Stimulation of Adenosine 5'-Triphosphate-Dependent In Vitro Deoxyribonucleic Acid Replication by Factors from the Periplasmic Space of Escherichia coli

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In vitro deoxyribonucleic acid (DNA) synthesis systems based on an earlier system using penicillin have been developed which use osmotic lysis of lysozyme-formed spheroplasts of Escherichia coli cells embedded in an agarose matrix. An adenosine 5'-triphosphate (ATP)-dependent semiconservative mode, or replicative mode, of in vitro DNA synthesis is exhibited which is sensitive to nalidixic acid. These systems require growth of the agar-embedded cells in a preincubation medium before spheroplast formation and osmotic lysis. Inhibitor studies suggest that one or more required macromolecular species are synthesized during this preincubation growth period. Osmotic shock fluid from E. coli contains macromolecular factors which preferentially stimulate the ATP-dependent semiconservative mode of in vitro DNA synthesis. In some cases, the ATP-independent mode of synthesis is inhibited by shock fluid. Evidence is presented that the stimulating factors found in the osmotic shock fluid come from the E. coli periplasmic space. This stimulation is observed using either toluene-treated cells or lysed agar-embedded ethylene glycol-bis-(beta-aminoethyl ether) N,N'-tetraacetate-lysozyme spheroplasts, and is thus independent of the in vitro DNA synthesis system used. Shock fluid obtained from a given E. coli dna mutant does not stimulate in vitro DNA synthesis by that mutant. However, in some cases, shock fluid from one class of dna mutants does stimulate ATP-dependent in vitro DNA synthesis by another class of dna mutants, in a thermosensitive reaction. Gently prepared cell extracts also stimulate ATP-dependent in vitro DNA synthesis, whereas cell extracts prepared by more severe procedures inhibit this in vitro synthesis. Several stimulating DNA replication factors may be present in the osmotic shock fluid, including products of E. coli dna genes.

Characteristics of an in vitro deoxyribonucleic acid (DNA) replication system using Escherichia coli cells immobilized in a matrix of agarose have been described (33, 34). DNA synthesis in this in vitro system closely resembles DNA replication in vivo in an adenosine 5'-triphosphate (ATP)-dependent process, as shown by the criteria of semiconservative synthesis, reduced synthesis in E. coli dna mutants (mutants thermosensitive for DNA replication [10]), and inhibition of in vitro DNA synthesis by specific inhibitors of DNA replication. In a similar DNA synthesis system using cells immobilized on a planar surface of cellophane, Schaller et al. (28) have shown that one or more nondialyzable components must be present at in vivo concentrations for replicative in vitro DNA synthesis to proceed. The agarose matrix thus appears to serve two purposes: (i) it maintains the intactness of the fragile DNA replication complex of DNA, intracellular site, and other components (31, 33); and (ii) it retains certain macromolecular components essential for replicative in vitro DNA synthesis at the required high concentration (28). In early experiments we used spheroplasts which form during growth in the presence of penicillin G (17) or by growing E. coli dap auxotrophs in the absence of diaminopimelate (1). Using the above considerations regarding the agar matrix, lysozyme systems have now been developed, and are described and used here.

The E. coli cell envelope consists of an inner membrane, a peptidoglycan layer, and an outer membrane (3, 25, 36). The inner membrane provides most of the permeability properties of the cell, and the rigid rod shape of the cell is maintained primarily by the peptidoglycan
layer, a single cross-linked mucopeptide molecule. Treatment of E. coli cells with chelating agents such as ethylenediaminetetraacetate (EDTA) or combined treatment with EDTA and lysozyme, disturbs the cell surface such that resuspension of such cells in low-osmotic-strength medium results in release of approximately 4% of the total cell protein into the medium (osmotic shock fluid). These released proteins, termed the periplasmic factors, represent a select class of the E. coli proteins, and are thought to be compartmentalized in the cell surface between the inner and outer membrane structures in the periplasmic space (11, 28).

Many hydrolytic enzymes, including alkaline phosphatase (EC 3.1.3.1), DNA endonuclease I (EC 3.1.4.30), and ribonuclease (RNase) I (EC 2.7.7.23), are present among the periplasmic factors. Osmotic shock fluid has been used as a first step in the purification of such enzymes and of binding proteins involved in sugar and amino acid transport (11, 27).

This report shows that osmotic shock fluid obtained from E. coli cells contains macromolecular factors which stimulate in vitro DNA replication. Stimulation of in vitro DNA replication by cell extracts prepared in a variety of ways has also been examined and comparison has been made with the stimulation obtained using osmotic shock fluid. We have used both toluenized cells (22) and cells lysed after agar immobilization (33, 34) as in vitro DNA synthesis systems to assay the effects of these factors on in vitro DNA replication. These two systems are representative of the two major classes of in vitro DNA synthesis systems capable of ATP-dependent semiconservative DNA synthesis in vitro (DNA replication in vitro; [15, 33]; T. Matsushita and H. Kubitschek, Adv. Microbiol. Physiol., in press).

