STUDIES ON THE IN VITRO SYNTHESIS OF TRANSFORMING DNA*

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DNA replication in bacteria is semiconservative[1] and proceeds with simultaneous replication of both strands in parallel,[2] starting from one end. Recently, Goulian *et al.*,[3] using highly purified *E. coli* polymerase and with single-stranded ϕX174 phage DNA as template, synthesized an infectious DNA product free of template. However, with double-stranded transforming DNA, this enzyme generates a branched, biologically inactive product.[4] The problem seems to be interference by the 5′-ended strand, which is not replicated *in vitro*.[5]

A less purified DNA-synthesizing system might contain additional factors permitting synthesis of a biologically active product. This approach is particularly attractive, since *B. subtilis* membrane fractions are rich in DNA polymerase activity. Moreover, nascent DNA appears to be attached to the membrane[6] as postulated by Jacob *et al.*[7]

In order to characterize the crude but highly active polymerase preparation from "membranes,"[8] it is important to study the assay conditions as well as the synthesized product. The current report describes some initial observations on a partially purified polymerase fraction from *B. subtilis* and the product it synthesizes from genetically marked transforming DNA.

Materials and Methods.—Transformable strains of *B. subtilis* and derivatives were used as a source of enzyme and template DNA. The genotypes are described in the figure legends. Unlabeled and labeled deoxynucleosides and deoxynucleoside triphosphates were purchased from Schwarz BioResearch. Polymerase was assayed according to Richardson *et al.*[9] Heavy $^{15}N^{2}H$ DNA labeled with ^{14}C- or ^{3}H-thymidine was isolated from bacteria grown as described earlier.[10] The average molecular weight of the DNA samples was 25–40 $\times$ 10^6 daltons.

Density gradient fractionation of the product: After polymerase reaction (see figure legends), NaCl was added to the assay mixture to a concentration of 0.15 *M*. The mixture was deproteinized[10] and dialyzed until more than 95% of the radioactivity in the nucleotides was removed. It was then centrifuged to equilibrium in CsCl with a standard DNA.[10]

The collected fractions were immediately checked for refractive index, acid-precipitable radioactivity of template and product, and biological activity.

Purified *E. coli* polymerase and T4-induced polynucleotide ligase were gifts of Dr. N. Cozzarelli; *E. coli* joining enzyme was kindly provided by Drs. B. Olivera and I. R. Lehman of the Biochemistry Department. The assays were performed as described previously.[11–13]

Partial purification of polymerase complex: Twenty grams of early log phase cells were converted into protoplasts in a 12% sucrose solution with lysozyme. All the purification steps were performed at 0–4°. The protoplasts were centrifuged at 7000 *g* for 25 min, and the pellet was resuspended in 80 ml of 0.05 *M* glycyl-glycine buffer,[9] pH 7.0, and sonicated for 30 sec to reduce the viscosity. The resulting lysate was centrifuged as above and the pellet containing whole cells, if any, was discarded. The specific activity[9] of the polymerase in the lysate was 0.8–1.2 units per mg of protein, with dAT-copolymer as template. The lysate was diluted with 100 ml of 0.01 *M* tris(hydroxymethyl)amino-

methane-HCl (Tris-HCl) buffer, pH 7.4, and precipitated with streptomycin sulfate.[9] The precipitate contained 80% of the activity and was dissolved in 0.05 *M* phosphate buffer, pH 7.34. This fraction (50 ml) was diluted with 230 ml of 0.05 *M* Tris-HCl buffer, pH 7.4. Ammonium sulfate (105 gm) was added gradually, and after 2 hr at 0°, the supernatant fluid was centrifuged at 20,000 *g* for 20 min. The resulting supernatant fraction had a specific activity of 80–110 units per mg of protein with dAT-copolymer as template, and 48 units with native DNA as template. The supernatant was precipitated with a saturating concentration of ammonium sulfate at 4° for 12 hr. The concentrated fraction of 15 ml was dialyzed against 0.01 *M* Tris-HCl buffer, pH 7.5, 0.001 *M* $MgCl_2$, and 0.001 *M* β-mercaptoethanol, and stored at 0°. During this procedure, 96% of the DNA was removed. The protein fraction was subjected to zone sedimentation at 36,000 rpm for 12 hr at 4°C in 5–25% sucrose gradients (containing 0.01 *M* Tris-HCl buffer, pH 7.5, 0.001 *M* $MgCl_2$, and 0.001 *M* β-mercaptoethanol), which removed all the residual DNA and eliminated 70% of the protein without activity. The active fractions had a specific activity of 98 units per mg of protein with native DNA as template, and 200 units with dAT-copolymer. The protein, active in DNA synthesis, obtained from gradients and used in the following studies was designated as the polymerase complex. The analysis of these fractions showed no transforming activity for at least three different genes.

