RIFAMPICIN RESISTANT DNA SYNTHESIS IN PHAGE T4 INFECTED ESCHERICHIA COLI

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SUMMARY

We have found that net DNA synthesis in T4 infected cells is rifampicin resistant. This finding implies that both the initiation of each T4 genome and its elongation are rifampicin resistant processes.

INTRODUCTION

The initiation of DNA replication for different types of DNA genomes is thought to be primed by RNA. In addition, there is some evidence that DNA Okazaki pieces found during ongoing DNA synthesis in E. coli are also RNA primed (1). Initiation of genome replication is thought to be primed by RNA in phages M13 (2,3), ϕx 174 (3,4), T7 (5), T4 (5,6), λ (7), in E. coli (8) and in certain episomes (9,10). For some of these systems RNA polymerase has been implicated as responsible for synthesis of RNA primer because the initiation of genome replication is inhibited by the RNA polymerase inhibitor rifampicin (2,3,7,8,9,10). For some of these systems sensitive to rifampicin, RNA covalently attached to DNA has been shown (2,3,10). RNA covalently attached to DNA can also be isolated from E. coli infected with phages T4 or T7 at the time when replication of the parental genome is initiated (5,6). It has not been reported, however, if synthesis of this RNA is sensitive to rifampicin.

An exception to the idea that RNA polymerase is solely responsible for genome initiation is the finding that RNA priming of ϕx 174 replication is rifampicin resistant and occurs by a different enzyme system (3,4). Also in contrast to the prevalent occurrence of rifampicin sensitive genome initiation is the finding that the ongoing synthesis of E. coli DNA (chain elonga-

tion) is rifampicin resistant (8) even though DNA Okazaki pieces have RNA covalently attached to their 5' ends (1).

Thus, to date, it appears that the initiation of genome replication is primed by RNA whose synthesis can be either rifampicin sensitive or resistant, while the elongation of an initiated genome (at least in E. coli) is rifampicin resistant.

In the studies reported here we investigated whether phage T4 DNA synthesis was sensitive or resistant to rifampicin. We have found that T4 genome replication and the elongation of each genome is rifampicin resistant.

MATERIALS AND METHODS

Phage $T4D^+$, an E. coli K12 rifampicin sensitive host described previously (W3110, Arg^-, Trp^-; ref. 11) and Strain B of E. coli were used. [Me-^{3}H]-thymidine was obtained from Schwarz/Mann and was purified before use by descending chromatography on Whatman No. 1 paper, using isopropanol, 28% NH_4OH, H_2O (7:2:1) as solvent. Non-radioactive thymidine was added to the medium to give the specific activities indicated in each experiment. ^{3}H-T4 DNA was prepared by incorporation of ^{3}H-thymidine and purified according to the methods of Thomas and Abelson (12). Rifampicin (Calbiochem) was prepared each day by mortar-and-pestle grinding in a small amount of water or medium. Undissolved material was pelleted by low speed centrifugation. Rifampicin concentrations in the supernatant were determined by extinction coefficient at 475 nm (a 1 mM solution at 475 nm gives an optical density of 15.4; ref. 13). DNase I (RNase free) was obtained from Worthington, RNase I from Calbiochem, phenol from Fischer Scientific, and sodium dodecyl sulfate from Matheson, Coleman and Bell. All reagents were of reagent grade.

Phage infection

The phage techniques used here have been described previously (14). T4 phage were added at a multiplicity of 5-10 to 2 x 10^8 cells/ml growing exponentially with aeration at 37°C in M9 medium (15) containing 0.25% casamino

