

DNA Synthesis *in vitro*

by

DOUGLAS W. SMITH*

HEINZ E. SCHALLER

FRIEDRICH J. BONHOEFFER

Max-Planck-Institut für Virusforschung und
Friedrich-Miescher-Laboratorium der Max-
Planck-Gesellschaft, Tübingen

DNA synthesis *in vitro*, using the classic purified DNA polymerase system of Kornberg¹, differs from replication *in vivo* in several respects. The two most important are that the *in vivo* rate is two orders of magnitude higher than the *in vitro* rate and that no net semiconservative synthesis is achieved *in vitro* with double stranded DNA as template². Furthermore, it now seems likely that DNA replication is mediated by a number of different proteins which may be assembled in a replication complex at the cell membrane. The search for such a complex in its functional state *in vitro*³⁻⁶ is, however, usually complicated by the presence of DNA polymerase, which effectively uses damaged DNA as a substrate for repair synthesis. In this and in the following article⁷ we describe two new systems for DNA synthesis *in vitro* which reduce the activity of this unwanted background reaction.

Table 1. CHARACTERISTICS OF THE INCORPORATION SYSTEM

System	dCMP incorporated into acid-insoluble nucleotides during 1 min at 37° C (pmoles/10 ⁸ cells)
Complete	14.5
Minus dTTP	0.4
Minus dATP	0.5
Minus dTTP, dATP	0.3
Minus dTTP, plus dUTP	14.1
Minus rATP	6.2
Precipitation without 3 dNTPs for 3 min, at 37° C	11.0
Plus pancreatic deoxyribonuclease (50 µg), venom phosphodiesterase (50 units)	
3 min before and during incorporation	0.7
After incorporation	1.2
Minus 0.06 M KCl, plus 0.032 M KPO ₄ (pH 7.5)	10.1
Minus 0.06 M KCl, plus 0.018 M KPO ₄ (pH 7.5)	1.2

E. coli W 3110 thy⁻ pol A₁ cells, prelabelled with ¹⁴C-thymine (about 300 c.p.m./10⁸ cells) were embedded in 2 per cent agar (2–6 × 10⁸ cells/ml.). The agar was fragmented; fragments were washed and incubated with aeration at 37° C in a spheroplast forming medium (66 ml/g agar) which consists of 'Penassay' broth containing penicillin (500 units/ml.), 0.5 M sucrose, and 0.01 M MgCl₂. After 2 h the agar was collected by filtration and washed five times with 10 ml. of ice cold lysis buffer (0.05 M Tris-HCl, pH 7.5, 0.005 M MgCl₂, 10⁻⁴ M tRNA, 5 per cent 'Brij 58'). Agar samples of about 0.05 g were assayed for nucleotide incorporation in 0.2 ml. of a reaction mixture containing 0.05 M Tris, pH 7.5, 0.005 M MgCl₂, 0.08 M KCl, 10⁻⁴ M dithiothreitol, 10⁻² M ATP, 2 × 10⁻⁵ M dTTP, dATP, dGTP, 2 × 10⁻⁵ M ³H- or ³²P-labelled dCTP (10² c.p.m./pmole), and 10⁻⁴ M deoxycytidine. After 1 min at 37° C the reaction was terminated by addition of 0.1 M EDTA (0.2 ml.), 1 M NaOH (0.5 ml.) was added and the agar was dissolved by heating to 100° C for 5 min. The acid-precipitable radioactivity was determined. The results were corrected for blank values (EDTA added at 0 min) which ranged from 0.5 to 1 per cent of the incorporation of the complete system. ¹⁴C-thymine prelabel was used to normalize the incorporation values with respect to the number of cells (except for the nuclease treated samples).

