ENZYMATIC SYNTHESIS OF TRANSFORMING DNA

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Received February 15, 1963

INTRODUCTION

The synthesis of DNA by the Escherichia coli polymerase requires, besides all four deoxyribonucleoside triphosphates, the presence of a DNA primer, whose base composition and nearest neighbor base frequencies are closely reproduced in the reaction product (Kornberg, 1960). We wish to present evidence that this enzymatic synthesis, carried out under the direction of a genetically transforming DNA primer, yields a biologically functional product. By using denatured, density-labeled transforming DNA as the primer, it was possible to isolate by density-gradient centrifugation the newly synthesized, primer-containing and primer-free molecules and to determine their biological activity.

METHODS

The DNAs, isolated by classical procedures, were denatured, when desired, by heating at a concentration of 200 μg/ml in 3×10^{-2} M NaCl at either 95 or 100° for a few minutes followed by rapid cooling on ice. Transformation to streptomycin-resistance in Diplococcus pneumoniae was assayed by a modification of the method of Lerman and Tolmach (1957), and in Bacillus subtilis transformation from auxotrophs to prototrophs was measured as described by Opara-Kubinska, et al. (in press). The preparation of the E. coli

* Contribution No. 203

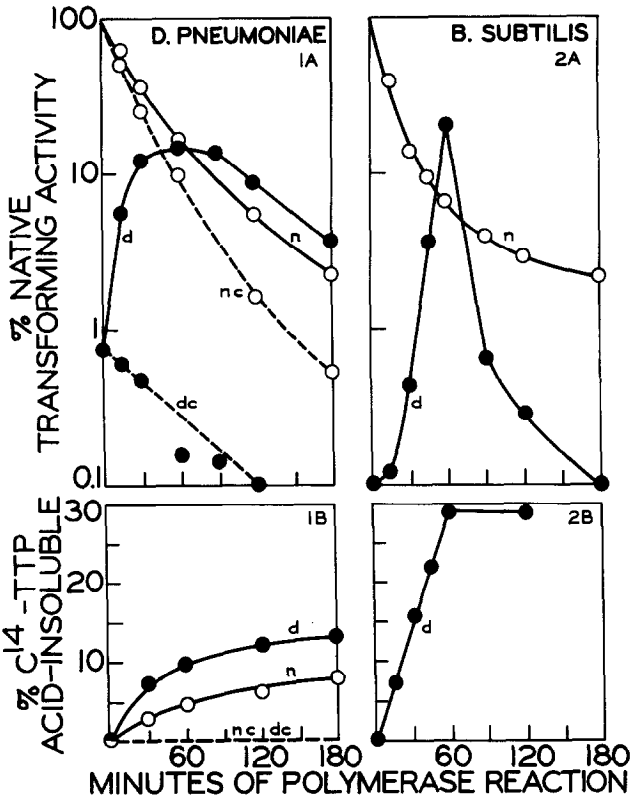
DNA polymerase and of the deoxynucleoside triphosphate substrates, the conditions of the DNA synthetic reaction, and the extent of DNA synthesis determined by the conversion of added 2C^{14}-thymidine triphosphate (C^{14}-TTP) into acid insoluble form are described by Lehman et al. (1958).

RESULTS

The kinetics of the reaction, utilizing as primers native and denatured transforming DNA, are presented in Figs. 1 and 2. With native DNA primers, transforming activity decreases rapidly (Figs. 1A and 2A), although DNA synthesis is obtained (Fig. 1B). When one triphosphate is omitted, there is no DNA synthesis, and the drop in transforming activity is more rapid (Figs. 1A and 1B). Traces of nucleases in the polymerase preparation (Lehman et al., 1962) are presumably responsible for the loss of activity, since transforming activity is extremely sensitive even to rare interruptions in the continuity of the DNA strands (Lerman and Tolmach, 1959). The lower rate of decline in the complete system, however, suggests that new active molecules are formed.

With the denatured primers in the complete system, the transforming activity (less than 1% that of native DNA at the onset) rises to a maximum after about 60 min. of reaction to yield an increase of 20 fold for D. pneumoniae (Fig. 1A) and 200 fold for B. subtilis (Fig. 2A), and there is an increase in total DNA (Figs. 1B and 2B). When one triphosphate is omitted, there is no DNA synthesis, and the transforming activity drops (Figs. 1A and 1B). The increase in transforming activity thus requires a simultaneous DNA synthesis. The rate of synthesis with the denatured primer is about twice that with the native primer (Fig. 1B) and is apparently sufficiently rapid to compete successfully with any nuclease activity.

