IDENTIFICATION OF A ϕX174 CODED PROTEIN INVOLVED IN THE SHUT-OFF OF HOST DNA REPLICATION

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SUMMARY: When Escherichia coli is infected with ϕX174, the host cell DNA replication ceases 10 - 12 minutes after infection. This shut-off is prevented by the addition of 30μg/ml chloramphenicol indicating a requirement for post-infection protein synthesis. Some amber mutants in ϕX174 cistron A, which codes for two proteins, do not shut off the host DNA replication. Amber mutants in all other ϕX174 genes shut off host replication. Mutants near the amino-terminal end of cistron A, which still produce the small 35,000 molecular weight cistron A polypeptide, shut off the host DNA synthesis whereas mutants near the carboxy-terminal end, which do not produce the small A polypeptide do not shut off the host.

INTRODUCTION: Infection of Escherichia coli by ϕX174 results in the shut-off of host cell DNA replication 10 - 15 minutes after infection (1, 2). Synthesis of host protein and RNA is not affected by ϕX174 infection (1, 3). The shut-off of host DNA replication is inhibited by 30μg/ml CAM[1] (1, 2) indicating a requirement for at least one phage induced protein. This communication reports the finding that some amber mutants in cistron A do not shut off host cell DNA synthesis whereas amber mutants in each of the other eight known cistrons (B-H and J) shut off the host. Cistron A of ϕX174 contains an internal initiator and codes for two polypeptides whose molecular weights are 60,000 and 35,000 daltons (4). Amber mutants near the amino-terminal end of the gene still synthesize a 35,000 molecular weight polypeptide and mutants near the carboxy-terminal end of the gene synthesize neither of these polypeptides. The function of the small A protein is not known, however, this report shows that the lack of the small A protein correlates with the lack of shut-off of host cell DNA synthesis indicating that this protein is involved in the shut-off.

[1] CAM = Chloramphenicol

MATERIALS AND METHODS: Bacteria and Bacteriophage: Escherichia coli C strains HF4701 (uvrA) (6) and HF4704 (thy⁻, tlr derivative of HF4701) (1) were obtained from Dr. P. Howard-Flanders. Phage φX174 and mutants am 86, am 50, am 8, am 8A, am 18 (all in cistron A), am 14 (cistron B), och 12 am 3 (double mutant; och in cistron C, am in cistron E) am 10 (cistron D), am 3 (cistron E), am 6 (cistron J), am S_5-10 (cistron F), am 9 (cistron G) and am 23 (cistron H) were obtained from Dr. Robert L. Sinsheimer. Am 8 is also temperature sensitive in cistron F (F. Funk, personal communication). Am 8A and am 8tr are temperature resistant revertants of am 8. φX174 mutants N14 and H90 (both in cistron A) were obtained from Dr. Masaki Hayashi.

Experimental Procedure: E. coli HF4704 was grown in TPG medium (1) supplemented with 2μg/ml thymidine and 1% casein hydrolysate, to a density of 1.3×10^8 cells/ml and infected at a multiplicity of 3. At the time of infection, and at 5 minute intervals thereafter, 1.5 ml of cells were pulsed for 2 minutes with [^{3}H] thymidine (10 μci/ml). The pulse was terminated by pouring the cells over 10 ml crushed ice containing 0.1 ml of 1M Na azide. The cells were pelleted by centrifugation at 5,000 rpm for 5 minutes in a Sorval SS34 rotor. The cells were resuspended in 1.0 ml of buffer containing 0.10 M Tris·HCl, pH 8.0, 0.01 M EDTA and 1 mg/ml of lysozyme (Sigma, eggwhite, grade 1), incubated at 0° C for 5 minutes, and lysed with 50 μl of 10% Sarkosyl. The DNA was extracted with phenol and precipitated with 2 1/2 volumes of ethanol at -20°C overnight. The precipitate was collected by centrifugation at 9,000 rpm for 10 minutes in a Sorval SS34 rotor and resuspended in 0.2 ml of 0.1 N NaOH. The absorbance at 260 mμ was measured and the samples were layered on a 5 ml 5 - 20% alkaline sucrose density gradient containing 0.01 M Tris·HCl, pH 8.0, 0.001 M EDTA, 0.15 M NaCl and 0.3 M NaOH. The gradients were centrifuged for 2 1/2 hours at 45,000 rpm in a Beckman SW50.1 rotor at 5°C. Fractions were collected from the bottom of the gradient and counted in a toulene based scintilation fluid.

Intracellular phage-coded proteins were labeled and analyzed on a 7 1/2% polyacrylamide gel slab as previously described (7) except that the [^{14}C] amino acid mixture was added at 5 and 20 minutes after infection. Luria broth was added at 30 minutes and the cells were harvested 4 minutes later.