We have tried to develop an *in vitro* system from *Escherichia coli* which maintains the structural integrity of DNA and membrane as far as possible, and which allows removal of the bulk of soluble components, including DNA polymerase. *E. coli* cells are embedded in a matrix of agar, the agar is fragmented, the cells are transformed into spheroplasts within the agar fragments and lysed carefully by osmotic shock. After extensive washing the agar still contains DNA and membranes. On incubation with deoxyribonucleoside triphosphates, DNA is synthesized

In a new *in vitro* system, DNA is synthesized semiconservatively at rates of chain growth comparable with replication *in vivo*. This DNA synthesis is also observed with a strain of *E. coli*, which lacks DNA polymerase activity *in vitro*.

within the agar fragments in a semiconservative manner. This enzymatic activity differs distinctly from that of DNA polymerase in several respects, and particularly in its rate of chain elongation. The *E. coli* mutant described by de Lucia and Cairns, W3110 thy⁻ pol A₁, which lacks DNA polymerase activity *in vitro*⁸, was extremely useful in further studies of this system. Only the repair replication was reduced, whereas the other activity remained unaltered. This supports the conclusion that our system characterizes a new DNA-synthesizing activity.

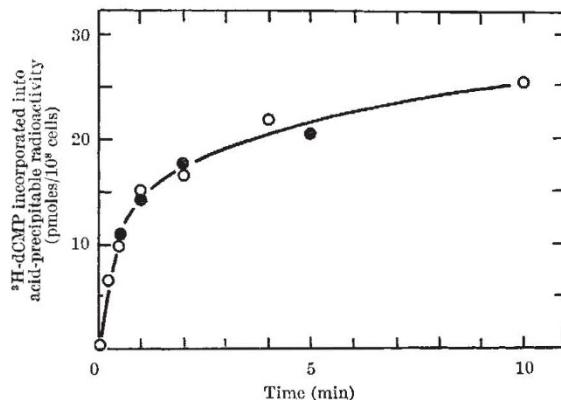
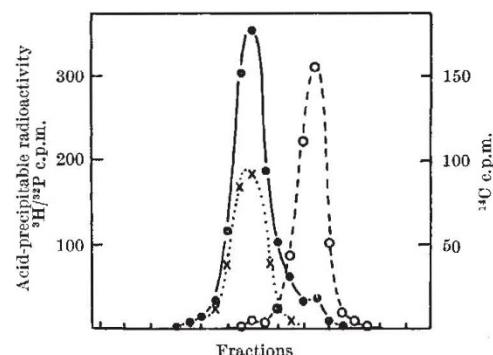


Fig. 1. Kinetics of DNA synthesis. ●, DNA synthesis in the complete system; ○, in the complete system with dUTP instead of dTTP.

Fig. 2. Density analysis of bromouracil-labelled DNA. DNA was synthesized in 0.5 g agar incubated in 0.75 ml. of the complete reaction mixture with dUTP replacing dTTP. After 60 s at 37° C, 0.5 ml. of a frozen solution of 0.06 M EDTA and 6 per cent 'Sarkosyl' was added and the agar was dissolved by addition of KI (1.2 g) and heating to 42° C for 15 min. The solution was diluted to 4 ml. and briefly sonicated. KI was removed by dialysis against 0.05 M Tris (pH 7.5)-10⁻⁴ M EDTA. The precipitated agar was removed by centrifugation. 2.8 g of CsCl was added to 2 ml. of the supernatant. The solution was centrifuged for 48 h at 37,000 r.p.m. in the Spinco 'SW 50' rotor. Acid-precipitable radioactivity was determined in fractions after a 4 min incubation at 100° C with 1 M NaOH (0.5 ml.). ○---○, ¹⁴C prelabelled DNA; ●—●, ³H DNA synthesized *in vitro*; ×···×, ³²P hybrid (bromouracil/thymine) DNA reference.

* Present address: Department of Biology, University of California, San Diego.