Transformation assays: The biological assays for single and linked genes were performed with limiting amounts of DNA.[14]

Results.—Properties of the polymerase complex: The polymerase complex, when incubated with saturating levels of deoxynucleoside triphosphates and with native *B. subtilis* DNA as a template, promoted a progressive synthesis with time, approaching 80 per cent of the template equivalents in two hours (Fig. 1). Additional incubation did not lead to further synthesis. During the first 90 minutes, only 7 to 10 per cent of the template was rendered acid-soluble. The amount of product synthesized was proportional to the amount of enzyme complex added, up to 15 μg of protein per assay. Mg^{++} and pH requirements were identical to those of purified *B. subtilis* polymerase.[15] Denatured DNA was six- to eightfold poorer as a template for the reaction. The complex possesses endonuclease activity, as shown by the loss of linkage between genes.[16] During the one-hour incubation, the single marker frequency dropped to 60 per cent of the initial amount and remained at this level, while the ability to transform all four markers

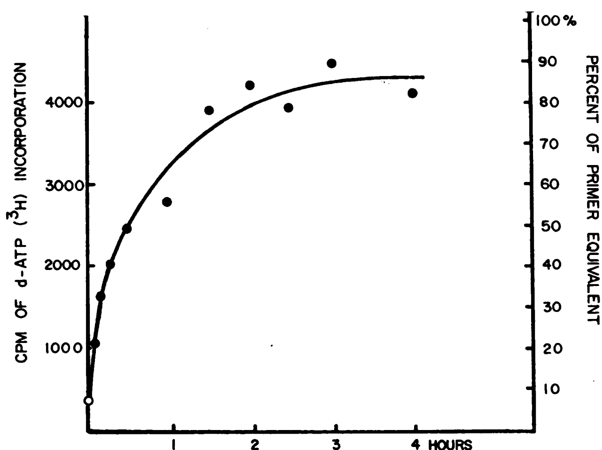

FIG. 1.—DNA synthesis as a function of time: 22 mμmoles of *B. subtilis* wild-type DNA was incubated in a volume of 1.1 ml with polymerase complex (100 μg of protein), 50 mμmoles of each deoxynucleoside triphosphate, and other required components[9] for a period of 1.5 hr. The specific activity of dATP ^{3}H was 8.5 × 10^3 cpm/mμmole. At specified time points, 100 μl of reaction mixture was withdrawn and assayed for acid-precipitable counts. Each aliquot contained 2.2 mμmoles of template and the ^{3}H-labeled product.

declined, rapidly reaching a minimum of about 10 per cent. In all the experiments, the incubation of the template DNA with the enzyme and no deoxynucleoside triphosphates led to loss of all the biological activity in five to eight minutes. This complex also had some contaminating ligase activity (Mr. P. Laipis, personal communication).

