Toluene-Treated *Escherichia coli* Replicate Only That DNA Which Was About To Be Replicated In Vivo

(bromodeoxyuridine triphosphate/semiconservative/N-ethylmaleimide)

RICHARD M. BURGER

Virus Laboratory, University of California, Berkeley, Calif. 94720

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ABSTRACT DNA density-transfer experiments with *Escherichia coli* strain B/r indicate that the DNA synthesized from exogenous substrates after treatment with toluene is made semiconservatively from that region of the chromosome about to be replicated at the time of tolune treatment. Up to 15% of the chromosome is replicated, at a rate 10-20% of that in vivo. A minor fraction of nonconservatively replicated DNA is readily distinguished.

It seems likely that DNA polymerase (1) (Pol I, EC 2.7.7.7) is involved not in the replication but in the repair of DNA in *Escherichia coli* (2,3). Experiments involving relatively gentle treatment of *E. coli* with toluene have recently revealed another polymerase activity (4). If bacterial mutants unable to synthesize DNA at an elevated temperature are treated with toluene, the new polymerase activity assayed in such toluneized cells is much reduced at the same restrictive temperature (4-6). Two further similarities between replication in vivo and in toluene-treated cells are reported below. We demonstrate the transfer of radioactive label from light chromosomal DNA to that of a hybrid density upon incubation of toluene-treated cells with a mixture of deoxynucleoside triphosphates containing bromodeoxyuridine triphosphate (BrdUTP) instead of dTTP. Only that part of the chromosome about to be replicated when tolune treatment begins exhibits this density transfer.

MATERIALS AND METHODS

The methods of Moses and Richardson (4) were adapted to a density-transfer protocol in which tolune-treated cells with specifically labeled chromosomes were incubated in a mixture of deoxynucleoside triphosphates containing BrdUTP. The cells were then removed, lysed, and their DNA density was analyzed by centrifugation in CsCl.

Nucleotides were purchased from P-L Biochemicals, except for [6-¹⁴C]dCTP (Schwartz/Mann) and [methyl-¹⁴C]thymine (New England Nuclear). BrdUTP was prepared from dUTP according to Chamberlain *et al.* (7), and purified on a DEAE-cellulose column eluted with triethylammonium b caricarbonate. Preparation and storage of nucleotide solutions has been described (8). Solutions of NAD were prepared weekly.

Pronase (Calbiochem) solutions of 100 mg/ml in 0.015 M NaCl-0.0015 M sodium citrate (1/10 SSC) were made and heated to 80°C for 10 min shortly before use. Toluene was redistilled and other chemicals were of reagent grade. CsCl was dissolved in 10 mM Tris-10 mM sodium EDTA buffer (pH 8.5); concentrations were determined and adjusted by the use of a Zeiss refractometer. Growth media contained minimal salts (9) plus 2 mg/ml of glucose, 50 µg/ml of N-ethylhistidine, and 3 µg/ml of thymine.

Cells (E. coli, B/r, thy-, his-) designated CW6, from C. B. Ward) were grown at 37°C with shaking, to a density of 2-3 × 10⁷ cells/ml, then harvested by centrifugation (2000 × g) at 4°C, and resuspended at 5 × 10⁶ cells/ml in 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM MgCl₂. The suspension was shaken with 1% tolune for 5 min at 37°C and then diluted 4.5-fold by the addition of a mixture yielding 13 mM MgCl₂, 0.13 mM NAD, 1.3 mM ATP, 33 µM (each) dATP, dGTP, and BrdUTP, and 20 µM [¹⁴C]dCTP (10 µCi/ml), in 0.9 ml of 70 mM potassium phosphate buffer (pH 7.4). Incubation at 37°C was halted after 20 min by precipitation with CH₃COOH (4) or by chilling if density analysis was desired. The chilled cells were sedimented three times at 5000 × g from 10 mM Tris-10 mM sodium EDTA buffer (pH 8.5), then resuspended in 1 ml, with Pronase (2 mg/ml) and sodium dodecyl sulfate (0.5%) added. Lysis occurred during 10 min at 45°C. After 2 hr more at 37°C, the lysates were passed 20 times through a 21-gauge hypodermic needle to shear the DNA, and clarified by centrifugation at 20,000 × g. Lysates (0.9 ml) were added to 9 ml of CsCl (density 1.80 or 1.82 g/ml) and centrifuged 30 hr at 25°C in a Beckman type 50 rotor at 34,000 or 39,000 rpm, respectively, to resolve light from hybrid DNA or each from heavy as well. Molecular weights of DNA after density gradient fractionation were determined by band sedimentation through isokinetic 15-28% sucrose gradients (10) for 8 hr at 41,000 rpm in a Beckman SW 41 rotor at 4°C. Purified λ phage DNA (from E. Jordan) was used as a standard. Radioactivity was determined in 0.3-ml fractions added to 5 ml of a 1:10 water: Aquasol (New England Nuclear) mixture.

