffer (0.1 M) contains 0.1 M NaCl–1 mM EDTA–0.01 M Tris·HCl (pH 8.0). NET (1 M) is the same as NET (0.1 M), except that the NaCl concentration is 1 M.

Sucrose gradients were fractioned by collection of drops from the bottom of the tube directly into scintillation vials. Scintillation fluid(Triton X-100–toluene base; 10 volumes) was added and the samples were counted in a Nuclear Chicago scintillation counter.

RESULTS

Conversion of SS to RF is inhibited by rifampicin but not by chloramphenicol

Upon infection of *E. coli*, the M13 phage DNA (SS) was promptly converted to the double-stranded replicative forms (Fig. 1*a*). Fully covalent forms (RF I) predominated over nicked ones (RF II). Some of the parental DNA, cosedimenting with free phage*, still remained. With *E. coli* treated with 200 μg/ml of rifampicin for 5 min before infection, there was very little conversion of parental SS to RF; most of the DNA sedimented in the position of intact phage (Fig. 1*b*). Adsorption of the phage to cells was not affected by rifampicin; about two parental phage equivalents per cell were recovered with or

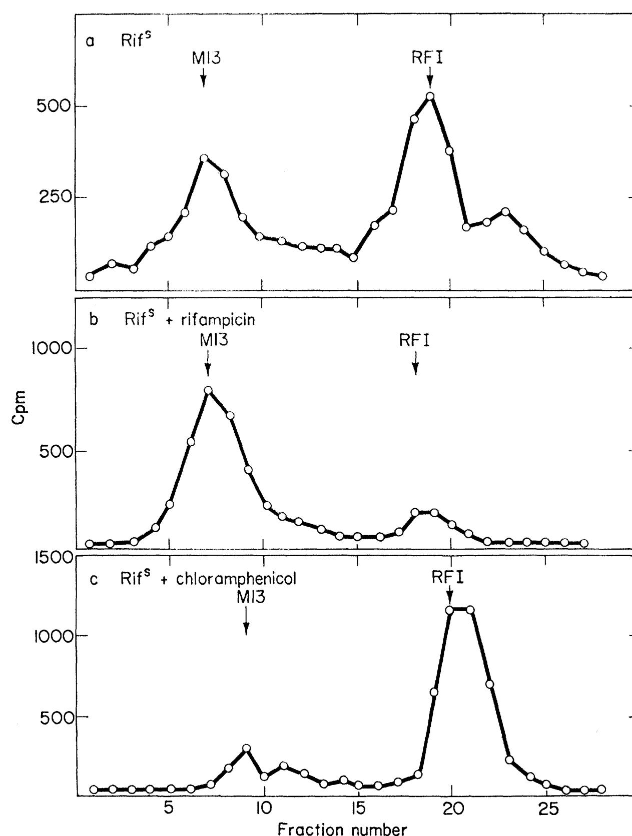

FIG. 1. Rifampicin inhibits the conversion of parental single-stranded DNA to the replicative forms. *E. coli* 5274 grown to 3×10^8/ml in 4 ml of medium at 37°C was either (*a*) untreated, (*b*) treated for 5 min with 200 μg/ml of rifampicin before infection, or (*c*) treated for 5 min with 100 μg/ml of chloramphenicol before infection. Cultures were then infected with M13-containing [^{3}H]DNA at a multiplicity of 100; the infection proceeded for 10 min at 37°C. Cultures were chilled on ice and 0.4 ml of 0.1 M KCN was added. Cells were collected by centrifugation at $5000 \times g$ for 10 min and resuspended with vigorous stirring in 1 ml of NET buffer (0.1 M) containing 10 mM KCN. The cells were washed one more time and resuspended in 1 ml of the same buffer. Lysozyme (0.1 ml of 4 mg/ml) was added and the cells were incubated at 37°C for 10 min. Sarkosyl (50 μl of 10%) was added to lyse the cells and the lysate was chilled and layered directly on a 5–20% sucrose gradient in NET buffer (1 M). After centrifugation for 15 hr at 23,000 rpm at 5°C in the SW27 rotor of the Spinco L2-65B ultracentrifuge, 0.8-ml fractions were collected and counted. Aliquots were titered for infectivity.

without rifampicin present. Uridine incorporation (presumably RNA synthesis) measured during the 10-min period of infection was reduced to 4–9% of control values by rifampicin treatment. Exposure of *E. coli* to 100 μg/ml of chloramphenicol did not inhibit the conversion of SS to RF (Fig. 1*c*, see also ref. 6).

To insure that the rifampicin inhibition is not a nonspecific effect due to the high concentrations of antibiotic used (and required for inhibition of intracellular RNA initiation, see ref. 11), a rifampicin-resistant mutant was obtained for study. This spontaneous mutant of *E. coli* 5274 possessed a rifampicin-resistant RNA polymerase as determined by assays of the enzyme in extracts (see *Methods*). With this mutant strain, conversion of SS to RF was not affected by

* This peak still possessed about 5% of the original specific infectivity of the infecting phage, and it also cosedimented with a phage-infectivity marker added to the lysate in other experiments. Uncoating of the viral DNA may be linked to the conversion of SS to RF (A. B. Forsheit and D. S. Ray, personal communication).

