Analysis of Host Range Restriction in *Escherichia coli* Treated with Toluene

(hydroxymethylcytosine/hydroxymethylcytidine triphosphate/T-even phages/DNA replication)

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ABSTRACT *Escherichia coli* cells treated with toluene replicate DNA when they are provided with deoxyribonucleoside 5'-triphosphates, ATP, Mg⁺⁺, and K⁺. However, when deoxycytidine 5'-triphosphate is replaced by hydroxymethyl deoxycytidine 5'-triphosphate, incorporation of nucleotides into acid-precipitable material by toluene-treated strains restrictive to nonglucosylated T-even phage is reduced to less than 5% of that normally observed. Even when dCTP is present in the reaction mixture, a similar effect of the hydroxymethyl analogue on DNA replication is observed. In contrast, toluene-treated *E. coli* K12r6-r2,4⁻, a strain permissive to the nonglucosylated T-even phage, incorporates hydroxymethyl deoxycytosine into its DNA, and replication proceeds at only a slightly reduced rate in the presence of the hydroxymethyl deoxycytidine 5'-triphosphate. The presence of the hydroxymethyl deoxycytidine 5'-triphosphate in the reaction mixture does not lead to degradation of preexisting DNA of the restrictive host, but it does lead to an irreversible inhibition of further DNA replication; the inhibition is observed only when the hydroxymethyl deoxycytidine 5'-triphosphate is present during replication. Thus phage-specific enzymes are not necessary for the incorporation of hydroxymethylcytosine into phage DNA, and the restrictive mechanism, present in the host cell before infection, can recognize hydroxymethylcytosine residues in its own DNA, as well as the DNA of the T-even phage.

The DNA of the T-even phages contains hydroxymethylcytosine (HMC) in place of cytosine (1). In addition, glucose is covalently linked to the HMC residues in ratios and configurations specific for T2, T4, and T6 phages (2). Kornberg, Zimmerman, and Kornberg (3) have shown that phage-induced glucosyl transferases catalyze the transfer of glucose from UDP-glucose to HMC residues in the phage DNA. T-even phage that contains nonglucosylated HMC, called T* phage, have been prepared by infection of *Escherichia coli* with phages defective in the (UDP-glucose:DNA α -glucosyltransferase (EC 2.4.1.26) (4), or by growth of wild-type phage in an *E. coli* strain lacking UDPG-pyrophosphorylase (EC 2.7.7.9) (5, 6).

Mature T* phage do not grow on *E. coli* B and K12 (7), strains that normally support the growth of T-even phage. T* phage DNA entering these restrictive hosts is rapidly, but incompletely, degraded to acid-soluble material (8, 9). In contrast, *Shigella dysenteriae* supports the growth of the T* phages. This permissive strain presumably lacks the restriction activi-

ties present in wild-type *E. coli*. Revel (10) has isolated mutants of *E. coli* B and K12 that are permissive to T* phages, and by genetic analysis has shown that the restriction of T* phages is controlled by two independent genes: r6 and r2,4. Attempts to demonstrate the restriction functions in cell extracts or in membrane preparations, however, have thus far failed (11). Although some of the deoxyribonucleases found in restrictive strains hydrolyze T*-phage DNA at a much greater rate than glucosylated DNA, these nucleases are present in normal amounts in permissive bacteria (12).

In this communication, we report detection of the r6 and r2,4 restriction activities in uninfected *E. coli* treated with toluene. *E. coli* cells treated with toluene are permeable to compounds of low molecular weight, including nucleoside triphosphates (13). Although these cells are no longer viable, they will perform extended semiconservative DNA replication when provided with the four deoxyribonucleoside 5'-triphosphates, ATP, K⁺, and Mg⁺⁺ (14). By using toluene-treated *E. coli* cells, we were able to introduce hydroxymethyl deoxycytidine 5'-triphosphate (HMdCTP) intracellularly, and to study the effect of HMdCTP on DNA replication in *E. coli* strains restrictive and permissive to the T* phages.

MATERIALS AND METHODS

Materials. Unlabeled deoxyribonucleotides, ATP, and [³H]TTP (11.1 Ci/mmol) were obtained from Schwarz BioResearch. Crystalline pancreatic DNase was purchased from Worthington. Dr. A. Kornberg generously provided us with HMdCMP, HMdCTP, and [¹⁴C]HMdCTP (1.2 $\times$ 10⁶ cpm/mmol).

