Synthesis of Phage 2C-DNA in Permeabilized B. subtilis

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Summary. Bacillus subtilis cells, infected with bacteriophage 2C and then permeabilized (plasmolysis, protoplast conversion, or treatment with organic solvents) incorporate dATP into DNA through a polymerization reaction requiring the 4 deoxyribonucleosidetriphosphates dATP, dGTP, dCTP and dTTP. While uracil is an in vivo precursor of phage 2C DNA (in which hydroxymethyluracil completely replaces thymine), neither uracil, nor dUTP, nor dUMP are incorporated into viral DNA by phage-infected permeabilized cells. Although the amount of dTTP incorporated under these conditions is small, this compound greatly enhances the incorporation of dATP into viral DNA.

Synthesis of 2C-DNA in permeabilized cells is discontinuous; however, the Okazaki fragments (2 × 10^6 daltons) which accumulate under these conditions, show no tendency to join and to form full strands, as they do in intact host cells. Finally, density shift experiments suggest that, in addition to repair synthesis, semiconservative duplication takes place within the permeabilized cells.

When phage-infected bacteria are permeabilized at different moments of the viral cycle, labeled precursors are mainly incorporated into cell DNA during the eclipse phase, and into viral DNA during the maturation phase. Moreover, viral DNA formation is prevented when cells are infected with virions previously irradiated with ultraviolet light.

Since most metabolic pathways and gene regulation patterns are not altered by the permeabilization process, allowing the use of direct DNA precursors, the systems of virus-infected permeabilized cells prove exceptional tools for a study of virus-host relationship.

Introduction

Although no evidence has yet been gathered for the synthesis of complete cell chromosomes in vitro, elongation and possibly initiation of DNA has been observed in bacteria which had been rendered permeable to deoxyribonucleosidetriphosphates by different treatments (Ben-Hamida and Gros, 1971; Matsushita et al., 1971; Mordoh et al., 1970; Moses and Richardson, 1970; Schaller et al., 1972).

Chromosomes isolated from 2C virions are double-stranded DNA molecules of about 10^8 daltons, in which thymine (T) is replaced by hydroxymethyluracil (HMU) (Pène and Marmur, 1964; Kallen et al., 1962). The 2 strands of viral DNA have different buoyant densities, and can be separated by ultracentrifugation in CsCl gradients (May et al., 1968; Truffaut, 1970; Hoet et al., 1975). We have previously shown that 2C-DNA replicates in a discontinuous fashion in vivo (Fraselle et al., 1976), and that after semiconservative duplication viral chromosome undergoes extensive genetic recombination (Hoet et al., 1976). The segments of DNA which are made during discontinuous replication (Okazaki fragments), and those which are exchanged during recombination, have comparable sizes (2 × 10^6 daltons) (Hoet et al., unpublished).

In the present work, the synthesis of phage 2C DNA in permeabilized cells of B. subtilis has been explored. For this purpose, three different systems of virus-infected permeabilized cells have been used, and the incorporation of labeled precursors into viral DNA has been followed. The biophysical analysis of the DNA synthesized under these conditions, has allowed a study of duplication and template function of viral DNA.

Materials and Methods

Bacteriophage Multiplication. Virus 2C was multiplied in the 168/2 strain of B. subtilis (leu- tryp-). Techniques for multiplication and titration of phage 2C (Cocito, 1969), and labeling and purification of virions (Cocito, 1974), have been previously described.

Permeabilization Techniques. Uninfected and phage-infected exponentially growing B. subtilis cells were submitted to one of the
the composition of the reaction mixture was as follows: 22.9 mM MgCl₂, 1.7 mM dithiothreitol, 0.06 mM of each of the 4 deoxyribonucleoside triphosphates dGTP, dCTP, dATP and dTTP, 2.3 mM ATP, and 143 mM K₂HPO₄/KH₂PO₄ buffer pH 7.4. To label DNA with (³H)-dATP (4 ~tCi, 22 Ci/mmoles), cold dATP was omitted from the reaction mixture. When labeling DNA with other radioactive precursors, these were added to the complete reaction mixture. The reaction was started by adding a permeabilized cell suspension (175 µl reaction mixture + 10⁸ cells in 100 µl NS buffer). Samples were taken after 0, 10 and 30 min incubation at 37°C, and reaction terminated with 0.3 N KOH. Bovine serum albumin (40 µg) and cold trichloroacetic acid (0.5 M) were added, and precipitates were collected on glass-fiber filters (Schleicher and Schüll, Dassel, West Germany), which were dried and counted in a liquid scintillation spectrometer. Values at time zero were subtracted from those obtained at 10 min (net cpm).