To demonstrate that the increase in transforming activity resides in newly synthesized DNA strands, and not in "renatured" primer, the reaction products were fractionated by exploiting differences in the densities of primer and product. Bifilarly-bromouracil-labeled DNA (BB-DNA, thymine in



Figs. 1 and 2. Kinetics of polymerase reaction with D. pneumoniae or B. subtilis native (n) and denatured (d) DNA primers. Reaction mixture as in Lehman et al. (1958), with total concentration of triphosphates equal to molar concentration of bases in the primer DNA; in the controls (nc, dc), deoxycytidine triphosphate was omitted. At the indicated times, samples were assayed for transforming activity (A) and for the per cent C^{14}-TTP in acid-insoluble form (B).

both strands replaced by bromouracil) was isolated from B. subtilis grown in the presence of bromodeoxyuridine and purified by centrifugation in a CsCl gradient as described in the legend to Fig. 3. This BB DNA (Fig. 3), the specific transforming activity of which (4×10^5 indole$^+$ transformants/ μg DNA) approximated that of the normal, bromouracil-free DNA (NN), did not appear to be contaminated with any appreciable amounts of NN or hybrid (NB) molecules, since only a single "heavy" BB band was observed in analytical CsCl or Cs_2SO_4 gradient centrifugations, even when the amount of BB DNA was many times higher than that depicted in Fig. 4A. By denaturation its den-

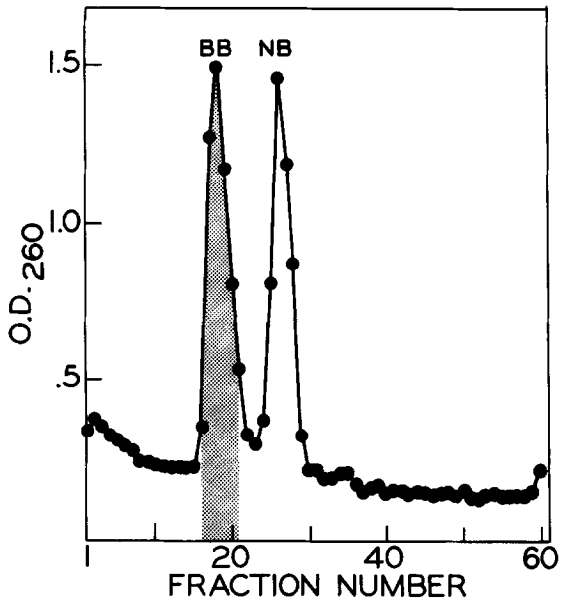


Fig. 3. Separation of the bifilarly (BB) from the unifilarly (NB) bromouracil-labeled DNA extracted from B. subtilis cells grown for 4 hr. in the presence of 100 μg bromodeoxyuridine and 4 μg 5-fluorodeoxyuridine per ml of medium, under conditions described by Opara-Kubinska et al. (in press). The CsCl density was adjusted to 1.75 g/cm^3, and 2.5 ml of the mixture overlayed with 1.5 ml of paraffin oil was centrifuged for 72 hr. at 35,000 rpm at 20°C (SW-39 rotor; Spinco model L centrifuge). 50 μl fractions were collected as described by Szybalski (1960). OD_{260} was determined for the undiluted fractions, using 20 μl microcuvettes. The sharp separation of the BB and NB DNA's was achieved by using much lower DNA concentrations (10 to 20 μg DNA per band) than those employed in the earlier described fractionations (Szybalski et al., 1960). The samples indicated by the shaded area were combined, diluted 1:1 with water, and the BB-DNA precipitated by adding two volumes of ethanol. The precipitate collected on a thin glass rod and dissolved in 0.03M NaCl was used in the subsequent experiments.

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sity was further increased (Fig. 4B), and its transforming activity reduced to 0.82% of the original value. This denatured (dB) DNA was used as primer in a synthetic reaction with the four natural triphosphates, including C^{14}-TTP. As this reaction proceeds, one and later a second new class of DNA should appear, both classes labeled with C^{14} and endowed with transforming activity: (1) hybrid molecules (NB), with one bromouracil- and thymine-containing strand, and (2) bromouracil-free molecules (NN). In two such experiments, one of which is illustrated in Figs. 4 and 5, these qualitative predictions were verified.