Bacterial Strains. The strain permissive to T* phage was the double mutant, *E. coli* K12r6-r2,4⁻, obtained from Dr. H. Revel. Restrictive strains were the single mutants, *E. coli* K12r6+r2,4⁻ and *E. coli* K12r6-r2,4⁺, and the wild-type *E. coli* K12r6+r2,4⁺, also obtained from Dr. H. Revel. Additional restrictive strains were *E. coli* W3110 (a thymine-requiring K12 derivative) and *E. coli* D110 (pol⁻endI⁻), a derivative of W3110 lacking DNA polymerase I and endonuclease I activity (14). Bacteria were grown in tryptone broth, supplemented with thymine (10 μ g/ml) when required.

DNA Synthesis. Synthesis of DNA in toluene-treated *E. coli* was measured as incorporation of [³H]dTTP into acid-precipitable material (14). *E. coli* D110 and W3110 were treated with toluene for 10 min; *E. coli* K12 strains were treated with toluene for 2 min, since maximal ATP-dependent DNA synthesis was obtained at this time.

Abbreviations: HMC, 5'-hydroxymethylcytosine; HMdCMP, 5'-hydroxymethyl deoxycytidine 5'-monophosphate; HMdCTP, 5'-hydroxymethyl deoxycytidine 5'-triphosphate; T* phage, T-even phage whose DNA contains 5'-hydroxymethylcytosine but no glucose; T* DNA, DNA isolated from T* phages.

FIG. 1. Effect of HMdCTP on DNA replication in toluene-treated *E. coli*. W3110 cells were grown in tryptone broth supplemented with thymine (10 μ g/ml) to 5×10^8 cells/ml, collected by centrifugation, resuspended in 0.07 volume of 0.05 M potassium phosphate buffer (pH 7.4), and agitated 10 min at 37°C with 1% toluene. The reaction mixture (0.3 ml) contained 70 mM potassium phosphate buffer (pH 7.4), 13 mM Mg^{++} , 1.3 mM ATP, 1.5×10^8 toluene-treated cells, and 33 μ M [3H]dTTP, dATP, dGTP, and dCTP, or HMdCTP, as indicated. The cells were incubated at 33°C, and at the indicated times cold 1 N trichloroacetic acid–0.1 M PP_i was added to a 0.3-ml aliquot of the reaction mixture to stop the reaction. After it was mixed, each sample was filtered through a Whatman GF/C glass filter (2.4 cm) and washed three times with 3 ml of cold trichloroacetic acid– PP_i , followed by three washes, of 3-ml each, with cold 0.01 M HCl. The filters were dried and the radioactivity was measured.

RESULTS

Effect of HMdCTP on DNA replication

Strains Restrictive to T* Phage. *E. coli* W3110 treated with toluene replicates its DNA in the presence of dATP, dGTP, dTTP, dCTP, ATP, Mg^{++} , and K^+ (14). In contrast, when HMdCTP is substituted for dCTP in the reaction mixture, incorporation of radioactivity into acid-precipitable DNA of high molecular weight is reduced to less than 5% of that normally observed (Fig. 1). The same reduction persists even when dCTP and HMdCTP are present together in equal amounts, but it is not observed in a reaction mixture containing dCTP and HMdCMP. Other *E. coli* strains

TABLE 1. Effect of HMdCTP on DNA replication in restrictive hosts

Strain	DNA synthesis	
	dCTP (%)	HMdCTP (%)
W3110	100	<5
D110	100	<3
K12	100	13

The reaction was as described in Fig. 1, except that incubation was for 30 min at 33°C. Control incubations in which ATP was omitted showed minimal DNA synthesis (<10%) and the values obtained were subtracted from the amounts of ATP-stimulated DNA synthesis to give the above results. 100% represents 100–125 pmol of [3H]dTTP incorporated per 30 min of incubation.

restrictive to T* phage *in vivo* show similar reductions in net DNA synthesis in the presence of HMdCTP (Table 1).

