FILAMENTOUS BACTERIAL VIRUSES

IX. PROTEIN SYNTHESIS INHIBITORS AND DNA REPLICATION

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Summary

DNA synthesis in filamentous virus-infected bacteria is stopped rapidly by withdrawal of required amino acids but not by addition of 180 μg/ml chloramphenicol. Initiation of DNA replication is not under control of the stringent response. DNA synthesis in chloramphenicol-treated bacteria is correlated with residual synthesis of a protein (probably the major viral coat protein) that is made in 180 μg/ml chloramphenicol but not in the absence of essential amino acids.

Introduction

Replication of DNA is thought to be controlled at the initiation step. In some systems (for instance the Escherichia coli chromosome) this step is sensitive to high concentrations of chloramphenicol or to amino acid starvation, and is therefore thought to require new protein synthesis; in other systems (for instance some bacterial plasmids) many rounds of DNA replication occur in the presence of chloramphenicol (for reviews see references 1, 2, 3, 4).

F-specific filamentous bacterial viruses (Ff) such as fd and M13 do not lyse or otherwise kill their host during normal productive infection: as virus is released from growing infected bacteria, viral DNA is continuously replicated If 180 μg/ml chloramphenicol is added to a culture that is maintaining such an established infection, viral DNA replication continues for a period of time, although the mode of replication changes from single-strand to double-strand synthesis (5, 6). However, if the temperature of the culture is raised from 32°C to 42°C at the time that chloramphenicol is added, DNA replication stops immediately (6). One explanation suggested for the temperature effect was that

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the synthesis of primer RNA required for initiation of viral DNA synthesis (7) may be under stringent control (8), since the stringent response is induced by an increase in temperature (9). We have investigated possible stringent control of the initiation of DNA replication.

Materials and Methods.

Most materials and methods have been fully described (10, 11 and 12). N16 medium is N11 without casamino acids (10). Bacterial strains DM 115 and DM 116 are CP78(F^+) *rel*$^+$ and CP79(F^+) *rel*$^-$, respectively, of Fiil and Friesen (13) and require Arg, His, Leu, Thr and Thi. The regulatory guanine nucleotides, "magic spots" (MS) I and II (ppGpp and pppGpp, respectively), were measured as follows. Cultures of bacteria were labeled with ^{32}P, and 0.1 ml samples removed into ice cold 2 N formic acid and stored at -20°C for no more than 9 days before analysis. Centrifugation to remove bacterial debris was not necessary to resolve MS II. Acidified samples were spotted in 10 µl aliquots on Macherey-Nagel cellulose 300 PEI sheets to give 50 µl in each spot, and analyzed by ascending chromatography in 1.5 M KH_2PO_4 adjusted to pH 3.0 with KOH. Internal markers of unlabeled nucleotides including ppppG were visualized by ultraviolet fluorescence. The chromatograms were autoradiographed and the labeled portions of the chromatograms removed and counted in toluene scintillant. There was less than 10% variation in quantity of MS I and II between duplicate chromatograms.

Identification of MS I and II was confirmed by two-dimensional chromatography (formate/phosphate) with unlabeled markers. Amino acid starvation of the isogenic strains DM 115 and DM 116 caused a reduction in RNA synthesis and an increase in MS I and II for the Rel^+ but not for the Rel^- strain, further confirming the identification of MS I and II.

Results and Discussion

In order to investigate possible involvement of the stringent response in initiation of viral DNA replication we have measured both total RNA synthesis and MS I and II levels since it was not known what levels might be significant for DNA replication in Ff-infected bacteria. RNA synthesis in Ff-infected