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The following E. coli strains were used: W3110 thy dna+ polA+ polB+ (obtained from J. Gross); p3478 thy polA1 (from J. Gross); D110 thy polA1 endA (from C. Richardson); MRE601 thy rnsA (from D. Düttig); CRT4636 thi his mal mtl strA polAl dnaA46(ts) (from Y. Hirota); CRT2967 thi his mal strA polAl dnaA266(ts) (from Y. Hirota); BT1026 thy polA1 endA dnaE1026(ts) (from F. Bonhoeffer); H10265 thy polA1 endA dnaE1026 polB (from M. Getler); and HMS83 thy lys rho lacZ polAl polB (from C. Richardson). A thymine auxotroph of MRE601 was derived using trimethoprim selection (35). Cells were grown in a tris(hydroxymethyl)aminomethane (Tris)-glucose-minimal salts (TG₃) growth medium (modified TPG medium [5] lacking sodium pyruvate), containing 0.1% Casamino Acids (Difco) and [³¹C]thymine (4 μg/ml, 0.03 μCi/ml; New England Nuclear Corp.) to 3 x 10⁸ cells/ml, as monitored using light scattering at 450 nm or by counting in a Petroff-Hauser counter. E. coli dna+ cells were grown at 37 C; E. coli dna mutants were grown at 30 C.

**In vitro DNA synthesis using agar-immobilized cells.** Exponentially growing cells (100 ml) were harvested by centrifugation (8,000 x g, 15 min, 4 C), washed with 20 ml 0.1 M KCl, 0.01 M MgCl₂, 0.01 M Tris-chloride, pH 7.4 (KMT buffer), centrifuged (4,000 x g, 15 min, 4 C), and resuspended in 0.1 ml of KMT buffer. In some experiments, TG₃ growth medium lacking glucose was substituted for KMT buffer, with no detectable difference in the results. One and one-half milliliters of melted 1.5% agarose (Calbiochem Corp.) in KMT buffer, cooled to 43 C, was added, the cells were rapidly dispersed in the agarose solution, and the agarose was permitted to gel at 0 C.

Agarose fragments were formed by forcing the agarose twice through a 100-mesh wire grid, the fragments were washed with 80 ml of KMT buffer, and then suspended in 10 ml of KMT buffer. Approximately 50% of the cells are immobilized in this procedure. [¹⁴C]Counts per minute per 10⁸ cells were determined from a comparison of acid-insoluble [¹⁴C]radioactivity with the number of cells per milliliter.

For growth of agar-immobilized cells in preincubation medium, fragments from 1.0 ml of agar preparation were grown in 25 ml of preincubation medium (0.33 M sucrose and 2 mM MgSO₄ in Penassay broth [Difco]) for the times indicated at 37 C for dna+ strains. The cells are viable, and form microcolonies in the agar fragments during preincubation. The number of cells were determined by slowly filtering an aliquot of the preincubation medium containing agar fragments through a 400-mesh screen, suspending the retained agar fragments in 0.5 ml of KMT buffer, incubating the suspension at 95 C for 2 min to melt the agar and release the bound cells, and counting of the cells in a Petroff-Hauser counter. Comparison of this number at any time of preincubation with that at zero hours of preincubation permits determination of [¹⁴C]acid-insoluble radioactivity per 10⁸ cells, as well as the average rate of growth of the immobilized cells during preincubation.

E. coli spheroplasts were formed using lysozyme and ethylene glycol-bis(beta-aminoethyl ether)-N, N'-tetraacetate (EGTA). Fragments from 1.0 ml of agar preparation were slowly collected onto a 45-mm membrane HA filter (Millipore Corp.), slowly washed with 20 ml of EGTA buffer (20% sucrose, 5 mM EGTA, 100 mM Tris-chloride, pH 7.4, 1 mM MgCl₂), resuspended in 0.5 ml of EGTA buffer, and frozen in acetone dry ice. The frozen suspension was thawed at room temperature, 0.02 ml of 2.5 mg/ml egg white lysozyme (EC 3.2.1.17; Calbiochem Corp.) in 50 mM Tris-chloride, pH 8, was added, and the solution was incubated at 0 C for 15 min. Conversion of cells to spheroplasts was monitored visually using phase contrast microscopy. When necessary, another 0.02 ml of 2.5 mg/ml lysozyme was added, with a further 15 min of incubation at 0 C. Spheroplasts formed by using a lysozyme and EDTA method similar to that used by Bazill et al. (2) exhibited in vitro DNA synthesis prop-
erties indistinguishable from those of the EGTA-lysozyme-former spheroplasts (data not shown).

To effect lysis, agar fragments containing spheroplasts were slowly collected onto a membrane HA filter (Millipore Corp.), drained slowly just to dryness, and washed consecutively with 13, 13, 8, and 13 ml of cold TM buffer (5 mM MgCl₂, 10 mM Tris-chloride, pH 7.4). Agar fragments from 25 ml of preincubation medium were gently resuspended in 0.5 ml of reaction mixture (0.13 M KCl, 7 mM MgCl₂, 50 mM Tris-chloride, pH 7.4, 4 mM beta-mercaptoethanol, 0.4 mM potassium phosphate, pH 7, 1.5 mM adenosine 5′-triphosphate (ATP), 10−4 M soluble ribonuclease acid [sRNA], 20 μM deoxyctydine 5′-triphosphate, 20 μM deoxyadenosine 5′-triphosphate [dATP], 20 μM deoxyguanosine 5′-triphosphate, and 20 μM deoxyxymidine 5′-triphosphate [dTTP], 0.1 μCi of [3H]dTTP per ml and 0.62 mM deoxyxymidine (dThd) and incubated at 36°C. Any residual DNA endonuclease I activity is competitively inhibited by the sRNA in the reaction mixture (18). The reaction was terminated by addition of the agar fragments to 1.0 ml cold NET buffer (0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-chloride, pH 8) containing 0.1 M sodium pyrophosphate, 0.01 M NaN₃, and 0.1% sodium sarcosyntate.