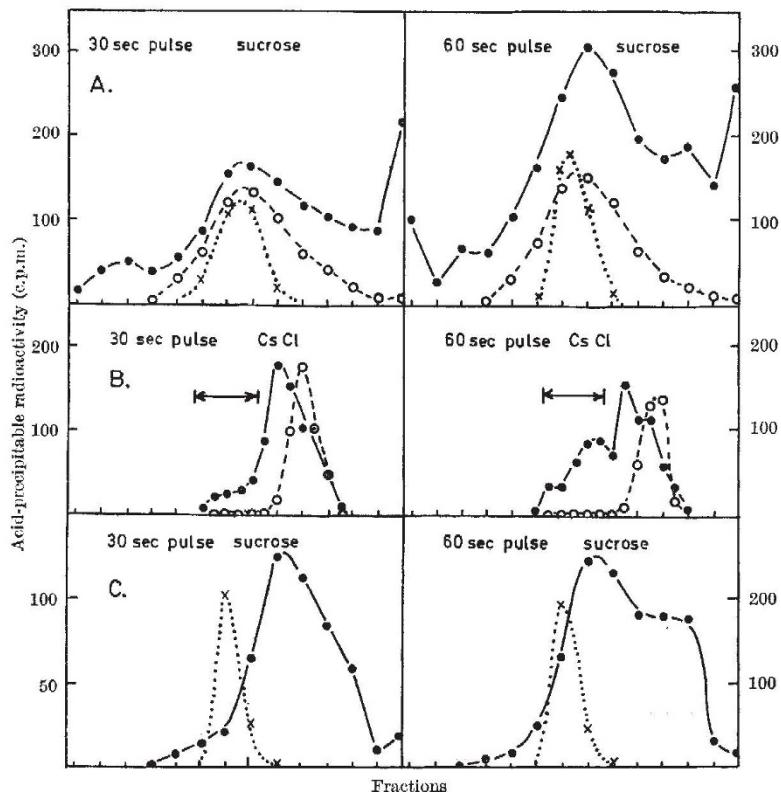


Fig. 3. Size determination. DNA was synthesized in the presence of dBUTP for 30 and 60 s. Except that sonication was replaced by mild shear the procedure was essentially as described in the legend of Fig. 2. A, Sedimentation analysis of a fraction of the sheared DNA. A sucrose gradient of 20 to 5 per cent sucrose in 0.01 M Tris and 2 M KI was layered on top of 2 ml. saturated CsCl. All solutions contained low molecular weight DNA (100 µg/ml.) and 'Sarkosyl' (0.1 per cent). Sedimentation was carried out in the Spinco 'SW 41' rotor for 13 h at 20° C and 30,000 r.p.m. The direction of sedimentation is from right to left. B, CsCl density gradient. The sheared DNA was separated from the agar by centrifugation into a cushion of CsCl. The CsCl concentration of the DNA containing fraction was adjusted to 57 per cent. After centrifugation in the 'SW 39' rotor at 30,000 r.p.m., 20° C for 48 h, fractions were collected and acid-insoluble radioactivity was determined in a sample of each fraction. The fractions containing hybrid DNA were pooled as indicated by the arrows. C, Sedimentation analysis of hybrid DNA. The hybrid DNA obtained from the above density gradient was dialysed against 10⁻² M Tris (pH 7.5) 10⁻⁴ M EDTA. Sucrose gradients were prepared as described for A except that 0.5 M NaCl was used instead of 2 M KI. Centrifugation was carried out in the 'SW 41' rotor for 5 h at 20° C and 36,000 r.p.m. ○—○, ¹⁴C-prelabelled DNA; ●—●, ³H-DNA synthesized *in vitro*; ×—×, ³²P-labelled fd DNA reference. Its sedimentation coefficient is $s_{20,w} = 31S$, determined in our conditions in an analytical ultracentrifuge.