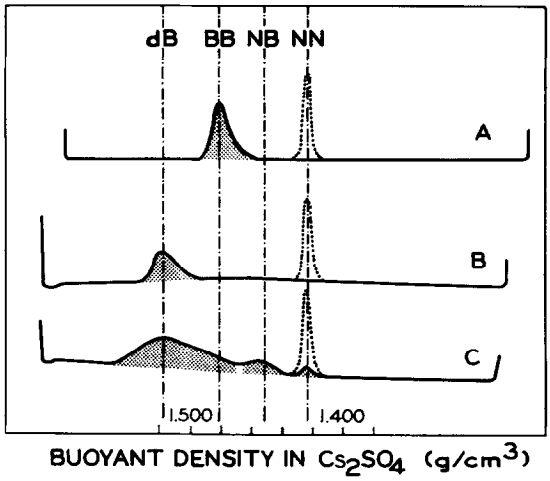

Fig. 4. Microdensitometer tracings of photographs taken after 20 hr. of Cs_2SO_4 equilibrium-density-gradient centrifugation (44,770 rpm, 25°) of native BB DNA (A), denatured dB DNA (B), and of the reaction mixture of Fig. 5 (C). The NN peaks (dotted lines) were obtained by repeating the centrifugations after the addition of native bromouracil-free DNA, as a reference density marker.

The distribution of UV-absorbing material, radioactivity, and transforming activity after centrifugation in a CsCl density gradient is shown in Fig. 5. The distribution of UV-absorbing material during centrifugation in the much steeper Cs_2SO_4 gradient is presented in Fig. 4, along with that of dB, BB, and NN DNAs. In addition to the broadened dB peak, two new peaks of UV-absorbing material appear (Fig. 4C), both containing C^{14} and both active in the transformation of the indole marker (Fig. 5), as well as of the unlinked leucine and phenylalanine markers. The larger of these two peaks has a density slightly higher than that expected for hybrid molecules (NB peak in Fig. 4C and fractions 20-45 in Fig. 5), while the smaller peak has the density position of bromouracil-free DNA (NN in Fig. 4C and fractions 48-55 in Fig. 5).

Over half of the transforming activity used for the preparative CsCl-gradient centrifugation was recovered in the collected fractions, about 16%

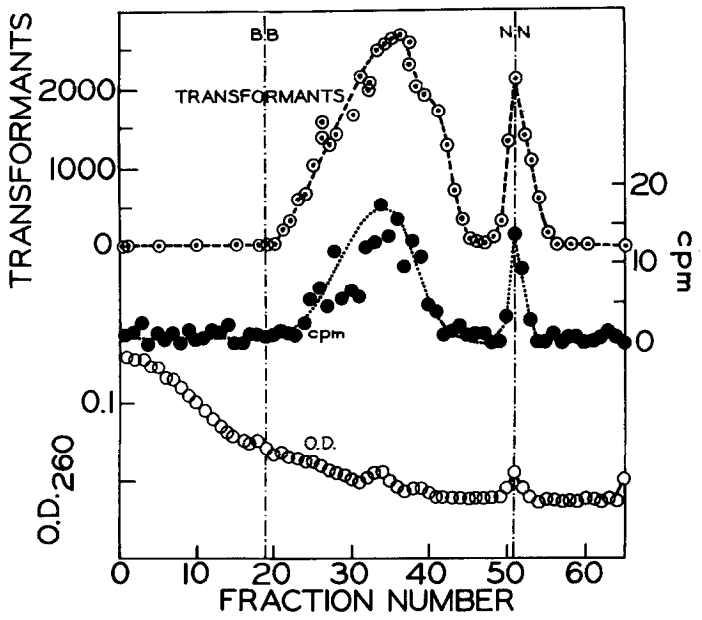

Fig. 5. Separation of primer from newly synthesized DNA. The reaction mixture (30 μg primer dB DNA per 0.8 ml total volume) as in Lehman et al. (1958), but with total triphosphates (including C^{14}-TTP) equal to 3.3 times the dB DNA primer concentration, was incubated 55 min. at 37°, heated at 60° for 10 min., and the unreacted triphosphates removed by dialysis. The amount of C^{14}-TTP in acid-insoluble form was 7.6%, which represents a 25.2% increase in DNA over the primer. The recovery of transforming activity was 25.4% as compared to 100% for the native BB DNA and 0.82% for the denatured dB primer. The CsCl density fractionation procedure and OD_{260} determination were the same as described in Fig. 3. CsCl was removed before assaying for radioactivity (in 20 μl samples) and for transforming activity (indicated as transformants per 2 μl). Expected DNA densities in CsCl in g/cm^3: dB = > 1.83; BB = 1.803; NB = 1.753; NN = 1.703. These were confirmed by micropycnometric determinations of density for the neighboring fractions and by analytical Cs_2SO_4 (Fig. 4C) and CsCl density-gradient centrifugations.