To assay acid-insoluble radioactivity, the termination mixture was heated to 97°C for 1 min, rapidly cooled and diluted by addition of 4 ml of cold NET buffer, and rendered 5% in trichloracetic acid by addition of 1 ml of 30% trichloracetic acid. The precipitate was collected onto Whatman GF/C filters, washed with 10 ml of water and 3 ml of ethanol, dried, and counted in a liquid scintillation counter using a toluene-2,5-diphenyloxazole scintillation fluid. The amount of DNA synthesized in vitro in picomoles per 10⁸ cells was determined from the ⁴H and ¹⁴C values, the specific activity of the [³H]dTTP, and the value of ¹⁴C counts per minute per 10⁶ cells.

In vitro DNA synthesis by using toluenized cells. Toluenized cells were prepared by a modification of the procedure of Moses and Richardson (22). Exponentially growing cells were harvested (10,000 × g, 10 min, 4°C) and suspended at about 5 × 10⁹ cells/ml in TRB buffer (0.24 M KCl, 0.10 M Tris-chloride, pH 7.5, 10 mM MgCl₂) containing an emulsion of 1% toluene. The emulsion was prepared before suspension of the cell pellet by briefly sonically treating the TRB-toluene solution. After the cell suspension was mixed for 2 min by using a Vortex mixer (Scientific Products, setting 5), the toluene was removed by blowing with a stream of nitrogen for 20 s. This solution formed 0.5 times the volume of the reaction mixture; beta-mercaptoethanol, ATP, and the four dATP’s were added to yield the standard reaction mixture described above. After incubation at 36°C for the indicated times, the reaction was terminated by addition of aliquots of the reaction mixture to 0.5 ml of cold NET buffer containing 0.1 M sodium pyrophosphate, 0.01 M NaN₃, and 0.1% sodium sarcosyntate, followed by addition of 5 ml of cold 5% trichloracetic acid. The precipitate was collected by filtration and assayed for radioactivity as described above.

Osmotic shock fluid. Using the procedure of Neu and Heppel (23), the indicated E. coli strain was grown in 1,000 ml of tryptone broth (20) at 37°C to 5 × 10⁹ cells/ml, harvested (10,000 × g, 10 min, 4°C), and washed twice with 400 ml of cold 30 mM NaCl, 10 mM Tris-chloride, pH 7.4. The cell pellet (0.5 g of wet cells) was suspended in 20 ml 33 mM Tris-chloride, pH 7.1, and 20 ml of 40% sucrose, 33 mM Tris-chloride, pH 7.1, was added with rapid stirring. Sodium EDTA (0.04 ml of 0.1 M), pH 7.1, was then added, and the solution was slowly stirred at 0°C for 10 min. After centrifugation (10,000 × g, 10 min, 4°C), the well-drained cell pellet was gently smeared over the inside of a chilled centrifuge bottle, forming a cell monolayer. Forty milliliters of cold 0.1 mM MgCl₂ was added and the cells were rapidly suspended. The cell suspension was stirred at 0°C for 10 min, centrifuged, and the supernatant fluid (the osmotic shock fluid) was concentrated to 1 to 2 ml overnight in dialysis tubing using polyethylene glycol. Control experiments indicated that about 95% of the treated cells retain colony-forming ability, that the DNA of such cells is insensitive to added pancreatic deoxyribonuclease (DNase) (EC 3.1.4.5), that approximately 5% of the total cell protein is recovered in the shock fluid, and that this shock fluid from E. coli endA⁺ lac+ cells contains the majority (90% or more) of DNA endonuclease I and alkaline phosphatase activity, essentially no beta-glucosidase (EC 3.2.1.23) activity, and less than 5% of the total cell DNA, properties previously described for such shock fluid (11).

Cell extracts. The freeze-thaw-lysozyme extract was prepared according to the procedure of Wickner et al. (39), as modified by Schekman et al. (29). The brij-lysozyme extract was prepared according to the procedure of DeLucia and Cairns (6). To prepare EDTA-lysozyme extracts, exponentially growing cells were harvested by centrifugation (10,000 × g, 10 min, 4°C), and suspended at 5 × 10⁹ to 10⁹ cells/ml in NET buffer. Lysozyme was added to 100 μg/ml from a fresh stock solution of 5 mg/ml prepared in 0.25 M Tris-chloride, pH 8. The cell suspension was incubated at 37°C for 15 min, frozen in acetone-dry ice and thawed at room temperature three times, mixed on a Vortex mixer for 2 min, and centrifuged (6,000 × g, 10 min, 4°C); the supernatant fluid was the EDTA-lysozyme extract. To prepare sonication extracts, exponentially growing cells were harvested by centrifugation (10,000 × g, 10 min, 4°C) and suspended at 2 × 10⁹ cells/ml in NET buffer. This cell suspension was sonically treated (five 15-s treatments at 90 W, Branson W140D Sonifier) and centrifuged (6,000 × g, 10 min, 4°C); the supernatant fluid was the sonication extract.

CsCl gradient analysis. Agar fragments in the termination mixture were homogenized 10 times in a small tissue grinder and incubated at 50°C for 30 min after addition of 3 mg of solid Pronase (Calbiochem). Solid KI was added to 2 M to solubilize the agar, the solution was incubated at 37°C for 10 min, diluted to 5 ml with NET/10 buffer (10-fold dilution of NET buffer), and dialyzed extensively at 4°C against NET/10 buffer. The volume was increased to 6.0 ml with NET/10 buffer, 7.95 g of CsCl (American Potash) was added, and the solution was centrifuged in the T50 rotor of a Spinco L3-50 ultracentrifuge for 36 h at 37,000 rpm at 20°C. After deceleration, 0.3- to 0.4-mL
fractions were collected and the CsCl density was determined in representative fractions via refractive index measurements (37) using an Abbe refractometer (Bausch and Lomb). Five milliliters of cold 5% trichloroacetic acid was added to each fraction, the precipitates were collected onto Whatman GF/C filters, washed with 10 ml of water and 3 ml of ethanol, and the filters were dried and radioactivity determined in a liquid scintillation counter using a toluene-2,5-diphenyloxazole scintillation fluid.