A Strain Permissive to T* Phage. Two independent genes control the restricting functions in *E. coli* K12 (10, 11). The *r6* function restricts all T* phages, while the *r2,4* function restricts T*2 and T*4 phages, but not T*6 phage. In contrast to the results described above for restrictive hosts, *E. coli* K12 $r6^-r2,4^-$, a permissive strain, replicates DNA after toluene treatment at only a slightly reduced rate in the presence of HMdCTP (Table 2). When HMdCTP and dCTP are both present in equal amounts, incorporation is 90% of that obtained with dCTP. When replication was measured with [^{14}C]HMdCTP, the radioactivity was incorporated into DNA at the same rate as [3H]dTTP, providing evidence that HMC was actually incorporated into host DNA. Both the *r6* and the *r2,4* restriction functions must be lacking, however, for DNA replication to occur normally with HMdCTP. As shown in Table 2, in K12 strains with single mutations, either in the *r6* or the *r2,4* restriction functions, incorporation of radioactivity into acid-precipitable material is reduced to about 20% of that obtained with dCTP only. These results suggest that the *r6* and the *r2,4* restriction functions are expressed independently in the toluene-treated cells, as well as in the restriction of T* phage *in vivo*.

Effect of HMdCTP on DNA repair

In addition to DNA replication, DNA repair synthesis can be observed in toluene-treated *E. coli* cells (14). Repair synthesis, as distinguished from replicative synthesis, is stimulated by DNase, is not inhibited by *N*-ethylmaleimide, persists at the restrictive temperature in DNA temperature-sensitive mutants, requires a *pol*⁺ strain for its demonstration, and does not require ATP. To study the effect of HMdCTP on DNA repair, pancreatic DNase (0.3 μ g/ml) was added (14) to toluene-treated *E. coli* W3110. Extensive and equal amounts of DNA synthesis occurred in the presence of HMdCTP as in the presence of dCTP, and this synthesis did not require ATP. Thus, in contrast to the results described for replicative synthesis (Fig. 1), under conditions of repair synthesis the cells do not discriminate between HMdCTP and dCTP. In the following experiments, *E. coli* D110, a strain lacking DNA polymerase I and endonuclease I, was used to eliminate the possibility of repair synthesis occurring in the presence of HMdCTP.

Effect of HMdCTP concentration

Equal concentrations of HMdCTP and dCTP are as effective as HMdCTP alone in reducing replicative DNA synthesis in a restrictive strain of *E. coli*. To investigate the effect of lower

TABLE 2. Effect of HMdCTP on DNA replication in restriction mutants

Strain	Phenotype	DNA synthesis	
		dCTP (%)	HMdCTP (%)
K12 $r6^-r2,4^-$	Permissive	100	70
K12 $r6^+r2,4^-$	Restrictive	100	21
K12 $r6^-r2,4^+$	Restrictive to T*2, T*4	100	22
K12 $r6^+r2,4^+$	Restrictive	100	13

The reaction was as described in Table 1.

concentrations of HMdCTP, *E. coli* D110 was incubated in a reaction mixture containing various concentrations of HMdCTP and a constant concentration of dCTP. As shown in Fig. 2, incorporation of radioactivity is still less than 10% of that observed with dCTP at a 10:1 ratio of dCTP to HMdCTP. Synthesis increases to 80% of the control value at a 100:1 ratio of dCTP, with a 50% reduction occurring at a 40:1 ratio in *E. coli* D110. These results, however, apply only to a strain of *E. coli* lacking DNA polymerase I. Preliminary experiments indicate that in a pol^+ strain, low concentrations of HMdCTP stimulate high levels of DNA repair synthesis.

Effect of HMdCTP on preexisting DNA

T* phage DNA entering a restrictive *E. coli* host is rapidly, but incompletely, degraded to acid-soluble material (8, 9). It has been suggested that the restricting functions of the host recognize nonglycosylated HMC residues in the T* DNA. To test this hypothesis in the toluene-treated cells, HMdCTP was added to toluene-treated *E. coli* D110 at 5, 15, and 25 min after DNA replication had begun. Incubation was continued for 25 min after the addition of HMdCTP. As shown in Fig. 3, HMdCTP inhibited the subsequent formation of acid-precipitable DNA. Furthermore, DNA synthesized before the addition of HMdCTP was not subsequently degraded. Thus, the effect of HMdCTP on DNA replication is specific to DNA synthesis occurring in the presence of HMdCTP. The following experiments present evidence that the incorporation of HMdCTP into DNA is a prerequisite for the expression of the $\text{r}\phi$ and $\text{r}\phi_{2,4}$ restriction activities.

