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# Synthesis of Bacteriophage and Host DNA in Toluene-Treated Cells Prepared from T4-Infected *Escherichia coli*: Role of Bacteriophage Gene D2a

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We investigated the synthesis of DNA in toluene-treated cells prepared from *Escherichia coli* infected with bacteriophage T4. If the phage carry certain *r*II deletion mutations, those which extend into the nearby D2a region, the following results are obtained: (i) phage DNA synthesis occurs unless the phage carries certain DNA-negative mutations; and (ii) host DNA synthesis occurs even though the phage infection has already resulted in the cessation of host DNA synthesis in vivo. The latter result indicates that the phage-induced cessation of host DNA synthesis is not due to an irreversible inactivation of an essential component of the replication apparatus. If the phage are D2a$^+$, host DNA synthesis in toluene-treated infected cells is markedly reduced; phage DNA synthesis is probably also reduced somewhat. These D2a effects, considered along with our earlier work, suggest that a D2a-controlled nuclease, specific for cytosine-containing DNA, is active in toluene-treated cells.

Recently, several systems have been developed for the study of bacterial DNA replication in subcellular or "in vitro" systems (for review, see reference 12). The DNA synthesis in some of these in vitro systems has been shown to be primarily replicative rather than repair. Extension of these systems to the examination of DNA synthesis in in vitro systems prepared from *Escherichia coli* cells that have been infected with bacteriophage T4 might be useful in the study of two problems. (i) The effect of phage infection on the synthetic apparatus for bacterial DNA synthesis could be studied. That is, the study of in vitro systems derived from T4-infected cells might provide information on the mechanism of the phage-induced shut-off of bacterial DNA synthesis (4). (ii) Phage DNA synthesis itself might be studied.

We examined DNA synthesis in uninfected *E. coli* bacteria, in bacteria infected with various mutants of bacteriophage T4, and in in vitro systems derived from both the uninfected and infected bacteria. The in vitro system we employed is basically the permeabilized cell system of Moses and Richardson (13) and Mordoh et al. (11): toluene-treated cells. Such cells are no longer viable, but they continue to make DNA, dependent on a supply of deoxyribonucleoside triphosphates (dNTPs) and ATP. Although toluene-treated cells are permeable to small molecules, they are rather impermeable to macromolecules. Our basic results concerning the two problems stated above are: (i) host DNA synthesis can occur in the in vitro system prepared from infected cells, even though host DNA synthesis has ceased in the cells from which the in vitro system is prepared; (ii) phage DNA synthesis can occur in the in vitro system. An unexpected finding is that the presence of D2a mutations (3, 16) in the infecting phage results in higher levels of DNA synthesis in vitro.

While we were preparing this paper, two other reports appeared which are worth noting here. Lavi and Marcus (9) used toluenized cells to study shut-off of *Bacillus subtilis* DNA synthe-

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sis by bacteriophage $\phi$e. In vivo, the shut-off phenomenon for the $\phi$e-*B. subtilis* system is quite different from that for the T4-*E. coli* system; likewise, their results for the former system in vitro are quite different from our results reported here for the latter system in vitro. Barry and Alberts (1) developed a different type of in vitro DNA synthesis system derived from T4-infected *E. coli*, and used it in an elegant study of phage DNA synthesis.

## MATERIALS AND METHODS

**Bacterial strains.** *E. coli* p3478 *polA1 thy*$^-$, which lacks DNA polymerase I (5, 6), was used in the DNA synthesis experiments. It is nonpermissive for phage amber mutants. p3478 was routinely checked for the presence of the *polA1* mutation by its sensitivity to methyl methanesulfonate (5) (0.1 ml of 10% solution added to the top agar).

Phage amber mutants were propagated on *E. coli* CR63. Stocks of *am*$^+$ phage were prepared on *E. coli* BB. CR63($\lambda$) was the nonpermissive host for phage *r*II mutants.

**Phage T4 strains.** The DNA$^-$ amber mutation used was *am*N82 in gene 44; it is referred to as *am*44. The *r*II deletion mutations have been described previously (3). Multiple mutants were constructed by standard phage crosses in strain BB bacteria.

**Media.** SMGCT defined medium and veal infusion yeast extract (VY) broth were described previously (3). The standard growth medium for p3478 was SMGCT plus thymine at 5 $\mu$g/ml (SMGCT+Thy). The bottom agar for minimal plates (used for bacterial assays) was 20 g of agar (Difco) per liter of SMGCT+Thy. The minimal top agar was 7 g of agar per liter of water. Standard saline citrate (SSC) consisted of 0.15 M NaCl and 0.015 M $Na_3$ citrate.