Other methods. Protein content was determined by the method of Lowry et al. (19). DNA content was determined by measurements of acid-insoluble radioactivity of cells labeled with [3H]thymine. Beta-galactosidase activity was determined using the procedure described by Miller (20), and alkaline phosphatase activity was determined using the procedure of Garen and Levinthal (7). Relative DNA endonuclease I activity was determined from the rate of sRNA inhibitable decrease in viscosity of 0.25 mg of salmon sperm DNA per ml (Calbiochem) in 0.05 M Tris-chloride, pH 8, 10 mM MgCl2, with or without 100 μg of yeast sRNA per ml (Calbiochem). Nalidixic acid (Calbiochem) was prepared as a 5 mg/ml solution in 0.5 M KOH, rifampin (Calbiochem) as a 10 mg/ml solution in 0.01 M KPO4, pH 5.4, and chloramphenicol (Sigma) as a 2 mg/ml solution in distilled water. All stock solutions were prepared immediately before use.

RESULTS

Preincubation requirement before lysozyme spheroplast formation. When lysozyme spheroplasts were formed with no preincubation after agar immobilization, the cells exhibited very little in vitro DNA synthesis ability and no ATP-dependent synthesis (Fig. 1). However, after incubation at 36 C in preincubation medium before lysozyme spheroplast formation, the in vitro DNA synthesis ability per cell increased dramatically. The cells grew during this incubation with a generation time of about 60 min, forming small colonies in the agar matrix. After a 1-h lag, the rate of in vitro DNA synthesis per cell increased nearly linearly with time, reaching a maximum at 3 to 5 h of incubation. The amount of in vitro synthesis in the presence of ATP was two- to three-fold higher than that observed in the absence of ATP (Fig. 1).

The need for growth in preincubation medium may be due to one or more of several reasons. First, the cells may be damaged during agar immobilization, requiring a recovery time. This is unlikely, however, since the cell treatments are decidedly less severe than cell lysis procedures. Second, one or more substances from the preincubation medium may interact with the cells so as to stabilize the replication apparatus during lysis. Third, a sufficiently high concentration of one or more molecules necessary for DNA replication may be synthesized during cell growth such that a high enough concentration of these molecules is maintained in the agar fragments during lysis and washing to permit the observed DNA synthesis in vitro.

To test these hypotheses, the effects of inhibitors of macromolecular synthesis were examined (Fig. 2). When present throughout the preincubation period, nalidixic acid, a specific inhibitor of DNA replication (9), rifampin, a specific inhibitor of initiation of RNA synthesis (38), and chloramphenicol, a specific inhibitor of peptide bond formation in protein synthesis (1, 14), all prevented the development of in vitro DNA synthetic ability per cell (Fig. 2). When added after 2 h of preincubation, nalidixic acid effects a decrease in ATP-dependent in vitro DNA synthetic ability within 30 min (Fig. 2) and a somewhat greater decrease is observed when nalidixic acid is also included in the in vitro reaction mixture (data not shown), dem-
preincubation medium. E. coli W3110 cells were grown in Agar-immobilized or presence of EGTA-lysozyme preincubation medium in aliquots from symbols: Aliquots or as quots were vitro DNA insoluble radioactivity incubated and ml h symbols: is sensitive with reaction mixtures all required. DNA is synthesized with the third hypothesis. In the absence of DNA and/or protein synthesis, already initiated rounds of chromosome replication are completed, but no new rounds are initiated (33). The decrease in in vitro synthetic ability per cell after exposure to these inhibitors for 90 min may reflect such effects on the DNA replication cycle.

Stimulation of in vitro DNA synthesis by added periplasmic factors. The effects of added osmotic shock fluid from two E. coli strains deficient in DNA polymerase I (EC 2.7.7.7) on the in vitro DNA synthesis capabilities of agar-embedded E. coli W3110 thy are shown in Fig. 3. In the absence of added shock fluid (Fig. 3a), a slight ATP-stimulated synthesis and a relatively low rate of synthesis were observed. Addition of shock fluid (Fig. 3b) from E. coli D110 thy endA polA, a strain (22) deficient in DNA polymerase I and DNA endonuclease I, slightly inhibited the in vitro DNA synthesis in the absence of ATP (ATP-independent synthesis) and stimulated the synthesis in the presence of ATP (ATP-dependent synthesis). Shock fluid from E. coli p3478 thy polA1, a strain (6) deficient in DNA polymerase I which was derived from E. coli W3110 and is the endA+ parent strain of E. coli D110 (22), stimulated synthesis in the presence of ATP and had little effect on the synthesis in the absence of ATP (Fig. 3c). Shock fluid from a

![Graph of figure 2](http://jb.asm.org/)

**Fig. 2.** The effect of inhibitors on development of in vitro DNA synthesis capability during preincubation. Agar-immobilized E. coli W3110 cells were grown in preincubation medium for the times indicated in the presence or absence of the indicated inhibitors. Aliquots were removed, the cells were converted into EGTA-lysozyme spheroplasts, lysed with TM buffer, and incubated in a standard reaction mixture at 36 C for 25 min. The reaction was then terminated, acid-insoluble radioactivity was determined, and the DNA synthesized in vitro was calculated from the 3H and 14C values and the number of cells present in the agar, as described in Materials and Methods. Circles: aliquots from the culture grown in the absence of all inhibitors, with the reaction mixture containing (O—O) or not containing (O—O—O) ATP. Other symbols: Aliquots from cultures containing inhibitors, with reaction mixtures all containing ATP. Open symbols: the indicated inhibitor was present in the preincubation medium for the entire time indicated. Closed symbols: the indicated inhibitor was added 2 h after growth in preincubation medium was begun. Symbols: O, no inhibitors; △, 100 μg of nalidixic acid per ml was added; □, 200 μg of chloramphenicol per ml was added; ▽, 200 μg of rifampin per ml was added.