### Synthesis of DNA in Permeabilized Cells

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### Centrifugal Analysis of in vitro Synthesised DNA

Suspensions of labeled cells in SSC buffer were incubated with lysozyme (100 µg/ml, 10 min at 37°C), and then treated with SDS (0.25% w/v). After extractions with water-saturated phenol and with ether, DNA solutions were dialysed against SSC, CsCl was added (ρ at 23°C = 1.75 g/cm³), and samples were centrifuged either in angular (R40 et R65) or in swing-out (SW 50.1) Spinco rotors at 19°C and 33,000 rpm for 65 h (Hoet et al., 1975). Fractions were collected on 3 MM filters (Whatman, Springfield Mill, England), treated with 0.3 N KOH, washed with 0.5 M trichloroacetic acid, dried and counted in a liquid scintillation spectrometer, using toluence containing 4 mg/ml of Omnifluor as scintillation fluid. Velocity centrifugations on alkaline sucrose gradients were described (Fraselle et al., 1976).

## Results

### 1. DNA Synthesis in Permeabilized Virus-Infected Bacteria

Exponentially growing bacteria were infected with phage 2C and submitted 25 min later to one of the 3 permeabilization techniques: plasmolysis, incubation with organic solvents, and protoplast conversion. Permeabilized cells were transferred to a reaction mixture containing 3 unlabeled deoxyribonucleoside triphosphates (dCTP, dGTP and dTTP) and (³H)-dATP, and the radioactivity incorporated into an acid-insoluble alcali-stable product was measured. As shown in Table 1, DNA synthesis occurred in all 3 systems of permeabilized host cells, and the reaction was sensitive to both DNAase and EDTA: note that viral DNA was the main reaction product under these conditions (v.i.). The polymerization reaction was linear for about 10 min, and the reaction product was proportional to the number of permeabilized cells within the range 1 × 10⁷–2 × 10⁸ cells/ml (not shown). The initial rate of (³H)-dATP incorporation into the DNA of permeabilized host cells was about 2 × 10² molecules/cell/min, i.e. approximately 1/6 of the incorporation rate of (³H)-uracil in virus infected non-permeabilized cells.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Proteoplas b</th>
<th>Toluenized cells b</th>
<th>Plasmonized cells b</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete</td>
<td>9,745</td>
<td>3,140</td>
<td>14,034</td>
</tr>
<tr>
<td>−ATP</td>
<td>9,995</td>
<td>3,883</td>
<td>10,726</td>
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<tr>
<td>−dTTP</td>
<td>6,859</td>
<td>503</td>
<td>−</td>
</tr>
<tr>
<td>+EDTA</td>
<td>511</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>−DNAase</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Composition of the reaction mixture as in Materials and Methods. Concentration of EDTA was 20 mM, and that of DNAase 100 µg/ml

b Permeabilization performed 25 min after infection (MOI = 15). Each sample contained 10⁸ infected cells.

### 2. Incorporation of Uracil and Thymine Derivatives into Viral DNA

Since uracil is precursor of 2C-DNA in vivo (Cocito, 1969, 1974), this base and its nucleoside mono- or triphosphates were tested as possible precursors under our experimental conditions. As shown in Table 2, however, neither uracil nor uracil derivatives were incorporated into DNA in virus-infected permeabilized cells. On the other hand, the strict requirement for dTTP, as shown in Table 1 was unexpected, since 2C-DNA does not contain thymine (Kallen et al., 1962; Truffaut, 1970; Roscoe and Tucker, 1964). It was thought, therefore, that dTTP might act catalytically (rather than as a direct precursor of viral DNA) in permeabilized host cells. To test this possibility, 2 experiments were performed. In the first experiment, labeled dTTP was incorporated into permeabilized