Synthesis of DNA with $^{15}N^{2}H^{3}H$ (thymidine)-labeled DNA: A DNA product

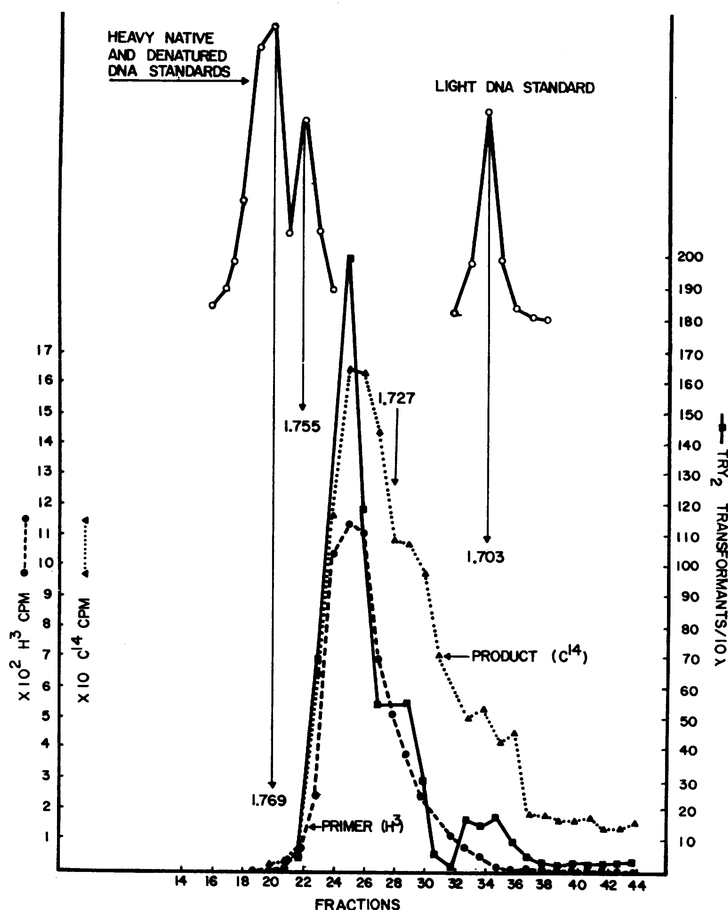

FIG. 2.—Synthesis of DNA with heavy template: $^{15}N^{2}H$- and ^{3}H-thymidine-labeled DNA (ρ = 1.755), prepared from cells of SB 532 (*aro-2, tyr-1*), 20 mμmoles of DNA with a specific activity of 6 × 10^{5} cpm/mμmole, was incubated with 50 mμmoles of each of the four deoxynucleoside triphosphates for 1.5 hr. Deoxycytidine and thymidine triphosphates were labeled with ^{14}C (dCTP = 10^{4} cpm/mμmole and dTTP = 5 × 10^{3} cpm/mμmole). The ^{14}C counts in the product were purposely kept low so that no serious spillage into the ^{3}H channel would occur during counting; 56 fractions from a 5.2-ml gradient were collected into tubes previously coated with 1 mg/ml of bovine serum albumin;[24] 10-μl samples were assayed for transformation of the *try-2*$^{+}$ activity, and 10 μl were acid-precipitated for radioactive counts with 100 μg of bovine serum albumin as a carrier. The standards were heavy native, heavy denatured, and normal light DNA's which were *try-2* and *tyr-1*$^{+}$ (SB 168). The proportion of denatured to native heavy DNA was 10:1. The biological activity of *tyr-1* was assayed in a medium with no phosphate so that the denatured DNA also transforms, but poorly.[24]

prepared with heavy template and light triphosphates was purified and run in a CsCl gradient with three DNA standards, native light (1.703), native heavy (1.755), and denatured heavy (1.769) (Fig. 2). Most of the heavy template molecules have reached the hybrid density stratum (1.730). The product counts (^{14}C) in the region between light and hybrid strata did not have the activity expected for the amount of DNA present, possibly because they represented the fragile replicating points.[17] In addition to the hybrid molecules, completely light molecules having 4 per cent of the total biological activity were found at the stratum of native light DNA. Completely light molecules could result from a second replication of a few semiconservatively replicated templates.

Since linkage experiments indicated the presence of endonuclease activity, it seemed likely that some of the breaks might be repaired by polynucleotide ligase. Figure 3 shows the result of one such experiment with a heavy template DNA labeled with ^{14}C-thymidine. The products were labeled with ^{3}H-dATP. The figure shows the ratio of biological activity to the template counts (^{14}C) of each fraction prior to ligase treatment. The light DNA region is devoid of any template counts but possesses significant amounts of biological activity. An aliquot of the same reaction mixture was incubated with ligase or joining enzyme. The hybrid and the light products underwent the same amount of increase in specific transforming activity after synthesis, suggesting that both strands are equally liable to breaks caused by the nuclease during replication. Addition of ligase during replication resulted in fourfold reduction in the extent of DNA synthesis. This observation suggested that DNA synthesis might be accompanied *in vivo* by endonucleolytic cleavage of the template molecules.