Effect of prior incubation with HMdCTP

From the preceding experiments, one can propose at least two mechanisms for the action of HMdCTP on DNA replication: (a) HMdCTP is incorporated into *E. coli* DNA, and subsequent to this incorporation the HMC-containing DNA is attacked by the restriction activities, or (b) HMdCTP is not incorporated into *E. coli* DNA, but blocks some other step in replication. To distinguish between these possibilities, the effect of prior incubation with HMdCTP on subsequent DNA replication with dCTP was studied (a) under conditions of replication and (b) under conditions of limited DNA synthesis.

DNA Replication. Toluene-treated *E. coli* D110 was first incubated for 2 min with HMdCTP, dATP, dGTP, dTTP, ATP, Mg^{++} , and K^+ . After this incubation, HMdCTP was effectively removed by adding dCTP, to give a final ratio of 400:1 of dCTP to HMdCTP. Synthesis was then measured for an additional 30 min. As shown in Fig. 4, the rate of incorporation of radioactivity in cells previously exposed to HMdCTP was less than 20% of that of controls. Prior incubation with dCTP and HMdCTP, in a 10:1 ratio, still resulted in over a 50% reduction in the rate of subsequent DNA synthesis. Thus, prior incubation with HMdCTP under conditions of replication leads to an irreversible reduction in further DNA replication.

Limited DNA Synthesis. Toluene-treated *E. coli* D110 was first incubated with HMdCTP, Mg^{++} , K^+ , and ATP. Extensive incorporation of HMdCTP was prevented by the absence of the other deoxyribonucleoside 5'-triphosphates necessary for DNA replication. The cells were then washed

FIG. 2. Effect of HMdCTP concentration on DNA replication. Toluene-treated *E. coli* D110 was prepared and incubated in the reaction mixture described in Fig. 1, except that 33 μM dCTP was present in all reactions and the concentration of HMdCTP was varied from 0.0 to 3.3 μM to give the ratios of HMdCTP to dCTP indicated. The cells were incubated for 30 min at 33°C, the reaction was stopped, and the acid-insoluble radioactivity was measured as in Fig. 1.

and incubated for 30 min in the standard reaction mixture with dCTP, dGTP, dATP, and dTTP, but without HMdCTP. As shown in Fig. 5, prior incubation with HMdCTP alone had no effect on subsequent DNA replication with dCTP. As expected, exposure to HMdCTP under conditions that allow extensive DNA synthesis reduced subsequent DNA replication to 25% of that observed after prior incubation with dCTP alone in control experiments. This result suggests that HMdCTP is incorporated into *E. coli* DNA, and that the synthesis of HMC-containing DNA is a prerequisite for the expression of the *E. coli* restriction activities.

FIG. 3. Addition of HMdCTP during DNA replication. *E. coli* D110 cells were grown, treated with toluene, and assayed for DNA replication as described in Fig. 1, except that the cells were initially incubated with 33 μM dCTP, dGTP, dATP, and dTTP, but no HMdCTP. At 0, 5, 15, and 25 min of reaction (arrows), 1 nmol of HMdCTP was added to parallel reaction mixtures. Incubation was continued at 33°C, and samples (0.3 ml) were removed from the reaction mixtures at intervals before and after the addition of HMdCTP. The reaction was stopped, and the acid-insoluble radioactivity was measured as described in Fig. 1.

FIG. 4. Effect of prior incubation with HMdCTP under conditions of DNA replication. *E. coli* D110 was grown and treated with toluene as described in Fig. 1. The reaction mixture (0.3 ml) contained 70 mM potassium phosphate buffer (pH 7.4), 13 mM Mg^{++} , 1.3 mM ATP, 1.5×10^8 toluene-treated cells, 33 μM [3H]dTTP, dATP, dGTP, and (a) 3.3 μM HMdCTP, (b) 3.3 μM HMdCTP and 33 μM dCTP, or (c) 33 μM dCTP. After two min at 33°C, the concentrations of all three reaction mixtures were adjusted to give a final concentration of 1.3 mM dCTP and 3.3 μM HMdCTP. Incubation was continued at 33°C, and 0.3-ml samples were withdrawn at each time point. The reaction was stopped, and the acid-insoluble radioactivity was measured as described in Fig. 1.