**Chemicals.** Dithiothreitol, egg white lysozyme, and bovine pancreatic RNase were from Calbiochem, dNTPs and ATP were from P-L biochemicals, and methyl methanesulfonate was from Eastman Organic Chemicals. Radiochemicals, from New England Nuclear Corp., were $^3$H-dATP (0.5 mCi/ml, 11 $\mu$g/ml) and thymidine-*methyl*-$^3$*H* (stock solution: 1 $\mu$Ci/ml, 0.18 $\mu$g/ml).

**DNA synthesis in vivo and in vitro.** Both measurements were performed on the same sample of cells. Cells were grown in SMGCT+Thy to a density of about $3 \times 10^8$/ml. The culture was then either used immediately or placed on ice. To start the experiment, the culture was divided into 10-ml samples (usually three, for two infected and one uninfected sample), and each sample was started shaking at 37 C at t (time) = $-5$ min. At t = 0 samples were infected (phage volume, 0.5 ml or less). At about t = 7 min all samples were assayed for viable bacteria. At t = 12 min an 8-ml portion was removed from each sample for the in vitro measurements (see below). At t = 15 min in vivo DNA synthesis was measured in a 1-ml portion of each sample, by the incorporation of $^3$H-thymidine in a 1.5-min pulse, as described previously (3).

**DNA synthesis in vitro.** This procedure is modified from Moses and Richardson (13) and Mordoh et al. (11). Cells (8 ml) were centrifuged at 5,000 $\times$ *g* and 0 C for 5 min. Pellets were washed with 8 ml of 50 mM Tris, pH 7.5, and recentrifuged. The pellets were then resuspended in 0.6 ml of 50 mM Tris, pH 7.5, to which was added 1 drop of toluene. The mixture was stirred on a Vortex-Genie for 10 to 15 s, allowed to stand for 20 min at 37 C, and then used without further storage. Toluene treatment leaves fewer than $10^{-6}$% colony-forming units in the uninfected sample. The reaction was started by adding 0.1 ml of toluenized cells to 0.1 ml of prewarmed reaction mix. The reaction mix is 50 mM Tris, pH 7.5, 30 $\mu$M each of the normal four dNTPs, 1.5 mM ATP, 100 $\mu$M dithiothreitol, 10 mM $MgCl_2$, and 160 mM KCl. The radioactive label is $^3$H-dATP (2.5 $\mu$Ci/ml). Samples were incubated for 30 min at 37 C. Incorporation was terminated by the addition of 3 ml of iced 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate (TCA-PPi). After 15 min on ice, samples were collected on membrane filters (Millipore Corp.) and washed with 15 ml each of cold TCA-PPi and 10 mM HCl. Filters were dried and counted as for the in vivo measurements (3).

The in vitro DNA synthesis system described above promotes primarily replicative, not repair, synthesis when used with uninfected *E. coli* (13, 11). Use of the *polA*$^-$ host, of course, reduces the likelihood of repair synthesis occurring. In most experiments we verified that the in vitro synthesis for uninfected cells is largely (about 90%) inhibited if ATP is omitted, as characteristic of replicative synthesis (12). We used the same procedure for in vitro DNA synthesis for infected cells as for uninfected cells, with no attempt to determine if these conditions are optimal for the infected cell systems. In vitro DNA synthesis in the infected cell systems is also largely inhibited by the omission of ATP from the reaction mix.

**Isolation of DNA for hybridization experiments.** Unlabeled *E. coli* BB DNA was isolated by the procedure of Marmur (10). Unlabeled T4 DNA was isolated from phage particles by phenol extraction (17). Radioactively labeled DNA made in vitro was prepared as follows. The usual method for the assay of in vitro DNA synthesis was followed except that the reaction was scaled up 10-fold, and the specific activity of the radioactive label was increased to 50 $\mu$Ci/ml. The labeled DNA was isolated from the reaction mix by a modification of the procedure of Smith (15). After the 30-min incubation for the in vitro reaction, EDTA was added to 20 mM and lysozyme to 25 $\mu$g/ml, and incubation at 37 C continued for 5 min. Sodium dodecyl sulfate was added to 0.5% and the incubation continued for another 5 min. The DNA was then extracted with phenol, treated with RNase (at 20 $\mu$g/ml) for 30 min at 37 C, and again extracted with phenol. After phenol extraction, DNA preparations were dialyzed extensively against SSC/100. DNA concentrations were determined by measuring absorption at 260 nm in a Gilford spectrophotometer. (1.OD unit corresponds to 55 $\mu$g of native glucosylated DNA per ml or 50 $\mu$g of native non-glucosylated DNA per ml; reference 17). Labeled DNA was sheared by passing it 10 times through a 20-gauge needle.