![Graph of figure 3](http://jb.asm.org/)

**Fig. 3.** Incorporation kinetics of periplasmic factor stimulation. Lysed agar-immobilized E. coli W3110 EGTA-lysozyme spheroplasts preincubated for 1 h and concentrated osmotic shock fluid, prepared from the indicated strain as described in Materials and Methods, were incubated in standard reaction mixtures with (O—O) or without (○—□—□—□) ATP at 37 C. The reaction mixtures contained (a) no added osmotic shock fluid, (b) shock fluid (90 μg of protein) from E. coli D110 thy polA1 endA, or (c) shock fluid (90 μg of protein) from E. coli p3478 thy polA1. After incubation for the times indicated, aliquots were assayed for trichloroacetic acid-insoluble radioactivity and picomoles of dTMP incorporated per 10^8 cells were determined as described in Materials and Methods.
variety of E. coli strains effects little or no stimulation, and often an inhibition, of the ATP-independent DNA synthesis, whereas stimulation of the ATP-dependent synthesis is always observed by using agar-embedded cells preincubated for 1 h (see Fig. 1) before lysozyme-spheroplast formation. Shock fluid from some, but not all, E. coli strains also stimulates the ATP-dependent in vitro DNA synthesis exhibited by agar-embedded cells preincubated for 3 h (see Fig. 1) before lysozyme-spheroplast formation.

The level of stimulation for a saturating amount of shock fluid varies with the E. coli source of the shock fluid. The strain whose periplasmic factors have produced the greatest stimulation is E. coli MRE601, a strain deficient in RNase I (8), an enzyme found in the periplasmic space (11). Concentration dependence of DNA synthesis in vitro by agar-embedded E. coli W3110 on shock fluid from E. coli MRE601 is shown in Fig. 4. No loss of acid-insoluble [14C]DNA was observed during incubation with or without ATP in the presence of shock fluid from any source. ATP-independent in vitro DNA synthesis showed little or no stimulation and some inhibition at high periplasmic factor concentrations. In contrast, the ATP-dependent in vitro DNA synthesis was strongly stimulated. The amount of stimulation increased nearly linearly with amount of shock fluid protein added to about 50 μg of protein, reaching a maximum at about 200 μg of protein. The combined effects on in vitro DNA synthesis in the presence and absence of ATP resulted in about a 15-fold stimulation by ATP when 200 μg of protein of shock fluid was added. The stimulation by the periplasmic factors thus appears specific for the ATP-dependent mode of in vitro DNA synthesis.

Stimulation by periplasmic factors of in vitro DNA synthesis by cells treated with toluene is shown in Table 1. The degree of stimulation is seen to be dependent on the concentration of cells used in the reaction mixture. At the high concentrations usually used, 3 × 10⁶ cells/ml, little stimulation of the ATP-dependent in vitro DNA synthesis was observed. However, as the cell concentration was reduced, a progressively greater stimulation per cell of the ATP-dependent synthesis was observed. At cell concentrations of 10⁴ to 3 × 10⁴ cells/ml of reaction mixture, a two- to three fold stimulation of the ATP-dependent in vitro DNA synthesis by osmotic shock fluid was observed. In contrast, DNA synthesis in the absence of ATP was inhibited by the addition of osmotic shock fluid. Thus, the stimulation by the periplasmic factors is also specific for the ATP-dependent in

![Figure 4. Concentration dependence of stimulation by E. coli MRE601 shock fluid. Lysed agar-embedded E. coli W3110 EGTA-lysozyme spheroplasts preincubated for 1 h and concentrated osmotic shock fluid, in the amounts indicated, from E. coli MRE601, prepared as described in Materials and Methods, were incubated in standard reaction mixtures with (O) or without (●●●) ATP for 25 min at 37 C. Termination of the reaction and determination of trichloracetic acid-insoluble radioactivity and picomoles of dTMP incorporated per 10⁸ cells were as described in Materials and Methods. The results are the average of duplicate experiments, presented as error bars shown.](http://jb.asm.org/)

![Table 1. Stimulation of tolueinized cells by periplasmic factors](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Cell concn in reaction mixture (cells/ml)</th>
<th>Periplasmic factors</th>
<th>[3H]dTMP incorporation (pmol/10⁸ cells)</th>
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<tr>
<td></td>
<td>+ ATp</td>
<td>− ATp</td>
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<tr>
<td>3 × 10⁴</td>
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<td></td>
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*E. coli W3110 cells were tolueinized, incubated in standard reaction mixtures in the presence and absence of ATP, with and without 100 μg of protein of osmotic shock fluid prepared from E. coli MRE601 cells for 20 min at 37 C, and the reactions were terminated and analyzed for picomoles of dTMP incorporated per 10⁸ cells as described in Materials and Methods. Cell concentrations were decreased as indicated by gentle dilution in TRB buffer after toluein treatment.
vitrone DNA synthesis when toluenized cells are used as the assay system.