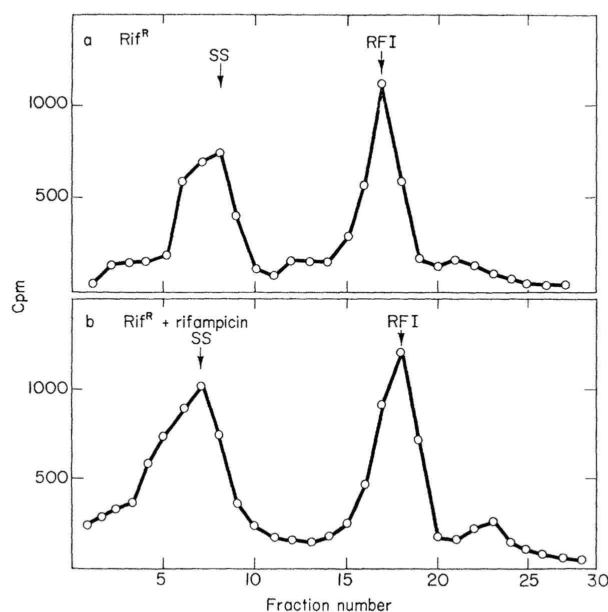

FIG. 2. Rifampicin does not inhibit conversion of parental DNA to RF in a rifampicin-resistant cell. This experiment was performed as described in Fig. 1, except that the *E. coli* 5274, rifampicin-resistant, mutant was used and the cells were lysed with 0.5% sodium dodecyl sulfate instead of Sarkosyl. (*a*) no treatment; (*b*) treated with 200 μg/ml of rifampicin for 5 min before infection.

rifampicin (Fig. 2). Thus, the primary effect of rifampicin in its inhibition of SS→RF appears to be on RNA polymerase.

Multiplication to RF is inhibited by rifampicin

Replication of RF requires at least one viral gene product, in addition to host-cell factors. The multiplication of double-stranded circular DNA molecules would appear to resemble chromosomal replication more than does the SS→RF conversion. In order to determine whether rifampicin would inhibit this step as well, *E. coli* 5274 was infected with phage M13 for 5 min, a time at which RF replication is well under way. Rifampicin was added to the culture at this point and the RF replication was observed by pulse-labeling of aliquots with [^{3}H]thymidine and measurement of its incorporation into RF by sucrose-gradient analysis. The rate of RF replication increased exponentially beyond 5 min after infection in the untreated control culture (12), and then leveled off at a constant rate about 5 min later (Fig. 3). Addition of 100 μg/ml of chloramphenicol at 5 min after infection did not inhibit the rate of RF replication for several minutes thereafter, even though the rate of protein synthesis, as judged by [^{3}H]leucine incorporation, had fallen to less than 2% after the first 3 min of treatment (Fig. 4). Thereafter, the rate of RF synthesis did fall, as expected from the requirement for continued protein synthesis during infection. Addition of rifampicin, however, produced an immediate inhibition of the rate of RF synthesis (Fig. 3), which fell exponentially for 20 min. In a separate experiment that measured the rate of [^{3}H]uridine incorporation under these conditions, the value fell to less than 4% within the first 3 min of treatment (Fig. 4). By 15 min after exposure to rifampicin, the rate of RF synthesis was nearly 100-times lower than that of chloramphenicol-treated cells. With the rifampicin-resistant strain, the rate of RF synthesis continued normally in the presence of rifampicin (Fig. 3).

DISCUSSION

Our findings demonstrate that rifampicin inhibits the conversion of parental single-stranded phage DNA to the double-stranded RF, as well as inhibiting the further multiplication of RF. Because mutants with an altered RNA polymerase resistant to rifampicin also resist these inhibitory effects, we conclude that this inhibition of DNA synthesis is caused by a specific interaction with RNA polymerase. What has not been excluded is the possibility that the rifampicin–RNA polymerase complex inhibits DNA replication by an attachment to DNA that prevents DNA chain initiation at that point, or extension beyond that point. Yet we know that rifampicin does not inhibit ongoing replication of the host DNA, which must also be undergoing transcription.

Should the rifampicin–RNA polymerase interaction prove to be an interruption of an essential function of the enzyme in DNA synthesis, then a transcriptional role would seem most likely. SS→RF conversion is not dependent on protein synthesis, and RF multiplication is nearly 100-times more

