On the Fidelity of Phage T_4-Induced Polynucleotide Ligase in the Joining of Chemically Synthesized Deoxyribooligonucleotides.+

by

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Summary: The fidelity of phage T_4-induced polynucleotide ligase has been studied by using chemically synthesized deoxyribooligonucleotide $p^{32}T_{12}$ and $p^{32}T_{11}C$. In the presence of polydeoxyadenylate, each substrate is joined by ligase to form longer chain deoxyribopolynucleotides. The ligase products have been characterized with respect to average chain length and nearest neighbour analysis.

Recently, several groups (1) have reported the discovery of polynucleotide ligase enzymes, which catalyze the synthesis of phosphodiester bond by the esterification of the 5'-phosphoryl group to the 3'-hydroxyl group of the interrupted DNA chains in the double-stranded DNA. The availability of such enzymes offers a novel synthetic tool in the building of DNA containing defined sequence for biological studies. In fact, a remarkable progress has been made in the successful application of this enzyme in the joining of chemically synthesized short deoxyribooligonucleotides in the presence of their complementary strand (2,3). These experiments along with others (Ib,c) make it clear that the organic synthesis of short-chains deoxyribooligonucleotides followed by enzymatic joining by polynucleotide ligase provide a promising approach for DNA synthesis.

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A very important consideration in any of these enzyme-catalyzed joinings, is the fidelity with which these enzymes make the desired 3'-5' link of deoxyribooligonucleotides possessing one or more mispaired bases. With this view in mind, we looked into the fidelity of phage T_4-induced polynucleotide ligase. The initial phase of our work reported here, involves the joining of 5'-phosphoryl group to the 3'-hydroxyl group of deoxyribooligonucleotides containing one mispaired base at the 3'-hydroxyl terminus(4).

Materials and Methods

Polyd-A was prepared from polydA:dT by separation of the strands in an alkaline cesium sulfate density gradient (5). Deoxyribooligonucleotides T_{12}, $T_{11}C$ were chemically prepared samples (6).

The polynucleotide kinase and ligase were isolated from Phage T_4-infected E. coli B cells (7) and prepared according to Richardson and his co-workers (8-9). The final kinase preparation, fraction VI was found to have 55,000 units per mg protein, while the final ligase preparation, fraction VII was found to have 2,200 units per mg protein. Both enzymes were found to be free from any deoxyribonuclease activity as determined according to Richardson (8-9).

Bacterial alkaline phosphatase, Micrococcal nuclease, spleen phosphodiesterase, snake venom phosphodiesterase and pancreatic DNase were purchased from Worthington. γ-p^{32}-ATP was purchased from International Chemical and Nuclear Corporation and New England Nuclear.

Results and Discussion

Preparation of 5'-P^{32}-deoxyribooligonucleotides

Phosphorylation of the 5'-hydroxyl end group of T_{12}, and $T_{11}C$ with γ-p^{32}-ATP by polynucleotide kinase was according to the procedure of Richardson (8). After incubation the solution was heated in a boiling water bath for 2 min, and the following methods were used for the isolation of the phosphorylated products.(1) The reaction mixture was chromatographed on a

DEAE-cellulose paper with 0.1 to 0.5 M triethylammonium bicarbonate pH 7.1 or 1 M ammonium bicarbonate as the eluents (2, 10). (2) The reaction mixture was chromatographed on a Whatman No. 3 paper with isobutyric acid - 1 M NH_4OH:0.1 M EDTA (100:60:1.6) (11). Both these paper chromatographic methods were used in the assay of kinase reactions. (3) The reaction mixture was concentrated to about 0.2 ml and applied on a Sephadex G-75 superfine column (90 cm x 1 cm) preequilibrated with 0.1 M tri-