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of which was in the NN band. Due to the attempt to remove CsCl before assaying for radioactivity, the recovery of C^{14} was low. In a previous experiment, however, in which C^{14} was counted prior to the removal of CsCl, the amount of C^{14} in all of the fractions was equal to 50-60% of the total added.

DISCUSSION AND CONCLUSION

To account for the transforming activity in the NN band, four suppositions may be considered. 1) The transforming activity results from the trailing of either active dB or NB DNA during the collection of the drops.

This explanation is unlikely, since a monotonic decline and not a sharp peak in transforming activity would have been observed (cf. Fig. 5). 2) The transforming activity is due to the incorporation of fragments of the dB primer into the NN DNA. The estimated limits of error in the density of the latter peak, as based on the analytical density-gradient centrifugation runs, with and without an added reference density marker (Fig. 4C), would exclude the presence of contaminating BB DNA in excess of 2% of the NN DNA. If the transforming activity is to be accounted for by the random inclusion of any fragments from the marker site of the dB primer, then for any one marker the specific transforming activity should be at most 2% of normal DNA. It was found, however, that the specific transforming activity of the DNA in the NN peak of Fig. 5 for the indole marker was equal to that of the original native DNA (BB) within a factor of two. 3) The transforming activity is accounted for by DNA entirely free of bromouracil, but one strand of which may have originated from some NB molecules present in the original BB DNA preparation. In the experiment of Fig. 5, of the total amount of radioactivity (synthesized DNA) 16% is in the NN band. Assuming that bromouracil- or thymine-containing DNA strands are equally active as primers (Wake and Baldwin, 1962, found that the rate of synthesis with the dAT or $d\overline{ABU}$ polymers was about the same), then the BB DNA would have had to contain at least 16% NB molecules. NB DNA after denaturation is separated by centrifugation in CsCl or Cs_2SO_4 into dB and dN strands of greatly differing densities. It can be seen from Fig. 4B that no dN DNA was observed; this was true also when much larger amounts of dB DNA were analyzed as in Fig. 4B, the limits of detection being considerably less than 5% of the total DNA studied. Finally, this supposition is rendered even more unlikely by the finding in a previous experiment referred to above, that the cpm/OD_{260} of the DNA in the NN band was twice that of the NB band, a finding incompatible with the suggestion that one of the strands of the NN DNA comes from unlabeled primer.

4) The transforming activity of the NN band is accounted for by DNA both strands of which are essentially newly synthesized DNA. This hypothesis

is compatible with all of the results and arguments presented. The appearance of NN DNA with a total synthesis of only 25% (Fig. 5) must indicate that only a fraction of the dB DNA served as active primer or that molecules containing newly synthesized strands are more active as primers. The major part of the dB DNA primer is still present in the dB region (Fig. 4C). In an analogous system, Wake and Baldwin (1962) using as primer the synthetic polymer $d\overline{ABU}$ and as substrates deoxyadenosine and thymidine triphosphates demonstrated the appearance of dAT-dAT polymers long before all of the primer had been converted into hybrid polymers of dAT-$d\overline{ABU}$. These authors found, for instance, that when 37% total synthesis had occurred, the distribution of polymers was 41% $d\overline{ABU}$-$d\overline{ABU}$ primer, 43% dAT-$d\overline{ABU}$ hybrid, and 16% dAT-dAT newly synthesized. In one of our experiments, the amount of DNA synthesized compared to the primer was about 40%; of the total DNA (as determined by OD_{260} in the fractions collected after centrifugation in CsCl), 40% was dB primer, 51% NB hybrid, and 9% NN newly synthesized DNA. On the basis of these considerations, it seems most plausible to attribute the transforming activity of the NN band of Fig. 5 to DNA both strands of which are newly synthesized in the course of the polymerase reaction, and to conclude that new, primer-free, active transforming molecules of DNA have been synthesized *in vitro* by the catalytic action of the *E. coli* polymerase with denatured transforming DNA primers.

ACKNOWLEDGMENTS

We are grateful for the help and advice of Dr. L. S. Lerman and of Mrs. Z. Opara-Kubinska, who also supplied us with the purified BB primer DNA. This work was aided by U.S.P.H.S. Grant No. GM-08894; and by a grant from The National Foundation; and by Grant No. CY-5215 of the National Cancer Institute to W. Szybalski.

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