**DNA-DNA hybridization.** The procedure of Kourilsky et al. (7) was used with modifications. Schleicher and Schuell B6 membrane filters (25 mm) were used. Each filter was loaded with 7 μg of unlabeled DNA (either *E. coli* or T4 DNA diluted in 2× SSC) and dried overnight at 80 C under vacuum (25 mm of Hg). Control experiments with radioactively labeled DNA showed 80 to 90% retention of the label on the filters. Filters were incubated in separate vials in 0.5 ml of 8 M urea-2× SSC containing 6 to 7 μg (2,000 to 6,000 counts/min) of labeled DNA. After a 12- to 16-h incubation at 42 C, filters were washed by suction (50 ml of 2× SSC on each side) and dried and counted as previously described.

## RESULTS AND DISCUSSION

The basic type of experiment from which most of our conclusions are derived is as follows. Samples of a culture of *E. coli* are infected with various T4 mutants. Three measurements are made on each infected sample and on an uninfected portion run in parallel: surviving bacteria, DNA synthesis in vivo, DNA synthesis in vitro. For convenience, the results for the infected cultures are expressed as a fraction of the uninfected control. The details of the methods are described in Materials and Methods.

The phage strains used in the experiments of Table 1 are divided into four phenotypic groups. DNA$^+$ phages are wild type for all genes involved in phage DNA synthesis. DNA$^-$ phages carry a mutation in gene 44, which is essential for phage DNA synthesis in vivo. It is assumed that this gene is also essential for phage DNA synthesis in vitro. Arguments that this assumption is correct will be presented later. D2a$^-$ phages carry *r*II deletion mutations which extend into the nearby D2a region (see Fig. 1, reference 3). D2a$^+$ phages are wild type for the D2a region: they may be either *r*II$^+$ phages or phages carrying *r*II deletions which do not extend into D2a.

Our primary observations based on the data in Table 1 are summarized in the following two points. These will serve as the basis of most of the following discussion. (i) In vivo: As expected, DNA$^+$-infected cells (groups B and D) make substantial DNA, whereas DNA$^-$-infected cells (groups A and C) make essentially no DNA. For the DNA$^-$-infected cells, the extent of DNA synthesis observed in vivo roughly corresponds to that expected from the surviving bacteria. (ii) In vitro: DNA$^+$-infected cells make more DNA than DNA$^-$-infected cells (cf. B versus A and D versus C). D2a$^-$-infected cells make more DNA than D2a$^+$-infected cells (cf. A versus C and B versus D).

**Shut-off of host DNA synthesis.** Is the phage-induced shut-off of host DNA synthesis which occurs in vivo (and is clearly seen in the in vivo data for DNA$^-$ phages, Table 1, groups A and C) reflected in the in vitro system? Examination of the in vitro data obtained for the same phages shows two different results, depending on the state of the D2a gene. Infection with DNA$^-$D2a$^-$ phages (group A) leads to DNA synthesis in vitro: host DNA synthesis, because of the mutation in the gene for phage DNA synthesis. However, infection with DNA$^-$D2a$^+$ phages (group C) leads to very low levels of DNA synthesis in vitro. Two possible explanations for this D2a effect should be considered. (i) The D2a gene product might be responsible for the shut-off of host DNA synthesis. (ii) The D2a gene product might have nothing to do with the shut-off phenomenon but in some other way inhibits in vitro DNA synthesis. Our earlier work (3, 16) allows us to choose between these alternatives. It is clear that D2a mutations do not prevent shut-off of host DNA synthesis in vivo (Table 1; more extensive consideration by Souther et al. in reference 16). Furthermore, the role we proposed for the D2a$^+$ product (3, 16) immediately suggests how it might lead to reduced in vitro DNA synthesis. We proposed that the D2a$^+$ product controls a nuclease involved, specifically, in the degradation of cytosine-containing DNA (C-DNA).