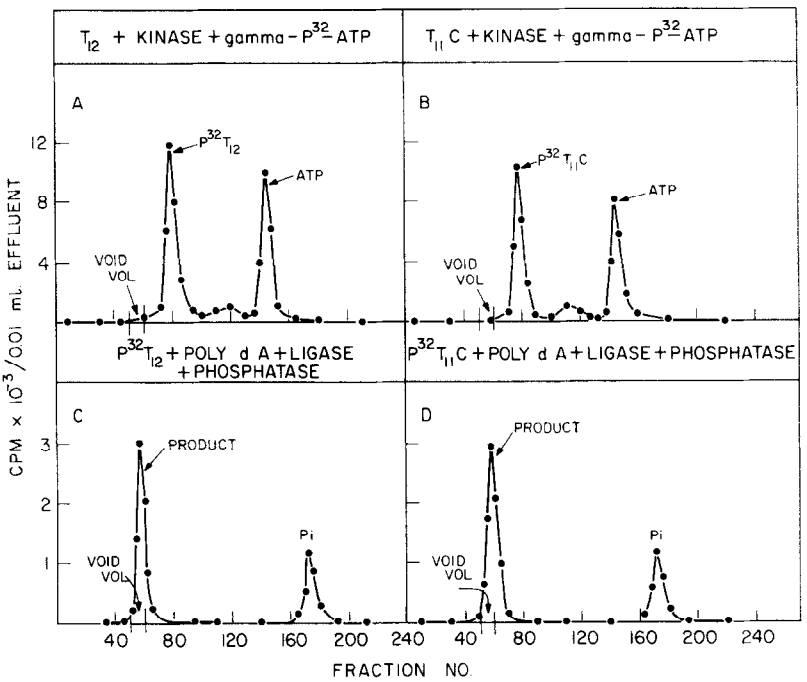

Fig. 1. Chromatography on a Sephadex G-75 superfine column (90 cm x 1 cm) of polynucleotide kinase and ligase products. In the case of the kinase reaction, Fig. 1A,B, the standard reaction mixture, after 2 min in a boiling water bath, was concentrated to about 0.2 ml and was applied to a Sephadex G-75 column equilibrated in 0.1 M triethylammonium bicarbonate buffer, pH 7.1. In the case of the ligase reaction, Fig. 1C,D, the standard reaction mixture (0.3 ml) containing 20 mμmoles of $P^{32}T_{12}$ or $P^{32}T_{11}C$ (specific activity 1.3 x 10^7 cpm per mμmole), 20 mμmoles polydA and 1 unit of ligase and other components according to Richardson (9), was incubated for 3 hr at 20°C. After 2 min in a boiling water bath, it was chilled and incubated for 45 min at 70°C with 20 μg alkaline phosphatase and applied to the column as described above. In each case, fractions of 0.5 ml were collected in 10 min and 0.01 ml of each fraction was counted for radioactivity as previously (12).

ethylammonium bicarbonate buffer, pH 7.1 at 5°C. On washing with the same buffer, the first radioactive peak corresponded to the product and was followed by another peak corresponding to the unused γ-P^{32}-ATP. The elution patterns of gel-filtrations of the phosphorylated products are recorded in Fig. 1A, B. The purity of these products was further checked using the paper chromatographic methods and also by the analysis of their products formed on degradation with snake venom phosphodiesterase, and with alkaline phosphatase.

Joining of deoxyribooligonucleotides in the presence of complementary deoxyribopolynucleotides

Joining of $P^{32}T_{12}$ or $P^{32}T_{11}C$ in the presence of stoichiometric amounts of poly dA by phage T_4-induced polynucleotide ligase was according to the procedure of Richardson (8). After 3 hr at 20°C more than 70% of the substrates appeared as high molecular weight products immediately after the void volume as examined by gel filtration on Sephadex G-75 (superfine) (90 cm x 1 cm) Figure 1C, D. The DEAE-cellulose paper strip assay (2) was also used to isolate and characterize the ligase products as recorded in Figure 2. Determination of the phosphatase-sensitive to phosphatase-resistant p^{32} indicated these products to be 400-450 nucleotide units long. Nearest neighbour analyses