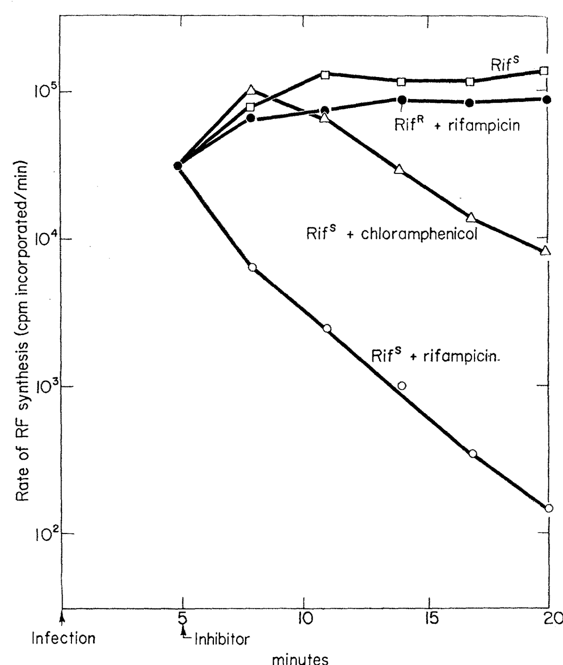

FIG. 3. Rifampicin inhibits the rate of RF synthesis. *E. coli* 5274 (or the rifampicin-resistant mutant) were grown at 37°C to 3×10^8/ml and infected with M13 at a multiplicity of 100. 100 μg/ml of chloramphenicol or 200 μg/ml of rifampicin were added 5 min after infection. At 3-min intervals, 9.5-ml samples were removed and exposed to 20 μCi of [^{3}H]thymidine for 1 min. Incorporation was stopped by pouring samples into 2 volumes of NET buffer (0.1 M)–10 mM KCN at 0°C. The cells were collected, washed, resuspended in 0.7 ml of 0.05 M Tris·HCl (pH 8.0)–1 mM EDTA, and lysed with 100 μg of lysozyme for 1–2 hr at 0°C. The M13 DNA was extracted by the modified Hirt procedure, precipitated by ethanol, and dissolved in 0.01 M Tris·HCl (pH 8.0)–1 mM EDTA. Samples were layered on 5–20% sucrose gradients in NET buffer (1 M) and centrifuged for 3 hr at 5°C in the SW56 rotor; 0.1-ml fractions were collected and counted. Data points are expressed as total counts in replicative forms I and II in each gradient.

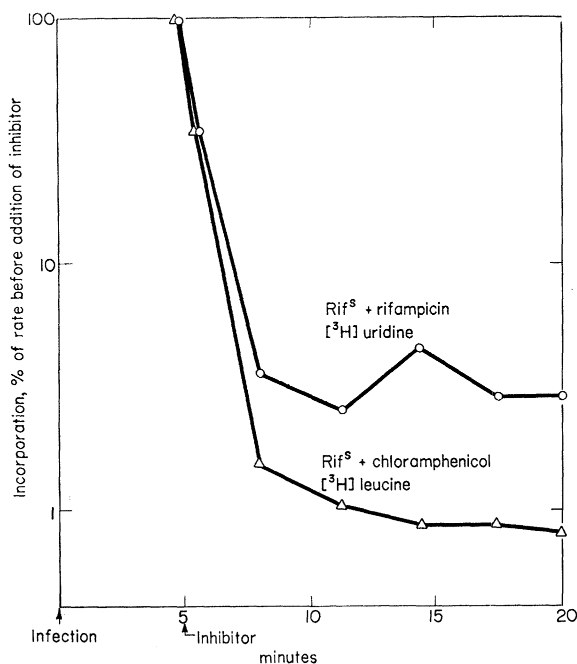

FIG. 4. The effect of chloramphenicol on protein synthesis and rifampicin on RNA synthesis in M13-infected *E. coli*. *E. coli* 5274 was grown to 3×10^{8}/ml at 37°C. At zero time, M13 was added at a multiplicity of 100. Just before 100 μg/ml of chloramphenicol or 200 μg/ml of rifampicin was added at 5 min after infection, 0.5-ml samples were labeled with 10 μCi of either [^{3}H]uridine or [^{3}H]leucine for 1 min and the labeling was stopped by the addition of cold 10% Cl_3CCOOH. Acid-insoluble material was determined by filtration on a Millipore HAWP filter and was counted in a scintillation counter. At 3-min intervals, samples were again taken and similarly pulse-labeled. The rates of incorporation are expressed relative to the initial rate without inhibitor added.

inhibited by rifampicin than by chloramphenicol. Thus, the RNA chains that are made by RNA polymerase would appear to serve some function other than as messengers for protein synthesis. Although these *in vivo* experiments cannot discriminate between transcription of the M13 DNA or *E. coli* DNA, preliminary experiments with cell-free extracts lacking almost all of the *E. coli* chromosomal material show a conversion of SS to RF that is inhibited by 5 μg/ml of rifampicin. Furthermore, this inhibition can be overcome by the addition of RNA polymerase purified from a rifampicin-resistant strain. These results suggest that RNA synthesis with the M13 viral strand as template is required for replication.

If such RNA, copied from the viral strand, is required for DNA synthesis, then this RNA could serve one of several functions. For example, RNA synthesis at a certain region on the parental DNA might disrupt some short, self-complementary regions that are known to exist in M13 DNA (13). Such disruption of helical structure might be necessary for proper initiation (14). The RNA synthesized might play a role itself, as in some macromolecular complex required for replication of M13 DNA (as has been proposed for the synthesis of *E. coli* DNA, K. G. Lark, personal communication). However, an attractive hypothesis for the function of such an RNA chain is that which originally prompted this investigation: to act as a primer terminus for covalent extension by a DNA polymerase.

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