RESULTS: E. coli HF4704 was infected with φX174 and samples were pulsed for 2 minutes with [^{3}H] thymidine at 0, 5, 10, 15 and 20 minutes after infection. The DNA was extracted and sedimented in alkaline sucrose. Figure I shows the gradients of the 5 (A), 10 (B), and 15 (C) minute pulses. This demonstrates the shut off of host cell DNA replication.

In a similar experiment, a culture was divided into three portions and the subcultures were treated as follows: 1) infected with am 3, 2) infected with am 3 in the presence of 30μg/ml CAM, 3) treated with 30 μg/ml CAM. The cells were pulsed and the DNA extracted and sedimented as before. The total counts in each of the host cell DNA peaks in these gradients were calculated and normalized by the absorbence at 260 mμ of the samples. These data were plotted as percent of the normalized counts incorporated into host DNA in the 0 - 2 minute pulse.

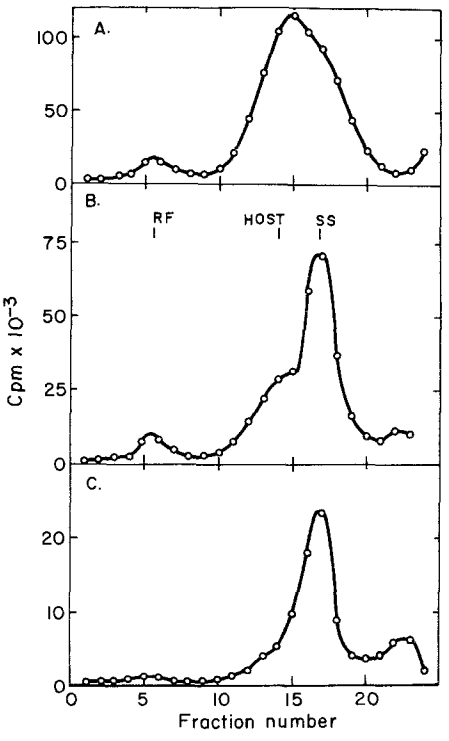

Figure 1: Alkaline sucrose density gradients of pulsed labeled, infected cells. E. coli HF4704 was infected with ϕX174 and portions were pulsed with [^{3}H] thymidine. The DNA was phenol extracted and sedimented in an alkaline sucrose density gradient as described. (A) 5 - 7 minute pulse ; (B) 10 - 12 minute pulse; (C) 15 - 17 minute pulse.

The results (figure 2) show that the shut-off of host DNA replication is prevented by CAM, indicating that post infection protein synthesis is required for this shut-off activity.

Figure 3 shows the results obtained when cells were infected with amber mutants in 7 of the 9 ϕX174 cistrons. The results of the experiment with wild type phage (figure 1) are included for comparison. The cistron E results are shown in figure 2. The shut-off activity is present in each of these mutants. Figure 4 shows the results obtained by infection with a series of mutants in cistron A. This demonstrates that some, but not all mutants in cistron A lack the shut-off activity. The results are not shown for am 8tr and N14 which did not shut off the host DNA replication and am 50 which did.

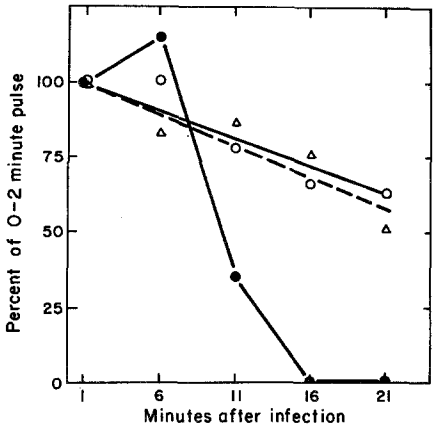

Figure 2: Sensitivity of shut off activity to chloramphenicol. A culture of E. coli HF4704 was divided into three parts. The first was infected with φX am 3 (cistron E). The second was infected with φX am 3 and treated with 30μg/ml CAM. The third was treated with 30μg/ml CAM. Each portion was pulsed with [^{3}H] thymidine and the DNA was extracted and sedimented as before. The total number of counts incorporated into host cell DNA was calculated from the gradients and normalized against the absorbance at 260mμ of the sample. These data were plotted as percent of the counts incorporated into the 0 - 2 minute pulse. O ——— O, uninfected plus CAM ; ● ——— ●, infected without CAM ; △ - - - △, infected plus CAM.