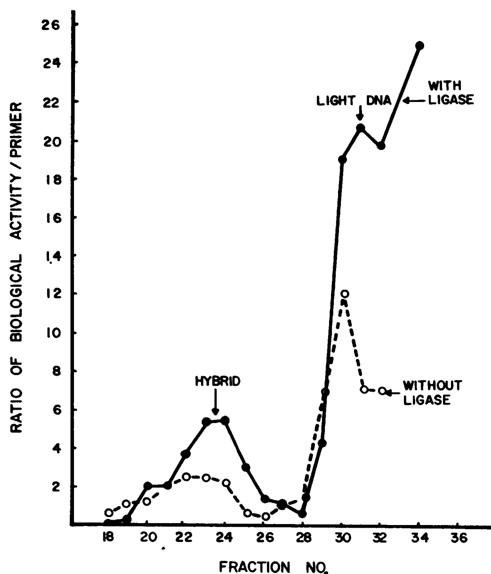

FIG. 3.—Effect of ligase on the reaction products: A reaction volume containing 100 mμmoles of $^{15}N^{2}H$- and ^{14}C-thymidine-labeled DNA (660 cpm/mμmole) from SB 1045 (*thy-1, his-2*) and 225 mμmoles of each deoxytriphosphate of which dTTP and dCTP were labeled with ^{3}H (3×10^{3} cpm/mμmole) was incubated with 200 μg of polymerase complex for 1.5 hr. At the end of the reaction, the mixture was divided into two aliquots. One was treated with ligase for 30 min, the other was used as control. In the case of ligase treatment, the deoxynucleoside triphosphates and polymerase were removed from the reaction mixture before incubation, since the T4 ligase had contaminating polymerase activity.[13] Joining enzyme from *E. coli*[6] does not have this activity. After treatment of the ligase, deproteinized DNA was centrifuged in CsCl with the control. The graph shows the ratio of the biological activity to the template (^{14}C) counts before and after ligase treatment. In the control, fraction 23 had 604 cpm and 1398 *try-2*$^{+}$ colonies, whereas fraction 30 had 7 cpm and 79 colonies. In the ligase-treated sample, fraction 23 had 412 cpm and 2221 colonies, whereas fraction 30 had 4.4 cpm and 82 colonies.

The products, hybrid and light molecules, were denatured and subjected both to CsCl centrifugation and to *E. coli* exonuclease-I, specific for denatured DNA.[18] At least 80 per cent of the product was denatured, and 92 per cent was degraded into an acid-soluble product by the nuclease.

Attempts to show strand separation: Hybrid molecules generated in the first cycle of synthesis were treated with the joining enzyme. These molecules have one heavy DNA strand labeled with ^{14}C and another strand of light ^{3}H-labeled DNA. On a second round of synthesis in the presence of unlabeled deoxynucleoside triphosphates, the light strand containing ^{3}H should give rise to completely light molecules, provided that the synthesis seen is semiconservative replication of DNA molecules and not a repair (Fig. 4). A small amount of biological activity is still found in the completely light stratum. These completely light DNA molecules were pooled from two experiments and rebanded in CsCl with a *B. subtilis* native DNA that carried wild-type genes not present in the original template (Fig. 5). There are no significant first-cycle template counts (^{14}C) present in the density strata of the light DNA molecules. The ratios of transformant colonies to template radioactivity for the original input DNA, hybrid product, and completely light (recentrifuged) product were 3.5, 1–2, and 45, respectively.[25] The distribution in CsCl suggests that they are large molecules.

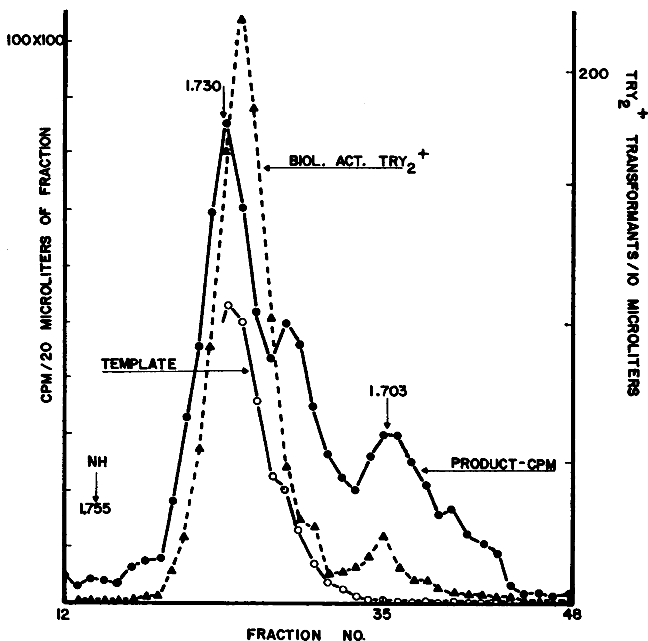

FIG. 4.—Second-cycle synthesis with cold deoxytriphosphates: 180 mμmoles of hybrid DNA (containing 6.8×10^{4} cpm of ^{14}C in the $^{15}N^{2}H$ strand and 2.3×10^{5} cpm of ^{3}H in the $^{14}N^{1}H$ strand) obtained from the first cycle of synthesis was treated with joining enzyme from *E. coli* and allowed to replicate in the presence of unlabeled deoxytriphosphates for 15 min, after removal of the joining enzyme. After polymerase reaction, the products were treated with joining enzyme and centrifuged in CsCl; 60 fractions were collected from a 5-ml gradient and 20 μl were acid-precipitated for ^{3}H and ^{14}C counts, while 10 μl were used for transformation.