The degree of stimulation of the ATP-dependent in vitro DNA synthesis using toluenized cells is less than that observed using agar-embedded cells. This is probably due (i) to lack of free entry of macromolecular species into toluenized cells (22), and (ii) to the presence of the stimulating species in nearly sufficient optimal concentrations in the toluenized cells. This latter possibility exemplifies the need in an assay system of a system initially defective or deficient in the functional activity under study.

Preferential stimulation of ATP-dependent semiconservative in vitro DNA replication. To further clarify the mode of DNA synthesis resulting from addition of periplasmic factors, an in vitro density labeling experiment, with analysis of the in vitro DNA products in CsCl gradients, was performed (Fig. 5). In the absence of added shock fluid, the DNA synthesized in the presence of ATP banded in two peaks (Fig. 5a), one at the position of hybrid DNA (bromouracil in one strand, thymine in the

![Graph](http://jb.asm.org/)

**Fig. 5.** Density labeling analysis of DNA synthesized in vitro. Lysed agar-embedded E. coli W3110 EGTA-lysozyme spheroplasts preincubated for 3 h and concentrated osmotic shock fluid from E. coli W3110 and MRE601 were incubated in reaction mixtures with and without ATP for 10 min at 36 C. The reaction mixtures were as described in Materials and Methods, except that [\(^{3}H\)] deoxyctydine 5'-triphosphate, CdR, and BrdUPT were substituted for [\(^{3}H\)]dTTP, TdR, and dTTP, respectively. The six reaction mixtures contained (a) ATP with no shock fluid, (b) no ATP and no shock fluid, (c) ATP with W3110 shock fluid (100 µg of protein), (d) W3110 shock fluid (100 µg of protein) but not ATP, (e) ATP with MRE601 shock fluid (100 µg of protein), and (f) MRE601 shock fluid but no ATP. The reactions were terminated, and the DNA was isolated and analyzed by equilibrium sedimentation in neutral CsCl gradients as described in Materials and Methods. The positions of duplex DNA containing only thymine (light, LL, DNA), containing thymine in one strand and bromouracil in the other (hybrid, HL, DNA), and containing bromouracil in both strands (heavy, HH, DNA), as determined by E. coli density marker DNA and refractometer determinations in representative gradients, are indicated by the arrows shown. ———- [\(^{14}C\)]DNA: ———- [\(^{3}H\)]DNA.
other), the other at a density slightly heavier than that of light DNA (thymine in both strands). In contrast, nearly all the DNA synthesized in the absence of ATP banded near the density of the parental light DNA (Fig. 5b). Thus, ATP is required for semiconservative DNA synthesis (DNA replication) in vitro, as observed previously (22, 28, 33). Shock fluid from E. coli W3110 cells stimulated the total DNA synthesis in vitro in the presence of ATP by E. coli W3110-lysed spheroplasts, with nearly an equal stimulation (about 2.5-fold) of both peaks observed in the CsCl gradient (Fig. 5c). In contrast, very little stimulation (about 70% increase) of the in vitro DNA synthesis in the absence of ATP was observed (Fig. 5d). Shock fluid from E. coli MRE601 cells resulted in a greater total stimulation of ATP-dependent in vitro DNA synthesis, with a preferential stimulation of synthesis of hybrid density DNA (about 14-fold stimulation) compared with the light density DNA (about six-fold stimulation) (Fig. 5e). In the absence of ATP (Fig. 5f), no hybrid density DNA was synthesized, although some stimulation of light density DNA (about three-fold) was observed. Thus, addition of periplasmic factors results in a preferential stimulation of the ATP-dependent semiconservative mode of DNA synthesis in vitro.

Periplasmic factors from E. coli dna and polB mutants. To further examine the specificity of the stimulation by the periplasmic factors, shock fluid from several E. coli dna mutants (10) and polB mutants (4, 12) was tested for ability to stimulate in vitro DNA synthesis in agar preparations of these same strains (Table 2). Agar-embedded cells preincubated for 1 h before lysozyme-spheroplast formation were used. The amount of shock fluid used, containing 100 to 200 μg of protein, provides maximal stimulation, thus permitting direct comparison of the results. Of the strains examined, in no case is stimulation observed at 30°C by shock fluid from a given dna mutant of in vitro DNA synthesis by that mutant. However, in some cases shock fluid from one type of dna mutant does stimulate in vitro DNA synthesis by another type of dna mutant. For example, periplasmic factors from E. coli BT1026 polA1 endA dnaE1026, deficient (24) in DNA polymerase I and DNA endonuclease I and thermosensitive for DNA polymerase III, stimulated the ATP-dependent in vitro DNA synthesis by immobilized E. coli CRT2667 polA1 dnaB266, about three-fold. However, when ATP-dependent in vitro DNA synthesis is assayed at the restrictive temperature of 43°C, no stimulation of immobilized E. coli CRT2667 or E. coli CRT-4636 by periplasmic factors from E. coli BT1026 is observed, whereas stimulation of this in vitro DNA synthesis by immobilized E. coli CRT2667 or E. coli CRT4636 by periplasmic factors from the wild-type E. coli MRE601 is similar at either 30 or 43°C. These observations suggest that (i) the stimulation by the periplasmic factors is specific and physiologically relevant to in vivo DNA replication, and that (ii) the stimulation can possibly be used as an assay for the purification of the product of the dna gene in question. Stimulation by E. coli MRE601 shock fluid is high in each of the mutants tested (Table 2) and represents a potential source for each of these gene products. The shock fluid used in these experiments may contain inhibitory as well as stimulatory factors. Since the observed stimulation is due to the total effect of all such factors, detailed analysis of these effects must await studies with the purified factors.