DISCUSSION

Strains of *E. coli* possess specific *restriction* and *modification* systems that enable them to distinguish between their own and foreign DNA (15). Meselson and Yuan (16) and Linn and Arber (17) have purified restriction endonucleases from *E. coli* that specifically attack phage λ and fd DNA, respectively. Smith and Wilcox (18) have purified an endonuclease from *Hemophilus influenzae* that can degrade various foreign DNAs, but not its own DNA. The enzymes that restrict nonglucosylated T-even DNA, however, have not been detected, except by *in vivo* phage assay and genetic analysis of the *r6* and *r2,4* gene loci in permissive mutants (11). Our studies relate the restriction mechanism *in vivo* to the observed effect of HMdCTP on DNA replication in toluene-treated *E. coli*. The results obtained clearly demonstrate that toluene-treated cells that are restrictive to the T* phages *in vivo* cease DNA replication when HMdCTP is substituted for dCTP, while similarly-treated cells permissive to the T* phages incorporate HMdCTP and continue DNA replication. This finding strongly suggests that the observed effect of HMdCTP results directly from the action of the restriction activities in toluene-treated *E. coli* cells.

Our studies also support the hypothesis that HMdCTP is first incorporated into *E. coli* DNA, and that subsequently the HMC-containing DNA is recognized by the restriction functions. HMC causes an irreversible reduction in DNA synthesis only if it is present under conditions that allow extensive DNA replication: HMC must be present as HMdCTP and it must be accompanied by the other three deoxynucleoside 5'-triphosphates. Thus, the synthesis of HMC-containing DNA is apparently a prerequisite to the expression of the restriction activities. Certainly, *in vivo*

FIG. 5. Effect of prior incubation with HMdCTP under conditions that allow limited DNA synthesis. *E. coli* D110 was grown and treated with toluene as described in Fig. 1. The reaction mixture contained 70 mM potassium phosphate buffer (pH 7.4), 13 mM Mg^{++} , 1.3 mM ATP, 1.5×10^8 toluene-treated cells, and (a) 33 μM HMdCTP, (b) 33 μM dCTP, or (c) 33 μM dATP, dGTP, dTTP, and HMdCTP. After 2 min at 33°C, the cells were washed and resuspended with the reaction mixture, as described in Fig. 1, except that 33 μM dCTP and no HMdCTP were present. Incubation was continued at 33°C, 0.3-ml samples were withdrawn at each time point, the reaction was stopped, and the acid-insoluble radioactivity was measured as described in Fig. 1.

the restriction enzymes recognize the nonglucosylated HMC residues in the phage DNA rather than the precursor to DNA synthesis, HMdCTP. Interestingly, it is unnecessary to postulate a requirement for new phage enzymes to incorporate HMC into phage DNA.

The presence of the unusual base, HMC, in the DNA of the T-even phages has led to the suggestion that the restriction enzymes must specifically recognize nonglucosylated HMC residues, rather than long base sequences unique to the T-even bacteriophage (11). Our results show that the restriction functions can recognize HMC residues in *E. coli* DNA. Furthermore, DNA synthesized before the addition of HMdCTP is not degraded in the presence of HMdCTP, suggesting that the restriction enzymes are truly specific for HMC. Not all nonglucosylated HMC residues can be recognized, however; phage T6 DNA contains 25% nonglucosylated HMC residues, and yet is not subject to restriction (11). Georgopoulos and Revel (19) have recently concluded that specific, nonglucosylated, nucleotide sequences are the substrate for restriction, based on studies with phage containing amber and other mutations in the glucosyl-transferase enzymes. Since DNA replication does not resume after replacement of HMdCTP by dCTP (Figs. 4 and 5), it appears that irreversible damage occurs at the replication site. For example, although HMC is present exclusively in the growing strands, the preexisting template strands might also be cleaved during restriction.

Our studies *in vitro* also show that the restriction activities are present in active form within the uninfected host. Still unexplained, however, is the observation that only *entering* nonglucosylated T* DNA is restricted. Bacteria lacking UDPG-pyrophosphorylase produce a full crop of T* phage,

even though exogenous T* DNA is restricted (20). Furthermore, it has been shown by isopycnic analysis that glucosylation does not become operative in wild-type cells until the ninth minute after T6 infection (21). To explain the intracellular protection of nonglucosylated T-even DNA, Fukasawa (9) proposed that the restriction functions might be located in the cell membrane, at the sites of phage entry. Our studies are consistent with a membrane site for restriction. The effect of HMdCTP is on newly-replicated DNA, a fraction of the cellular DNA that has been shown to sediment with membrane components (22, 23). Furthermore, the conditions of repair synthesis do not discriminate between HMdCTP and dCTP. If correct, this model of T* restriction would explain the failure to detect the restriction activities in cell-free extracts when exogenous T* DNA substrates are used (11).

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