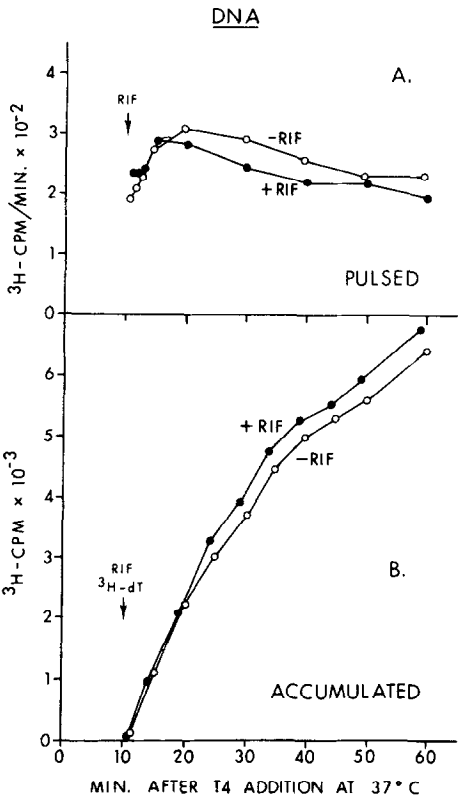

Figure 1. The effect of rifampicin on accumulation and pulses of ^{3}H-thymidine. A culture infected with T4 @ 37°C (time zero) was split into two parts (A and B). A: Rifampicin (final concentration = 400 µg/ml) or an equal volume of medium was added 10 minutes after infection and one minute incorporations of ^{3}H-thymidine determined as described in Methods. B: ^{3}H-thymidine to give 3.47 µC/ml (421 mC/mmole) and rifampicin (final concentration = 400 µg/ml) or an equal volume of medium were added 10 minutes after infection. Samples of 20 µL were taken from the cultures and added to 3 ml 5% TCA at the indicated times. Acid-insoluble radioactivity was determined as described in Methods. Data obtained in A were corrected for specific activity and sample size to make them equivalent to specific activity and sample size of B. The initial point (minus rifampicin) in A without correction was 2960 counts/min. Symbols: O, -rifampicin; ●, +rifampicin.

acids (M9S). In all experiments reported here, at least 95% of the cells were infected two minutes after phage addition.

Pulse labeling

One minute periods of incorporation were accomplished by mixing, at indicated times, 0.2 ml aliquots of culture with 0.2 ml medium of identical composition containing, in addition [Me-^{3}H]-thymidine (5 µC/ml, 606 mC/mmole). Incorporation was stopped after one minute by the addition of 5 ml of 5% TCA.

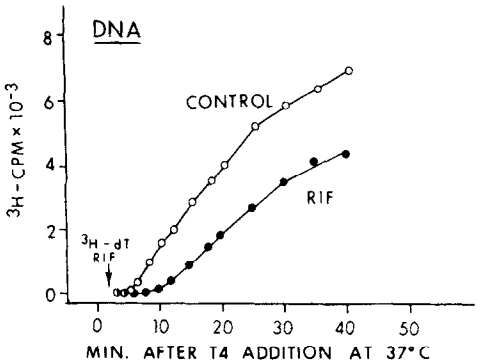

Figure 2. Thymidine incorporation into DNA when rifampicin is added two minutes post infection. An infected culture was divided and to each at two minutes ^{3}H-thymidine was added to give 0.7 μC/ml (53 mC/mmole). To one, rifampicin was also added to a final concentration of 400 μg/ml. Samples of 20 μL were removed at the indicated times to determine acid-insoluble radioactivity. Symbols: O, -rifampicin; ●, +rifampicin.

Precipitates were collected on Whatman GF/C filters, washed, and counted as described previously (11).

Assays for nuclease lability of in vivo labeled DNA

DNase. DNase was dissolved to give a final concentration of 50 μg/ml in 50 mM Tris-Cl (pH 7.3), 20 mM $MgCl_2$. A volume of the sample to be tested was mixed with 20 volumes of the DNase solution and incubated at 37°C for one hour. Thirty volumes of 5% TCA were added. After precipitation at 0°C for 15 minutes the precipitate was filtered and radioactivity counted as described previously (11). This sample was compared to two other aliquots mixed similarly with buffer except that neither contained DNase; one was incubated at 37°C and the other was not. The radioactivities recovered in the latter two samples were the same and were used as the control (minus DNase) sample.

RNase. RNase in water at a concentration of 1 mg/ml was heated for five minutes at 100°C to destroy DNase activity. The RNase was then diluted to give a final concentration of 25 μg/ml in 50 mM Tris-Cl (pH 7.3), 0.1 mM EDTA. Samples to be tested were mixed and treated in the same way as described for DNase above.

Preparation of DNA

A sample of infected cells at 37°C (0.6 ml or less) was added to an equal

volume of presaturated phenol containing one percent sodium dodecyl sulfate in an 18 x 150 mm glass tube at 100°C. The mixture was agitated in a 100°C water bath for one minute from the time of mixing (such mixtures usually lost turbidity and became clear after 30 seconds). The mixture was quickly cooled to room temperature (20°C), gently mixed for 15 minutes, and then centrifuged at low speed to separate the phases. The aqueous phase was removed without disturbing a large amount of cell debris at the interface. The aqueous phase was evaporated to dryness and resuspended in 0.015 M sodium citrate containing 0.15 M NaCl and 1 mM EDTA (SSC-EDTA). Approximately 80% of the ^{3}H-material which was acid-precipitable from infected cells was recovered in the aqueous phase.