Synthesis of DNA

Experimental details of the system are described in the legend to Table 1. The system shows properties expected for template dependent DNA synthesis *in vitro* (Table 1). Synthesis requires all four deoxyribonucleoside triphosphates and the product is sensitive to degradation by deoxyribonuclease. α -³²P-dCTP is incorporated just as well as ³H-dCTP. Pyrophosphate ions inhibit the incorporation while phosphate ions have little influence. This suggests that deoxyribonucleoside triphosphates are the precursors for the DNA synthesized. The system is accessible to deoxyribonuclease during incubation. This finding, and the incorporation of α -³²P-labelled nucleotides, indicate that the incorporation takes place *in vitro* rather than in any non-disrupted cells.

Mg^{2+} is essential for the activity of the system. Excess EDTA inhibits the system reversibly, whereas the same amount of ethylene glycol-bis-(2-aminoethyl ether)N,N'-tetraacetic acid (EGTA) has no influence. The dependence of the described enzymatic activity on pH, ionic strength, Mg^{2+} concentration, temperature and especially on ATP is quite different from that of the activity of purified DNA polymerase. These differences do not prove, however, that the synthesizing enzyme is different from DNA

polymerase. They might rather reflect properties of other enzymes present in the crude system and which are indirectly involved. The optimal conditions for the system are given in the legend to Table 1 and closely resemble intracellular conditions.

When repair replication by DNA polymerase is avoided by using the polymerase-negative mutant, the agar procedure is unnecessary, for DNA synthesis is observed even in ordinary lysates of spheroplasts. Shear by vortex-mixing has only little influence, whereas extensive breakage by sonication reduced this synthesis by at least a factor of ten.

DNA synthesis levels off after 1 to 2 min at 37° C (Fig. 1). The reason for this is not yet known. It seems unlikely that the short lifetime of the system is the result of exhaustion of precursors or heat inactivation. Repeated addition of 5'-deoxyribonucleoside triphosphates during incubation and preincubation of the system at 37° C without deoxyribonucleoside triphosphates has little influence on the final amount of DNA synthesized. The initial rate of synthesis calculated from incorporation after 30 s corresponds to the incorporation of 5×10^6 nucleotides per min per spheroplast.

Characteristics of DNA Synthesis

To demonstrate semi-conservative synthesis, bromodeoxyuridine triphosphate (dBUTP) was used as a density label: dTTP can be replaced by dBUTP without a reduction in DNA synthesis (Table 1 and Fig. 1). This is to be expected, for *in vivo* dBUTP and dTTP seem to be equally suitable for DNA polymerization (unpublished results of H. E. S. and Voss). The DNA synthesized *in vitro* in the presence of dBUTP instead of dTTP was broken by sonication and analysed in CsCl density gradients. The newly synthesized material had the hybrid density expected of semiconservatively synthesized DNA, which consists of one old light strand and one heavy strand containing the

³H label (Fig. 2). On denaturation, these strands separate in a CsCl gradient and band at positions of heavy and light single strands. As Dr Herrmann has shown, the amount of the parental DNA which is transferred to hybrid density is about 0.5 per cent.

The rate of chain elongation can be calculated from the size of the pieces of newly synthesized DNA and the time required for their synthesis. To determine the size, DNA was synthesized in the presence of dBUTP for 30 or 60 s. The agar was dissolved in KI and the solution sheared. Sedimentation analysis of the sheared DNA showed a broad distribution of molecular weights (Fig. 3A). The average molecular weight is about 3×10^7 daltons as calculated from the sedimentation coefficient of 30S (ref. 9). To determine the size of the newly synthesized hybrid molecules the DNA was first removed from the agarose by a preparative sucrose gradient centrifugation and collected on a CsCl cushion. It was then subjected to CsCl density gradient centrifugation. The density distribution is shown in Fig. 3B. That fraction of the newly synthesized DNA which banded at the position of hybrid DNA was analysed for size distribution in a sucrose gradient (Fig. 3C). The largest molecules which had banded at hybrid density sedimented at about 30S, which corresponds to a

molecular weight of 3×10^7 daltons. When the sheared DNA was denatured and the heavy single strands were isolated from a CsCl density gradient, the largest molecules found had the same sedimentation rate as denatured λ DNA (which has a molecular weight of 15×10^6 daltons). From these data the rate of chain elongation is calculated to be 1.5×10^3 nucleotides per second—very close to the rate of DNA replication *in vivo*¹⁰.