RESULTS

Toluene-treated cells incorporated [¹⁴C]dCTP into acid-precipitable material for at least 30 min at a nearly constant rate of 1-3 × 10⁶ nucleotides per cell per minute, in contrast to a rate of 10⁴ in vivo. Subsequent rapid diminution of activity was not forestalled by enzymatic regeneration of nucleoside triphosphates or periodic addition of fresh substrates. Half the nucleoside triphosphates remained intact after 1 hr, as determined by paper chromatography. The incorporation rate is halved at concentrations of 5-10 µM.
dCTP or dATP, the only substrates so tested. The uptake of [3H]dCTP from a mixture containing 33 μM dTTP was unaffected by addition of BrdUTP. When the reaction mixture contained equal concentrations of dTTP and BrdUTP, the density of the product indicated that neither nucleotide was incorporated preferentially, as previously observed (11).

Fig. 1 depicts a density transfer experiment indicating semiconservative replication in toluene-treated cells containing uniformly labeled DNA. In Fig. 1A, 6% of the originally light [14C]DNA appears at hybrid density after incubation for 20 min with a mixture containing BrdUTP. Its new density coincides with that of the dCTP label with this exception: some 3H label also appears at lighter densities. When N-ethylmaleimide (NEM), an inhibitor innocuous to Pol I, is present (Fig. 1B) no density transfer is seen and the small amount of tritium incorporated appears in the predominantly light-density material typical of repaired DNA (12). Omission of ATP from the reaction mixture has the same result as does addition of NEM. This NEM-resistant incorporation accounts for the lighter 3H “tail” in Fig. 1A, and may be due to Pol I (4). This light material contains many single-strand breaks, since it ranges in size from 15 × 10^6 to <10^6 daltons in alkaline sucrose. In contrast, the DNA replicated semiconservatively, either before or after toluene treatment, showed a molecular weight of 8 × 10^6 in alkali and 30 × 10^6 when native, which rules out the possibility that the hybrid density material was composed of small fragments.

To determine whether label incorporation occurred anywhere but at the preexisting, growing fork, density transfer experiments were done with chromosomes previously labeled only “behind” the growing fork. It was found that no density transfer could be detected if radioactive label was absent from the region about to be replicated in vivo before toluene treatment. In Fig. 2, chromosomes were labeled with 14C for various periods before toluene treatment, but no label was subsequently transferred to hybrid density (A, B) unless the period of prelabeling was one generation (50 min) or more (C, D). Otherwise, the extent of density transfer was unaffected by the period of labeling. Thus, none of the density transfer in Fig. 2 resulted from a prematurely initiated cycle of DNA replication or DNA repair, or any phenomenon occurring anywhere except in that chromosomal region immediately preceding the preexisting growing fork. By contrast, when premature initiation was induced by removal of nalidixic acid (13) from cells that had been exposed for 50 min and then treated with toluene, density transfer occurred even in cells labeled for only 20 min before addition of the DNA-replication inhibitor (manuscript in preparation).

**DISCUSSION**

The demonstration that toluene-treated cells replicate only that chromosomal region that was about to be replicated in vivo is insufficient to establish that the mechanisms regulating replication in vivo are intact, but the absence of such derangements as reinitiation and extensive repair encourages that hope. Our experiments cannot determine whether the DNA segments synthesized just before and just after toluene treatment are covalently joined, nor can they rule out the

**Fig. 1.** Density analysis in CsCl of DNA replicated after treatment with toluene. Cells were grown over four generations in a medium containing [methyl-14C]thymine, 0.2 μCi/ml. (●). The reaction mixture furnished after toluene treatment contained BrdUTP and [3H]dCTP (○) (A) plus 1.3 mM NEM (B).

**Fig. 2.** Density transfer from chromosomes labeled for different periods. Before toluene treatment, cells were grown in [methyl-14C]thymine, 0.1 μCi/ml (●) for (A) 20 min, (B) 35 min, (C) 50 min, and (D) 65 min. Their doubling time was 50 min, and cell titers at harvest were 1.5–3 × 10^8/ml. The reaction mixture contained BrdUTP and [3H]dCTP (○).
possibility that reinitiation of the DNA replication cycle may occur in cells about to reinitiate at the time of toluene treatment.

Each property of DNA replication that can be shown to be the same before and after toluene treatment adds strength to the argument that this synthesis results from the action of the \textit{in vivo} replicase. The replication we have observed is extensive and rapid, resulting in transfer of label from light chromosome to newly synthesized DNA of hybrid density. Of special interest is the finding that the specific site of replication is maintained and that no new replication is initiated anywhere else. Perhaps the cells have lost the capacity to reinitiate chromosome replication, but there remains the possibility that exogenously supplied substances may induce initiation (6).

The details of how initiation and consequent DNA replication are controlled may become susceptible to study \textit{in vitro} by the use of toluene-treated cells or other preparations (11, 14).

Recent studies of toluene-treated \textit{Bacillus subtilis} give results consistent with those in this report (15).

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