A second method was also used. An equal volume of cold 10% TCA was added directly to the infected culture, the mixture incubated at 0°C for 15 minutes and then centrifuged at 10,000 x g for 20 minutes. The supernatant was discarded and the precipitate dissolved in water. The two methods yielded the same results.

RESULTS

Ongoing T4 DNA synthesis is rifampicin resistant. T4 DNA synthesis was measured in two ways: 1) by a one minute incorporation of ^{3}H-thymidine and 2) by a long-term (60 minutes) incorporation of the same DNA precursor. The addition of rifampicin is seen to have little effect upon the pulse labeling of T4 DNA (Fig. 1A) or the accumulation of the thymidine precursor (Fig. 1B). The DNA synthesis observed is quantitatively the same whether it is measured by pulse (Fig. 1A) or by accumulation (Fig. 1B). Such ^{3}H-thymidine labeled material was shown to be DNA by its sensitivity to DNase I (> 80% sensitive) and by its resistance to RNase I (< 2% sensitive). The same results were obtained with purified ^{3}H-T4 DNA. Elsewhere (16) we have shown in similar experiments that the addition of rifampicin is effective within 10 minutes of drug addition as demonstrated by a $\geq$ 96% inhibition of one minute pulse incorporations of ^{3}H-uridine. This sensitivity of T4 RNA synthesis to rifampicin

has been reported previously (17). These results indicate that DNA synthesis is resistant to rifampicin when the drug is added ten minutes after infection.

The early addition of rifampicin only partially inhibits DNA synthesis. Rifampicin was added two minutes after T4 infection to determine the effect upon DNA precursor incorporation. As shown (Fig. 2) there is a delay in incorporation into DNA. The rate and extent of incorporation are also less in the presence of rifampicin than in its absence. It is clear, however, that there remains considerable DNA synthesis resistant to rifampicin. What is surprising is that the incorporation of precursor label is delayed perhaps indicating that synthesis of the accumulated product does not begin until ten minutes after infection. If rifampicin is added at the time of phage addition there is no incorporation into DNA (< 1% of the control curve at any time, Fig. 2). This finding suggests that rifampicin resistant DNA synthesis in T4 infected cells can occur when rifampicin is added two or more minutes after infection.

The addition of rifampicin inhibits phage production. The burst size of T4 was measured in EH medium 60 minutes after infection as previously described (14). The addition of rifampicin at ten, two, or zero minutes after phage addition gave burst sizes of 3%, $\leq$ 0.2%, and $\leq$ 0.2% respectively compared to an untreated culture (burst size = 160).

DISCUSSION

We have found that T4 DNA synthesis is completely resistant to rifampicin if the drug is added ten minutes after infection. This is shown by the observation that rifampicin resistant ^{3}H-thymidine incorporation appears in a product which is sensitive to DNase but insensitive to RNase. The effectiveness of rifampicin in this experiment is indicated by our observations that the rate of RNA synthesis is inhibited $\geq$ 96% and that phage yield is decreased 30 fold. An earlier addition of rifampicin two minutes after infection (Fig. 2) was relatively ineffective in inhibiting T4 DNA synthesis, the resistant incorporation beginning approximately ten minutes after infection. Thus, resistant DNA synthesis is observed (whether rifampicin is added two or ten minutes after

infection) at a time when net synthesis from parental DNA is occurring. We therefore conclude that ongoing T4 DNA synthesis is resistant to rifampicin. In addition, since the ongoing synthesis of T4 DNA entails the production of many T4 genomes, this finding suggests that both the initiation of each T4 genome and its elongation are rifampicin resistant processes. Our results do not comment on whether RNA primers are needed for either of these processes. However, our results do indicate that if RNA primers initiate individual genomes, concatemers (19) or T4 Okazaki pieces (18), their synthesis is rifampicin resistant. That is, the synthesis of such RNA primers is either not directed by RNA polymerase, or such primers are synthesized prior to the onset of net T4 DNA synthesis. We would like to add that we have obtained results like those reported here using strain B of E. coli as host.

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