TABLE 1. *In vivo and in vitro DNA synthesis in cells infected with various T4 strains*

| Phage strain | Surviving bacteria[a] | In vivo DNA synthesis[a] | In vitro DNA synthesis[a] |
|---|---|---|---|
| A DNA$^-$D2a$^-$ phages | | | |
| *am*44-*r*IIH23 | 0.03 | 0.04 | 1.63 |
| *am*44-*r*IIPT8 | 0.03 | 0.07 | 1.19 |
| B DNA$^+$D2a$^-$ phages | | | |
| *r*IIH23 | 0.05 | 1.59 | 2.60 |
| *r*IIPT8 | 0.05 | 1.81 | 2.84 |
| C DNA$^-$D2a$^+$ phages | | | |
| *am*44 | 0.08 | 0.03 | 0.23 |
| *am*44-*r*IIA105 | 0.04 | 0.02 | 0.25 |
| *am*44-*r*II1241 | 0.06 | 0.03 | 0.26 |
| D DNA$^+$D2a$^+$ phages | | | |
| wild type | 0.04 | 2.79 | 1.22 |
| *r*IIA105 | 0.09 | 3.30 | 0.98 |
| *r*II1241 | 0.09 | 2.89 | 1.23 |

[a] The data are expressed as a fraction of the uninfected control. Typical values for the uninfected control are 2 × 10$^8$ bacteria/ml, 1,500 $^3$H-thymidine counts/min incorporated (in vivo), and 800 $^3$H-dATP counts/min incorporated (in vitro).

Since both template and product host DNA contain cytosine, it is easy to envision that, if the D2a-controlled nuclease is active in vitro, it could reduce observed host DNA synthesis in vitro.

Therefore, based on the above analysis, we conclude that host DNA synthesis occurs in toluenized cells prepared from T4-infected cells, even though shut-off has already occurred in vivo. In other words, the shut-off of host DNA synthesis which occurs in vivo is reversed during preparation of the in vitro system. We also conclude that a D2a-controlled nuclease is active in our in vitro system.

Results virtually identical to those in Table 1 are obtained (data not shown) if all the infecting phages carry the mutation *denA*S112, which leads to absence of T4 endonuclease II and to defective host DNA degradation in vivo (14). Although it might seem surprising that *denA*$^+$-induced degradation does not lead to reduced in vitro DNA synthesis, the result is not unreasonable. We estimate that the amount of host DNA synthesis observed in vitro corresponds to about 10% of the host chromosome. Analyses of the kinetics of degradation of the host chromosome in *den*$^+$-infected cells (8, 18) indicate that, when we prepare our in vitro system, most of the host chromosome is still macromolecular and that some of it is probably in pieces as large as 10% of the chromosome. Those analyses do not allow any statement about the size of the particular segment of the DNA containing the replication fork.

**Phage DNA synthesis.** Infection with DNA$^+$ phages consistently leads to more DNA synthesis in vitro than does infection with DNA$^-$ phages (Table 1). It thus appears that phage DNA synthesis occurs in vitro, if the phage are DNA$^+$.

What is the effect of D2a mutations on phage DNA synthesis in vitro? We have not really investigated this question in any detail; however, the data of Table 1 suggest that the D2a$^+$ product inhibits phage DNA synthesis less than it inhibits host DNA synthesis. If that is so, it might well be because the template phage DNA contains not cytosine, but instead hydroxymethylcytosine; thus, the template is resistant to the D2a nuclease specific for C-DNA, and the presence of the odd base in one strand of the product might protect it from degradation.

**Role of phage gene 44 product in vitro.** In the above it has been assumed that the presence or absence of a gene 44 mutation provides a control over phage DNA synthesis in vitro, as it does in vivo. Since the role of the gene 44 product in phage DNA replication is not understood (1), one cannot be certain that it is necessary in vitro. We carried out two kinds of experiments to test whether the gene 44 mutation does prevent phage DNA synthesis in vitro.

First, we carried out experiments similar to those shown in Table 1 by using either a gene-43 (phage DNA polymerase) or a gene 45 (unknown function) mutation instead of a gene 44 mutation. The results were similar in each case (data not shown). The simplest interpretation is that the products of genes 43, 44, and 45—all necessary for phage DNA replication in vivo—are also all necessary for phage DNA replication in our in vitro system.