Effects on in vitro DNA synthesis by added cell extracts. To provide evidence that the stimulating factors are in fact located in the periplasmic space of the surface of the bacterial cell and do not arise from the released cytoplasm of a small percentage of lysed cells, the effects of cell extracts prepared in several ways was examined (Fig. 6). Cell extracts of E. coli MRE601 prepared by procedures which extensively shear the bacterial DNA strongly inhibit the ATP-dependent in vitro DNA synthesis, with perhaps some stimulation at low concentrations of added extract (Fig. 6). Such extracts include the EDTA-lysozyme extract and the sonication extract. Essentially no effect of these extracts on the ATP-independent in vitro DNA synthesis was observed (Fig. 6).

In contrast, cell extracts prepared by gentle procedures, permitting removal of most of the high-molecular-weight bacterial DNA by centrifugation, show stimulating characteristics similar to those of the osmotic shock fluid. These extracts include the freeze-thaw-lysozyme extract (29, 39) and the brij-lysozyme extract (6). In both cases, no effect on the ATP-independent in vitro DNA synthesis was observed, whereas a several-fold stimulation of the ATP-dependent synthesis was seen (Fig. 6).

The degree of stimulation per microgram of protein by these gently prepared extracts was very similar to that observed using osmotic shock fluid (Fig. 6). If the stimulating activity observed in the osmotic shock fluid were due to cytoplasmic factors released from a small percentage of lysed cells, the degree of stimulation per microgram of protein found in the total cell
<table>
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<th>E. coli mutant in agar</th>
<th>[³H]dTMP or [³²P]adenosine 5'-monophosphate incorporation (pmol/10⁶ cells) at:</th>
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<tr>
<td></td>
<td>30 C</td>
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<td>43 C</td>
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<tr>
<td>None*</td>
<td>H3883(polB)</td>
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<td>190-150*</td>
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<td>MRE601(polB DNA⁺)</td>
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* Lysed agar-embedded E. coli EGTA-lysozyme spheroplasts of the indicated strains, preincubated for 1 h, and concentrated osmotic shock fluid from the indicated E. coli strains, were incubated in reaction mixtures with and without ATP for 25 min at 30 C or at 43 C as indicated. The reaction mixtures contained either [³H]dTTT and TdT, or alpha[³²P]dATP and AdR, and an amount of shock fluid containing 100 to 200 µg of protein. Termination of the reaction, determination of acid-insoluble radioactivity, and picomoles of dTMP or dAMP incorporated per 10⁶ cells were as described in Materials and Methods.

* E. coli source of periplasmic factors.

* The pairs of numbers represent in vitro DNA synthesis in the presence and absence of ATP, respectively, and are each the averages of duplicate experiments.
extracts should be considerably greater than that observed for the osmotic shock fluid. For example, if the stimulating activity came from the cytoplasm of 3% lysed cells, then the degree of stimulation per microgram of protein should be approximately three-fold higher for a gently prepared extract than for osmotic shock fluid. Such a stimulation from the gently prepared extract is not observed, thus providing evidence that the stimulating factors are released from the periplasmic space.

On the other hand, if all of the stimulating factors were found in the periplasmic space, the degree of stimulation per microgram of protein for the osmotic shock fluid should be about 20-fold higher than that of a total cell extract, since the osmotic shock fluid contains only about 5% of the total cell protein; this also is not observed. The constancy of the stimulation activity per microgram of protein thus suggests that some stimulating activity, released by the gentle lysis procedures, is not found in the osmotic shock fluid. To further test this possibility, cells which had suffered the osmotic shock procedure were further extracted using the freeze-thaw-lysozyme procedure. This gentle extract obtained from shocked cells exhibited a stimulating activity per microgram of protein nearly identical with the other gentle extracts (Fig. 6). Further, the sum of the protein so released per cell and the protein released by the osmotic shock procedure per cell was within experimental error of being identical to the amount of protein per cell released by freeze-thaw-lysozyme extraction of intact cells. Thus, stimulating factors are released by gentle procedures such as the freeze-thaw-lysozyme procedure which do not appear to be released by the usual osmotic shock procedure.

**DISCUSSION**

Evidence is provided that macromolecular species, or factors, can be obtained from the periplasmic space of *E. coli* cells which preferentially stimulate the ATP-dependent semiconservative mode of in vitro DNA synthesis. This stimulation has been observed in two classes of in vitro DNA synthesis systems: (i) in toluene-treated cells (22), in which the permeability properties of the cells are altered but the cells remain intact; and (ii) in agar-embedded cells which have been converted into spheroplasts using EGTA and lysozyme and then osmotically lysed (33, 34). Thus, the observed stimulation is independent of the in vitro DNA synthesis system used to assay the stimulating activity.

Osmotic shock fluid from *E. coli dnaA* cells contains factors capable of stimulating in vitro DNA synthesis by at least three classes of thermosensitive *E. coli dna* mutants, as well as the ATP-dependent synthesis exhibited by *E. coli polA polB* mutants (Table 2). In contrast, shock fluid obtained from a given *E. coli dna* mutant does not stimulate in vitro DNA synthesis by the same strain. However, shock fluid from *E. coli BT1026 polA1 dnaEl026* stimulates the ATP-dependent in vitro DNA synthesis exhibited by agar-embedded *E. coli CRT4636 polA1 dnaA46* and *E. coli CRT2667 polA1 dnaB266* at 30°C, but not that exhibited at 43°C. Thus, cross complementation of this type between different classes of *dna* mutants occurs in some cases in a thermosensitive reaction. The *dna* mutants exhibit a somewhat greater thermosensitivity for in vitro DNA replication in this system than is observed in vivo, and the results are complicated by the presence of inhibitory activities as well as stimulatory activities in the osmotic fluid.