A culture of E. coli HF4701 was U.V. irradiated and portions were infected with the various cistron A mutants. The intracellular φX174 coded proteins were labeled with [^{14}C] amino acids and electrophoresed on a 7 1/2% polyacrylamide gel. An autoradiograph of the gel is shown in figure 5. The gel shows that none of these mutants synthesize the large protein coded for by cistron A, and two mutants, H90 and am 18, also fail to make the small A protein. Am 8 has been mapped in the region of the genome that codes for both A proteins (8) however, am 8tr and am 8A, temperature resistant revertants of am 8,both synthesize the small A protein.

Both mutants which lack the small A protein, H90 and am 18, fail to shut-off host replication, indicating that this protein plays a role in the shut-off. However, am 8tr and N14 synthesize the small A protein and fail to shut-off the host DNA replication, suggesting that the relationship between the small A protein and the shut-off activity is not direct.

DISCUSSION: Infection of E. coli with φX174 results in the shut-off of host

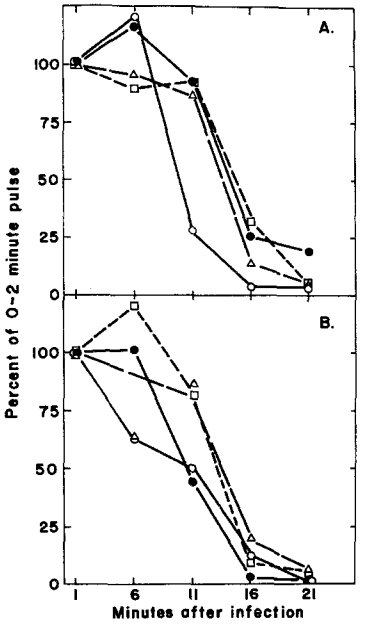

Figure 3: Host cell shut off by mutants of ϕX174. Cultures of E. coli HF4704 were infected with amber mutants of ϕX174 and pulsed with [^{3}H] thymidine. The DNA was extracted and sedimented as described and the data was calculated and plotted as in the legend to figure 2. (A) O ——— O, wild type ; △---△ , am14 (cistron B); □-----□, och 12, am 3 (cistrons C and E); ● ——— ●, am 10 (cistron D). (B) O ——— O, am S_{5-10} (cistron F); △----△, am 9 (cistron G)□----□, am 23 (cistron H) ; ● ——— ●, am 6 (cistron J).

cell DNA replication at about 10 - 12 minutes after infection, as has been observed by others (1, 2, 9). The results of Lindqvist and Sinsheimer (1) and Stone (2) that this shut-off is prevented by CAM have been confirmed. These results are in conflict with the observations of Ishiwa and Tessman (9) who found that the shut-off was not prevented by CAM. As Stone (3) has suggested, this discrepency is probably due to Ishiwa and Tessman working at high multiplicities of infection (10 p.f.u./ml). Stone has observed a nonspecific inhibition of all macromolecular synthesis at high multiplicities of infection (3). This nonspecific inhibition is observed in the presence of CAM.

Mutants in each of the 9 known cistrons of ϕX174 have been tested for the ability to shut-off host cell DNA replication. The only mutants which did not show the shut-off activity were located in cistron A. This cistron codes for

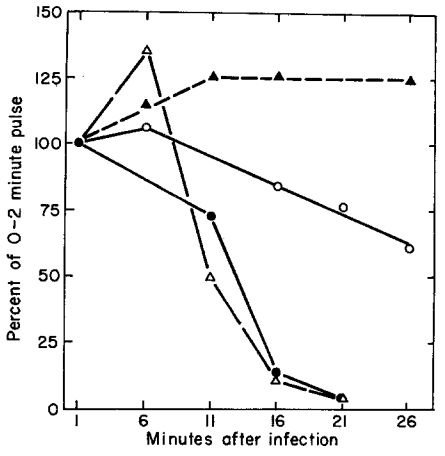

Figure 4: Host cell shut off by mutants in cistron A. Experiments were performed as described in legend to figure 3. O ——— O, H90; △-----△, am 8A; ●——● am 86; ▲----▲ am 18.

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two polypeptides of molecular weights 60 - 65,000 and 35,000 (4). Both mutants which do not synthesize the small A protein failed to shut-off the host DNA replication, indicating that this protein has a role in the shut-off. However, two other cistron A mutants, N14 and am 8tr, which do synthesize a 35,000 molecular weight polypeptide also failed to shut-off the host replication. Thus, the relationship is not clear. However, there is no evidence that the 35,000 molecular weight polypeptides synthesized by these mutants are active small A proteins.

The status of the am 8 mutation is confused. This double mutant carries a temperature sensitive mutation in cistron F as well as the amber in cistron A. Two independently isolated temperature resistant revertants of am 8 (am 8A and am 8tr) have been used in this study . Although am 8 has been mapped in the region that codes for both A proteins (8), both of the temperature resistant strains synthesize a 35,000 molecular weight polypeptide. Also, one of these revertants, am 8A, shuts off the host, but the other does not. Stone also observed shut-off of host cell replication with am 8 (2). Undoubtly, he used a temperature resistant revertant also, since the phage was grown at 37°C.