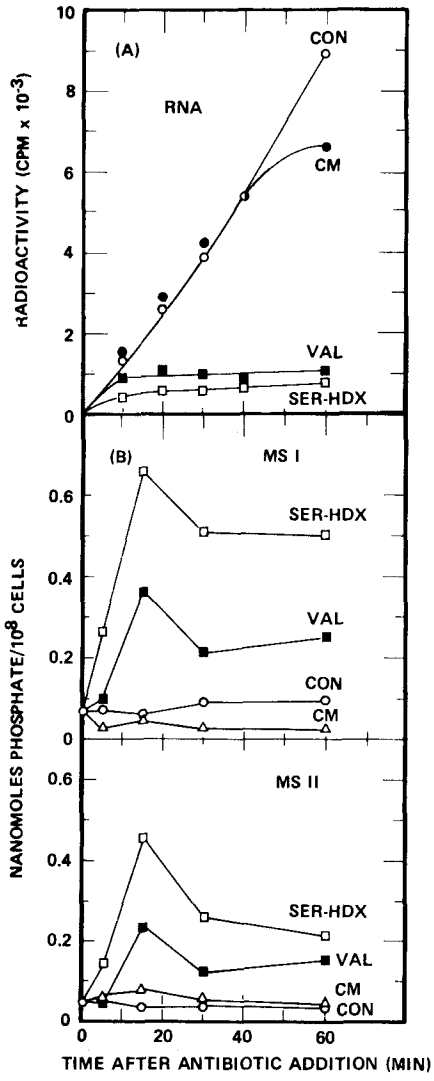

Figure 1. Induction of the stringent response in Ff-infected bacteria. (A) RNA synthesis. Exponentially-growing bacteria of strain DM 117 in N13 medium plus 50 µg/ml Ile were infected with fd (at a multiplicity of 500 PFU per bacterium) and incubated for a further 30 min. The cells were then centrifuged, resuspended in N13 medium plus 50 µg/ml uridine and 5 µCi/ml ^{3}H-uridine and the suspension divided into 4 parts. Each part immediately received one of the following: 50 µg/ml Ile (CON), 50 µg/ml Ile plus 180 µg/ml CM (CM); 50 µg/ml Ile plus 180 µg/ml serine hydroxamate (SER-HDX), or 300 µg/ml Val (VAL). Incubation of the suspensions was then continued and 0.5 ml samples removed as indicated and analysed for cold TCA-precipitable radioactivity. (B) MS I and MS II determination. Essentially the same procedure was followed for MS I and MS II synthesis, except that N16 medium plus 50 µg/ml Ile was used. ^{32}P-orthophosphate (100 µCi/ml) was added at the time of virus addition. Portions of the labeled culture were added to the inhibitors 30 min after infection, and 0.1 ml samples removed at the times indicated for MS I and MS II determination, as described in Materials and Methods.

Rel^+ bacteria continued after the addition of chloramphenicol, but ceased after valine-induced isoleucine starvation due to stimulation of the synthesis of MS I and II (Fig. 1; reference 8). The Ff DNA synthesis that takes place in the presence of chloramphenicol was also specifically stopped by isoleucine starvation (Fig. 2b; Fig. 2a shows that the inhibition of viral DNA synthesis

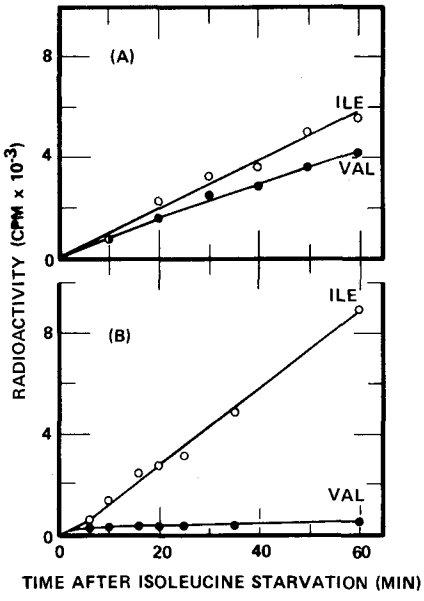

Figure 2. Effect of isoleucine starvation on viral and host DNA synthesis. (A) Effect on host DNA synthesis. Cells of strain DM 117 growing exponentially in N13 plus 50 µg/ml Ile at 37°C were centrifuged for 3 min at 12,000g and resuspended in warm N13 containing 250 µg/ml deoxyadenosine, 40 µg/ml thymidine and 5 µCi/ml ^{3}H-thymidine. The culture was then divided into 2 parts. To one portion was added Ile (50 µg/ml) and to the other Val (300 µg/ml). Samples were removed as indicated and the cold TCA-insoluble material determined. (B) Effect on viral DNA synthesis. The experiment was essentially the same as in (A) except mitomycin C-treated bacteria (which cannot replicate chromosomal DNA) that had been infected with fd and incubated for 20 min before imposing isoleucine starvation were used. The "cold" thymidine concentration was reduced to 5 µg/ml.