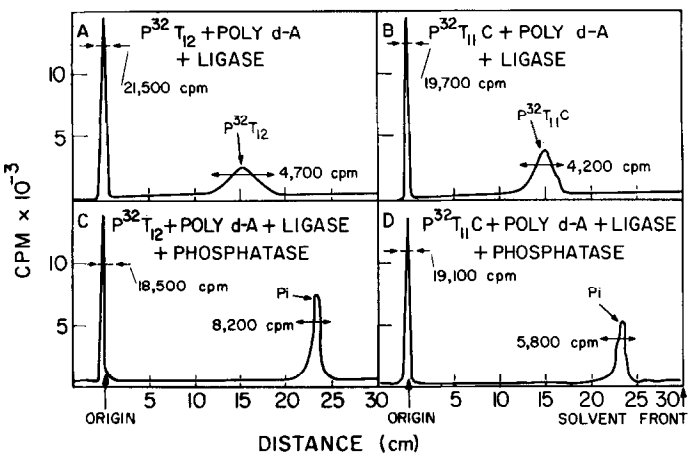

Fig. 2. Analyses of reaction products formed with $P^{32}T_{12}$ or $P^{32}T_{11}C$ in the presence of poly dA and polynucleotide ligase. Chromatography was DEAE-cellulose strips with 0.35 M ammonium formate and 7 M urea as the eluent.

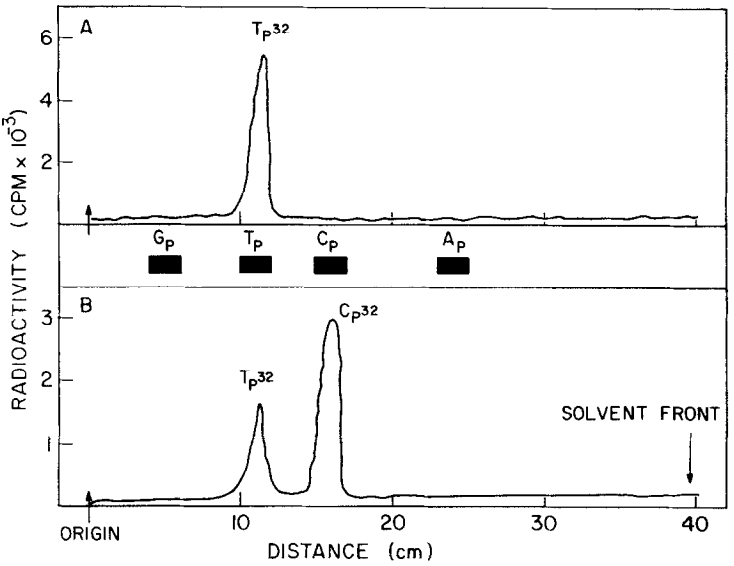

Fig. 3. Nearest-neighbour analysis on the products of ligase obtained after removal of the 5'-phosphate group. Chromatography was on Whatman No. 3 paper strips with isobutyric acid - NH_4OH (conc.) - water, 66:1:33 (v/v) as the eluent as previously described (12).

on the products obtained after the removal of the terminal 5'-phosphate group are recorded in Fig. 3 (12). It is clear that in the case of the ligase product of $p^{32}T_{12}$, all of the radioactivity is present in Tp, while in the case of $p^{32}T_{11}C$, most of the radioactivity is present in Cp, and some in Tp. The amount of Tp may reflect the extent of contamination of $T_{11}C$ with T_{11} or the presence of traces of nucleases.

The present work has shown that T_4-induced polynucleotide ligase can catalyze the covalent joining of interrupted deoxyribooligonucleotide strands, with one mispaired base at the 3'-hydroxyl terminus of a bihelix.

Considering the magnitude of the synthetic task, the fidelity of enzymes which catalyze the covalent joining of interrupted poly- and oligodeoxyribonucleotide strands of a bihelix becomes of paramount importance in the building of a DNA from chemically synthesized deoxyribooligonucleotides of defined sequence.

Similar experiments, with deoxyribooligonucleotides,

containing more than one mispaired base at both termini as well as in the middle, are currently underway.

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