Second, we carried out preliminary analyses by DNA-DNA hybridization of the DNA produced in the various in vitro reaction mixes. In these experiments, the radioactively labeled DNA synthesized in vitro is tested for retention on filters containing bacterial DNA, phage DNA, or no DNA. The data from these experiments are presented in Table 2. It is seen that, as expected, the DNA synthesized in vitro in uninfected cells is entirely host DNA: no more of the labeled DNA binds to the phage DNA filter than to the blank, control filter. The DNA synthesized in vitro in the DNA$^-$D2a$^-$-infected cells, likewise, is entirely host DNA. On the other hand, the hybridization experiments indicate that the DNA made in vitro in cells infected with DNA$^+$ phages contains phage DNA. Thus, the hybridization experiments confirm the previous identifications of host and phage DNA in the in vitro reaction mixes. It is also noted from the data in Table 2 that the relative amount of host DNA, compared to phage DNA, is greater with the DNA$^+$D2a$^-$-infected cells than with the DNA$^+$D2a$^+$ (wild-type)-infected cells. This again suggests that the D2a$^+$ product is inhibiting host DNA synthesis more than the phage DNA synthesis.

Our main conclusions are: (i) host DNA synthesis can occur in an in vitro system derived from T4-infected cells, even though host DNA synthesis has been shut-off in vivo; (ii) phage DNA synthesis can occur in vitro, if the genotype of the infecting phage permits; and (iii) D2a mutations in the infecting phage stimulate DNA synthesis in the in vitro system.

Our in vitro system in all of these experiments has been toluenized cells. It would be quite interesting if different results were obtained in different in vitro systems.

Our first conclusion puts some constraints on models to explain phage-induced shut-off of host DNA synthesis. It is consistent with ideas such as a phage-induced shut-off protein that is lost in the in vitro system, or a conformational

TABLE 2. *DNA-DNA hybridization of DNA synthesized in vitro*

| Source of radioactively labeled in vitro DNA[a] | DNA retained on filters[b] | | |
|---|---|---|---|
| | Bacterial DNA filters | Phage DNA filters | Blank filters |
| Uninfected cells | 0.11 | 0.03 | 0.03 |
| DNA⁻D2a⁻-infected cells | 0.12 | 0.02 | 0.03 |
| DNA⁺D2a⁻-infected cells | 0.07 | 0.09 | 0.01 |
| DNA⁺D2a⁺-infected cells | 0.06 | 0.18 | 0.03 |

[a] The DNA⁻ mutation is *am*44, and the D2a mutation is *r*IIH23.

[b] The data are expressed as a fraction of the input radioactive DNA which bound to the filter.

change that is reversed in the in vitro system. It is not consistent with the irreversible inactivation of an essential component of the replication apparatus (unless that component is no longer essential in the in vitro system). In a sense, a system which shows the alternative result (no host DNA synthesis in vitro with infected cells) might be more interesting. Then biochemical analyses of and complementation between the uninfected (on) and infected (shut-off) systems might lead to useful information about shut-off.

Concerning the second conclusion, we might note that those DNA⁻ phages whose only defect is in the production of dNTPs would be expected to make DNA in vitro, since the dNTPs are supplied. We verified this prediction in one case (gene 56 mutants; unpublished data).

The fact that D2a effects are observed in vitro opens the possibility of purifying and characterizing the active component in a D2a⁺ cell. The ability of that component, which may or may not be the D2a gene product itself, to inhibit DNA synthesis in vitro would serve as an assay. Of course, such experiments would require use of in vitro systems permeable to macromolecules (see reference 12).

One feature of the D2a effects observed here might appear to contradict earlier results. Souther et al. reported previously that the D2a-controlled nuclease plays only a minor role in degradation of the host chromosome in vivo (16). We now find that it is able to inhibit host DNA synthesis in vitro nearly completely. This difference in effect on host DNA in vitro and in vivo might have any number of explanations, many trivial. But one possible explanation strikes us as particularly interesting. We note that one difference between the two systems is that in vivo the host DNA is not replicating (the shut-off phenomenon), whereas in vitro it is replicating (reversal of shut-off, as shown in this report). Thus, we wonder whether the greater effect of the D2a-controlled nuclease on host DNA in vitro might reflect some preference of that nuclease for nascent DNA or for DNA near the replication fork. This suggestion can be tested by variations of the experiments reported here.

As in our earlier work, we must again note that all the D2a mutations used here are extended *r*II deletions. Therefore, we cannot exclude the possibility that the effects we ascribe to D2a (here, and references 3, 16) may require, also, *r*II or D1 mutations, or both. However, we recently isolated presumptive D2a point mutants, free of *r*II mutations, which show, in all cases tested, the phenotypes we ascribed to D2a (2).

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