during amino acid starvation did not result from a decrease in the pool sizes of nucleoside triphosphates, since near normal synthesis of bacterial DNA occurred). This difference between chloramphenicol addition and amino acid withdrawal initially supported the idea that Ff DNA synthesis is under stringent control. However, two further experiments showed clearly that this is not the

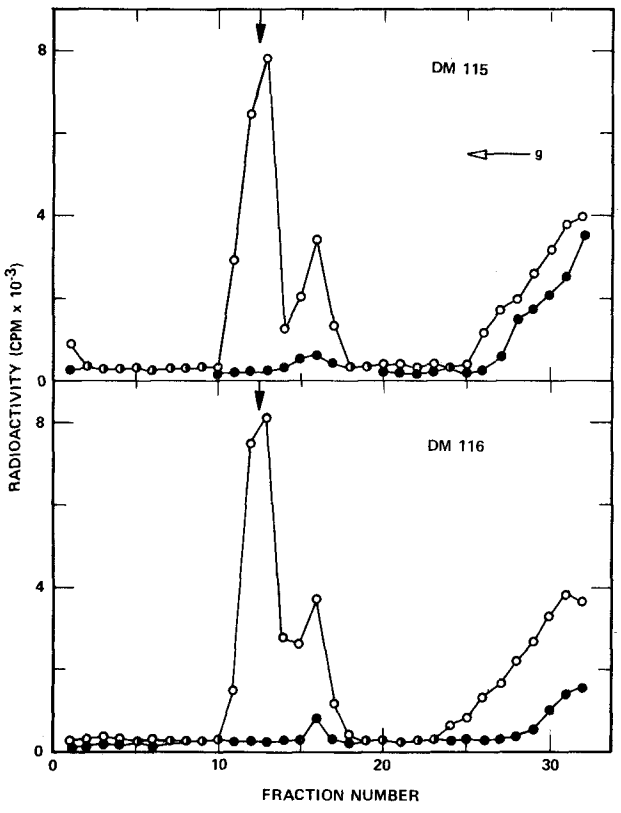

Figure 3. Effect of amino acid starvation on viral DNA synthesis in Ff-infected Rel^+ and Rel^- bacteria. DM 115 (rel$^+$) and DM 116 (rel$^-$) were grown at 37°C to exponential phase in N13 medium plus 50 µg/ml each of Arg, His, Leu, Thr, Ile. At a bacterial density of 1 x 10^8/ml the cultures were infected with fd. After incubation for 40 min the bacteria were centrifuged and resuspended in N13 medium plus 50 µg/ml each of Arg, His, Leu, Thr. Each culture was then divided into 2 portions and each portion treated with either Val or Ile as in Fig. 2. After incubation for 6 min, 2 ml portions of each culture were pulsed for 5 min with radioactive thymidine (10 µCi/ml, 2 µCi/µg, plus 250 µg/ml 2 -deoxyadenosine). After the pulse, bacteria were washed and lysed and the lysates analysed by centrifugation through high-salt neutral sucrose gradients (10). The vertical arrows indicate the position of marker ^{32}P-labeled fd-DNA in the sucrose gradients Open symbols: Ile; closed symbols: Val.

case. First, Ff DNA synthesis was stopped by amino acid withdrawal in both Rel^+ and Rel^- bacteria (Fig. 3), although RNA synthesis was stopped only in the Rel^+ strains. Second, serine hydroxamate (an antibiotic that stops protein synthesis by blocking seryl-t-RNA synthetase) behaves like amino acid withdrawal in inducing a stringent response in Rel^+ bacteria (Fig. 1), but it