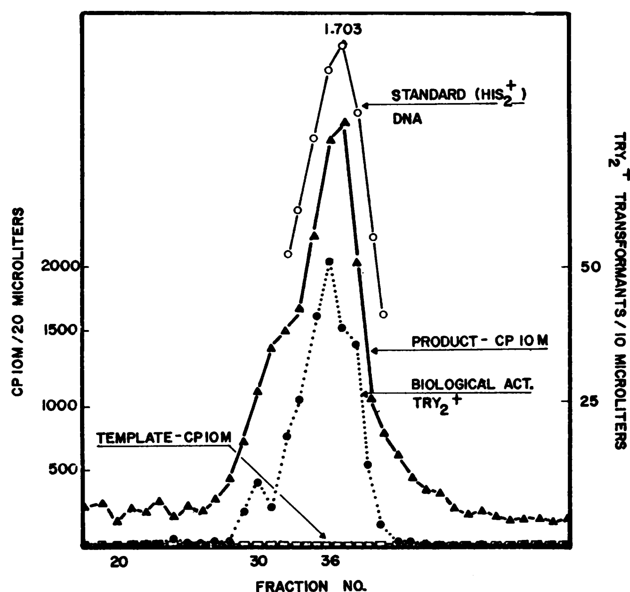

FIG. 5.—Refractionation of light product: Pooled light DNA obtained from two experiments in which the template was *his-2* was run together with a standard *B. subtilis* DNA carrying *his-2*$^+$ activity. The peak of standard corresponds to 2.2×10^3 colonies/ml.

It was shown previously that the analyzed gene cluster in the *B. subtilis* chromosome was at least 10 million in molecular weight.[14] The genes used in these studies occupy at least 80 per cent of that linkage group. The genetic analysis (Table 1) shows that at least 8–12 per cent of the molecules carry activity for more than one marker, although a very few (less than 3%) carry the entire linkage group.

TABLE 1. *Genetic analysis of the light product.*

Template genotype = *aro-2*$^+$ *try-2*$^+$ *tyr-1*$^+$ D: 111
Recipient genotype = *aro-2 try-2 tyr-1* D: 000

Classes of transformants	Per cent of total
111	2
110	7
011	12
010	79

The aromatic linkage group is the same as described by Nester *et al.*[14] Transformants for *try-2*$^+$ were selected; 200 of such transformants were stroked onto nutrient agar plates and then replicated to appropriately supplemented media to determine the remainder of the genotype.

Discussion.—Our present studies suggest that DNA replication in *B. subtilis* requires an enzyme complex containing nuclease, polymerase, and ligase activities. We do not know whether the fraction is a natural complex or several separate enzyme activities whose joint action is needed for replication. These different activities do remain together throughout the limited purification procedures described. The complex possesses a small amount of exonuclease activity that is suppressed during synthetic conditions, as is the case with T4 polymerase.[19] The presence of endonuclease activity is suggested by the disruption of pre-existing linkage. Breaks caused by endonuclease(s) during replication are presumably repaired by polynucleotide ligase. The kinetic data on

synthesis suggest that although several activities are present, they are coordinated to some extent during synthesis.

Endonuclease activity may function in replication by nicking the template molecules ahead of the replicating fork, as suggested earlier by Watson and Crick.[20] This would relieve the stress imposed on the unreplicated region of the chromosome. The recently discovered ligases might also be crucial in replication. Nagata[21] proposed a discontinuous model of DNA replication, and some recent observation by Okazaki *et al.*[22] might support such a mechanism functioning *in vivo*. Several of these models have been discussed recently.[23]

Summary.—A polymerase complex isolated from *B. subtilis* synthesizes a small proportion of biologically active DNA molecules from an appropriate template. These were separated by differential density labeling. Some of these product molecules carry several genes known to be linked in the template DNA. The nature of the complex and the roles of endonuclease and ligases in these reactions are discussed in the light of our present knowledge of DNA replication.

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[25] As indicated in the text, template DNA undergoes endonucleolytic attack during the reaction and therefore suffers a loss of biological activity. Thus the template DNA in hybrid product is an appropriate standard for an estimate of residual template biological activity in the light product. The ratio of ^{14}C-specific transforming activity in light and hybrid product (>20) provides a figure of merit for the extent to which template information has been transferred to a product free of template atoms.