![Fig. 6. Concentration dependence of stimulation by *E. coli MRE601* cell extracts. Lysed agar-embedded *E. coli* W3110 EGTA-lysozyme spheroplasts preincubated for 1 h and cell extracts, in the amounts indicated and prepared as described in Materials and Methods, from *E. coli MRE601* were incubated in standard reaction mixtures with (open symbols) or without (closed symbols) ATP for 4 min at 37°C. Termination of the reaction and determination of acid-insoluble radioactivity and picomoles of dTMP incorporated per 10^6 cells were as described in Materials and Methods. The results are the average of duplicate experiments. O, osmotic shock fluid; △, freeze-thaw-lysozyme extract; ▽, freeze-thaw-lysozyme extraction of cell pellet resulting from osmotic shock treatment; ◀, brij-lysozyme extract; ◇, EDTA-lysozyme extract; □, sonic extract.](http://jb.asm.org/ on May 18, 2021 by guest)
shock fluid. Nevertheless, the observed thermosensitivity and complementation imply that the observed stimulation is not a nonspecific property of the shock fluid, and is probably physiologically related to the process of DNA replication in vivo.

The shock fluid from E. coli MRE601 rnsA stimulates the ATP-dependent in vitro DNA synthesis by all E. coli dna mutants examined (Table 2), suggesting that the replication factors deficient in these mutants may all be found to some extent in osmotic shock fluid from wild-type E. coli cells. Further, initial studies have shown that the stimulating activity found in the osmotic shock fluid is heat-labile, nondialyzable, and partially RNase sensitive (data not shown). Thus, more than one factor stimulating in vitro DNA replication is probably present in the shock fluid. Since osmotic shock fluid obtained from RNase I-deficient cells exhibits the greatest stimulating activity, and since the activity is partially RNase sensitive, one or more of the factor(s) may be RNA species. Further, the shock fluid may contain inhibitory activity, and the relative content of these different factors in shock fluid obtained from different E. coli strains may vary. Purification of the factors is required for definitive clarification of their respective activities. The functional roles of these factors in the steps found in the process of DNA replication (15, 33; T. Matushita and H. Kubitschek, in press) have not yet been determined.

Evidence has been presented showing that the stimulating activity found in the osmotic shock fluid does not arise from the cytoplasm of a small percentage of lysed cells (Fig. 6), but is in fact found among the periplasmic factors. On the other hand, studies with extracts obtained by gentle lysis procedures indicate that additional stimulating activity, not found among the periplasmic factors, is present in these extracts. The relative content of different replication factors in osmotic shock fluid and in these extracts can best be determined by purification of the factors.

Cell extracts obtained using comparatively severe lysis procedures inhibit rather than stimulate the ATP-dependent in vitro DNA synthesis. Since these extracts contain nearly all intracellular DNA and β-galactosidase activity (data not shown), they probably also contain most of the cellular stimulating activity. The observed inhibition then is probably due to the simultaneous presence of inhibiting activity. When added to the reaction mixture, purified sheared E. coli DNA does not by itself inhibit the ATP-dependent in vitro DNA synthesis (data not shown). Cell extracts prepared by gentle procedures contain only 50 to 80% of the total cell protein, 30 to 70% of the total β-galactosidase activity, and less than 5% of the total cellular DNA found in cell extracts prepared by more severe procedures (data not shown). Thus, it is possible that the gently prepared extracts do not contain much of the putative inhibitory activities found in the more severely prepared extracts. The gently prepared extracts, however, do appear to contain more inhibitory factors than are present in the osmotic shock fluid, since kinetic experiments similar to those of Fig. 3 show a comparatively rapid decrease in the initial rate of the synthetic reaction when gently prepared extracts are included in the reaction mixture. Whether the variation in relative amounts of stimulating and inhibitory factors found in these different preparations is due to an intracellular compartmentalization of some form is not known, although it is likely that the gently prepared lysates would contain a greater fraction of intracellular soluble protein than protein found localized in the cell surface.

Indirect evidence accumulated over the past decade suggests that at least the origin and growing fork of a semiconservatively replicating DNA molecule are noncovalently attached to a large intracellular structure, possibly a site at the cell surface, in bacteria and in phage-infected bacteria (31-33). Recently, the development of methods for separation of the inner and outer membranes of the E. coli cell surface (26) has permitted the demonstration that phage M13 parental replicative form DNA is attached to the inner membrane (13), whereas in contrast, phage φX174 parental replicative form DNA is associated with the outer membrane (16). Also, the E. coli nucleoid, or folded chromosome, when isolated at 4 C, appears to be associated with a membrane fragment characterized by outer membrane markers (40). Further, treatment of toluenized cells with 1% Triton X-100, a mild detergent which preferentially alters the inner membrane of the E. coli cell surface (30), increases the permeability of these cells to large molecules, yet without disruption of the in vitro DNA synthetic capability of these cells (21). These observations suggest that, at most, only part of the inner membrane is involved in replication of the bacterial chromosome and that the outer membrane may, in addition or alone, be involved in this process. Such ideas provided the rationale for examination of the periplasmic factors for DNA replication stimulating factors, and it will be of interest to delineate the precise location and function in
DNA replication of these factors.

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LITERATURE CITED