The size distribution of the hybrid DNA (Fig. 3C) does not permit an exact calculation of the average size of the newly synthesized piece of DNA, mainly because the selection of hybrid DNA is a preferential selection of small molecules. As expected, the average sedimentation velocity of ^3H -labelled DNA which bands at light density was greater than that of hybrid DNA.

The rate of total incorporation (5×10^5 nucleotides/min/spheroplast) is about five times higher than the estimated rate of chain elongation (10^3 nucleotides/min). If all the DNA were synthesized at a rate of 10^3 nucleotides/min, the rate of total synthesis could be explained by synthesis at two to three growth points. This number is not unreasonable. There may well be more growth points per spheroplast, but, on the other hand, not all of them might be active in the *in vitro* system.

Relationship with Replication *in vivo*

Our *in vitro* system can synthesize DNA at rates comparable with replication *in vivo*. The total amount synthesized *in vitro* within 1 min is of the same order of magnitude as the amount of DNA synthesized *in vivo* by replication within the same time. Although DNA synthesis continues for only about 1 min, the short lifetime of the system seems less disturbing because the size of the DNA which is copied within this time is very large and corresponds to 10^5 base pairs. It has not yet been shown that both DNA strands are synthesized in this system or that DNA synthesis really occurs on double stranded DNA. We believe, however, that we are observ-

ing a limited continuation of real replication at the original growth point.

This system is still very crude. Preliminary experiments indicate that it may be difficult to purify the synthesizing apparatus, free it from its original DNA and use it for synthesis on external template DNA. As demonstrated by Knippers and Sträling⁷, a possible first step in purification is the isolation of membrane fractions which display a similar DNA synthesizing activity.

Because DNA synthesis is also observed in sheared bacterial lysates neither integrity of the bacterial chromosome nor an active swivel mechanism of the kind postulated for replication¹¹ can be necessary for this kind of DNA synthesis. That DNA synthesis is observed *in vitro* when a DNA polymerase-negative mutant is used does not exclude the possibility that the synthesizing enzyme is DNA polymerase associated with other components in a special complex. The more likely interpretation, however, is that our system makes use of another distinct replicating activity, responsible for replication *in vivo*.

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The DNA Replicating Capacity of Isolated *E. coli* Cell Wall-Membrane Complexes

by

ROLF KNIPPERS

WOLF STRÄLING

Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft,
Tübingen

The only DNA synthesizing activity left in a bacterial mutant lacking DNA polymerase is associated with the cell membrane.

THE DNA polymerase described by Kornberg^{1,2} has afforded many clues as to the nature of DNA polymerization, but it is not certain whether it constitutes part of the cellular machinery for the replication of genetic material *in vivo*. Moreover, many of its properties³ can be explained in terms of a DNA repair enzyme. Attempts to characterize the process of DNA replication in a cell free system by measuring the incorporation of radioactive deoxyribonucleoside triphosphates into acid-precipitable DNA^{4,5} have been obscured by the high activity of the DNA polymerase. Some clarification of these difficulties has been obtained, however, by the use of the mutant of *Escherichia coli* described by de Lucia and Cairns, which

possesses almost no DNA polymerase activity but nevertheless grows like wild type strains in the usual conditions⁶.

We have used the DNA of bacteriophage ϕX 174 to determine the DNA replicating potential of cell extracts and of cell-wall membrane complexes prepared from a derivative of the DNA polymerase mutant. The various intracellular forms of ϕX DNA are small enough (mol. wt. 3.4×10^6) to be handled without much danger of breakage. *E. coli* DNA (mol. wt. about 3×10^9), on the other hand, is broken into many pieces during conventional extraction procedures.

Early in the reproduction cycle of phage ϕX 174, the parental circular double stranded DNA molecule (replica-