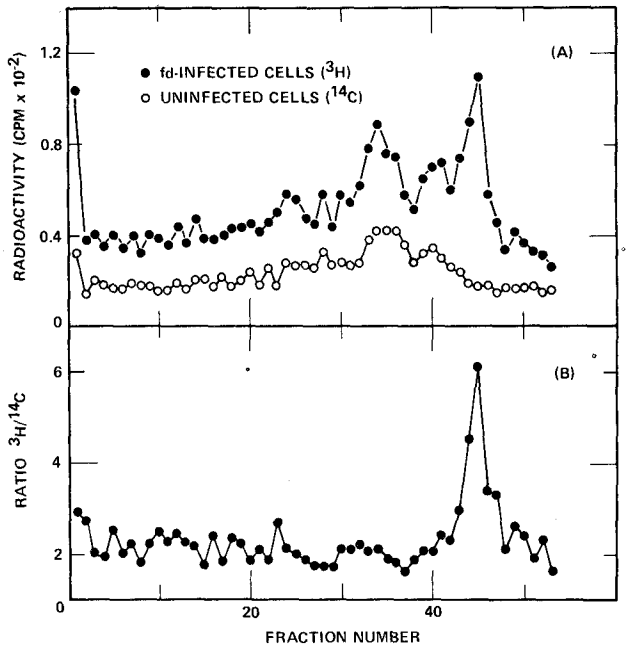

Figure 4. Gel electrophoresis of cell lysates of Ff-infected and uninfected bacteria labeled with amino acids in the presence of chloramphenicol. DM 117 was grown at 37°C to exponential phase in N13 medium plus 50 µg/ml Ile. At a cell density of 2 x 10^8/ml the culture was divided into two 5 ml portions and one infected with fd. After incubation for 40 min chloramphenicol (final concentration 180 µg/ml) was added to both portions and after a further 5 min ^{3}H-leucine (final activity 10 µCi/ml) was added to the infected culture and ^{14}C-leucine (final activity 1 µCi/ml) added to the uninfected culture. After incubation of both cultures for 30 min, incorporation of label was stopped by addition of 10 ml amounts of ice-cold NET (10) plus KCN (0.01M). The cultures then were mixed together, the cells were washed twice with ice cold NET/KCN, were resuspended in 0.4 ml ice- cold H_2O and were lysed by addition of 20 µliters of lysozyme (4mg/ml) and brief sonication. The proteins were then dissociated and analysed by SDS-polyacrylamide gel electrophoresis (A), as previously described (12). Virus-specific polypeptides are revealed by plotting the ratio of ^{3}H:^{14}C (B).

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did not stop Ff DNA synthesis (10). Furthermore, the formation of parental RF-DNA from infecting single strand DNA, a step known to require primer RNA synthesis (7), occurs in amino acid deprived bacteria (10). Therefore the primer RNA needed to initiate Ff DNA synthesis is not under stringent control, and the fact that Ff DNA is synthesized in the presence of chloramphenicol but not after amino acid starvation is not related to the stringent response.

Another possible explanation for the difference between these two methods

of blocking protein synthesis would be that proteins necessary for DNA synthesis are still made in the presence of 180 μg/ml chloramphenicol, but not after withdrawal of amino acids. Such leaky protein synthesis has been suggested (14) to explain the chloramphenicol-resistant protein (15) that is necessary for ϕX174 DNA synthesis. Indeed, in the presence of chloramphenicol, Ff-infected bacteria, but not uninfected bacteria, specifically incorporated radioactive amino acids into material that showed a specific peak on SDS-polyacrylamide gels (Fig. 4). This material has an amino acid composition similar to that of the major coat protein (gene 8 protein) of the virion and co-migrated in gel electrophoresis with authentic gene 8 protein (experiment not shown). The synthesis of minute amounts of gene 8 protein might be relatively insensitive to chloramphenicol because multiple copies of the corresponding mRNA and many ribosomes are probably needed to synthesize the 10^4 to 10^5 molecules/min/bacterium of this protein necessary to produce completed virions. The correlation between residual gene 8 protein synthesis and the continuation of viral DNA synthesis suggests a hitherto unsuspected role for gene 8 protein in DNA synthesis.

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