The mechanism by which φX174 shuts off the host DNA replication is unknown and very little is known about the properties of the small A protein. It

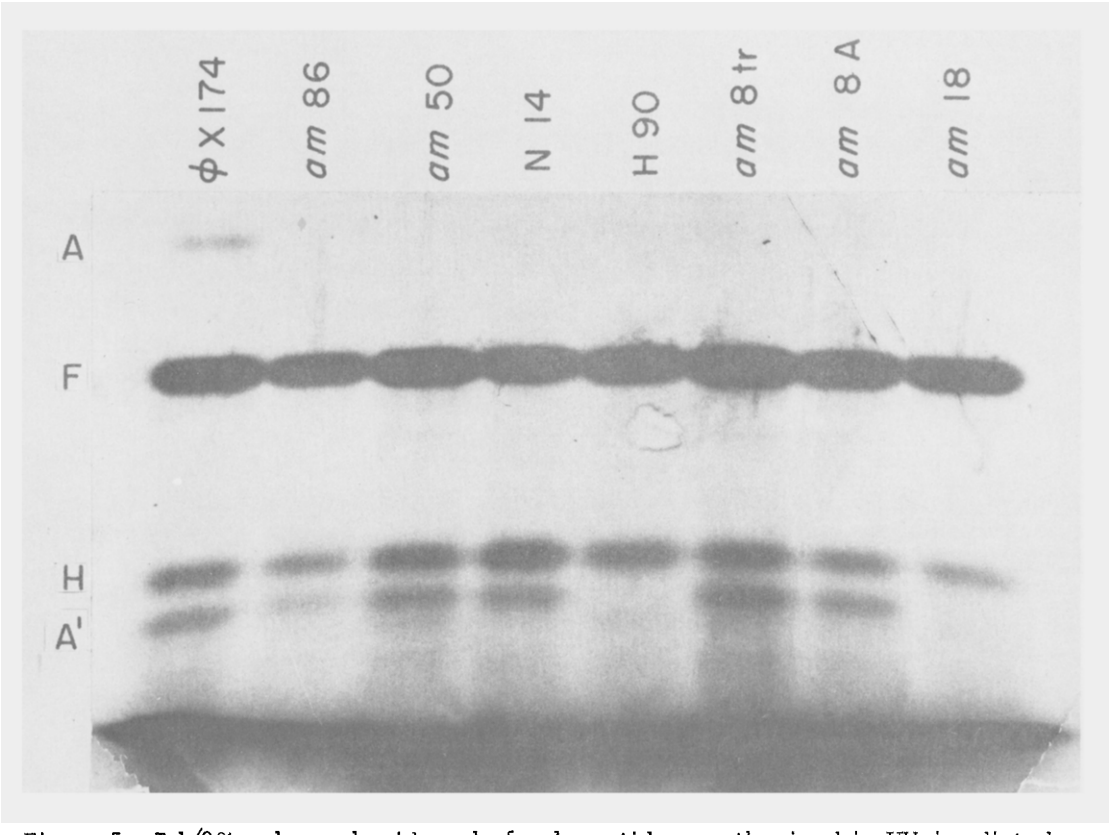

Figure 5: 7 1/2% polyacrylamide gel of polypeptides synthesized in UV-irradiated E. coli HF4701 infected with φX174 and cistron A mutants. Preparation and electrophoresis of samples was as described in methods.

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has been shown to bind strongly to DNA cellulose columns (5). φX174 virus particles contain one copy of a 30,000 molecular weight polypeptide (6) which coelectrophoreses with the small A protein (4). When vaccinia virus infects L cells, a single-stranded DNA specific endonuclease is released from the virus core in the nucleus concomitant with the inhibition of host cell DNA synthesis (10). It is possible that the small A protein interacts with the host DNA at the single-stranded regions of the replication forks, perhaps degrading the single-strands. This would explain the reduction in size of E. coli DNA observed in φX174 infected cells (Martin and Godson, unpublished results). The large cistron A protein is known to have endonucleolytic activity (11), but whether this activity resides in the portion of the molecule that is common to

both large and small proteins is not known. Alternatively, the shut-off activity may be due to interactions with another protein involved in host DNA replication.

It is interesting to note that the replication of double stranded ϕX174 replicative form DNA ceases at the same time that host replication is shut-off (12). The shut-off of viral double stranded DNA replication, which resembles E. coli DNA replication, at least superficially, is also prevented by CAM (13, 14). It is quite possible that the shut-off of host cell DNA replication is a side product of a mechanism designed to shut-off viral double-stranded DNA replication.

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