CATALYSIS OF DNA JOINING BY BACTERIOPHAGE T4 RNA LIGASE

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SUMMARY: RNA ligase, purified extensively from Escherichia coli infected with wild-type or DNA ligase mutants of bacteriophage T4, catalyzes the joining of 5'-phosphoryl terminated DNA to DNA and RNA acceptors. This was shown by the conversion of [5'-^{32}P]deoxyoligomers to a form resistant to phosphatase, the increased chain length of the joined RNA-DNA copolymers, the circularity of the DNA joined product, and the transfer of the 5'-^{32}P label of the donor DNA to the 3'-end of both RNA and DNA acceptors. The novel DNA joining activity is intrinsic to RNA ligase since it co-purifies with RNA joining activity and has the same requirements, inhibitors, and thermolability. These results suggest that RNA ligase should be a useful reagent for the synthesis of defined sequence DNA and RNA-DNA copolymers and raise the possibility of a role for RNA ligase in DNA metabolism.

RNA ligase, induced by coliphage T4, joins together RNA chains intramolecularly and intermolecularly in a reaction requiring ATP (1-5). RNA ligase is distinct from T4 DNA ligase, the gene 30 product, and differs from this enzyme in the lack of a template requirement and in substrate specificity (1). While it has been reported that the enzyme is specific for RNA (1), in this communication we show that RNA ligase catalyzes the joining of a DNA donor molecule to a DNA or RNA acceptor. The lack of a template requirement makes RNA ligase an attractive reagent for the synthesis of nucleic acids with a defined sequence and was used in this report to make circular oligo(dT). The results also raise the possibility of a physiological role of RNA ligase in DNA replication, such as in the formation of DNA-RNA copolymers; a DNA ligase other than the well-characterized phage and host enzymes has been the subject of continuing speculation. A future publication will describe mutants of T4 which lack detectable RNA ligase activity.

MATERIALS AND METHODS

Nucleic Acids. Concentrations of nucleic acids are expressed in terms of 5'-termini unless otherwise indicated. Poly(rA), poly(rU), and poly(rC) were purchased from Miles Laboratories, Inc. and $d(T)_n$ from Collaborative Research. [^{3}H]$d(T)_{50}$, [^{3}H]poly(rU), and poly(dA) were synthesized enzymatically (6). The nonadeoxynucleotide, pG-C-T-T-C-C-C-G-A, was synthesized

chemically, and 3',5'-OH terminated $r(A)_{20}$ and $[5'-^{32}P]r(A)_{20}$ were prepared by the method of Silber et al. (1). DNA oligomers were similarly labeled with polynucleotide kinase and $[\gamma-^{32}P]ATP$. All labeled polymers were purified by Sephadex G-50 or G-25 filtration in 50 mM triethylammonium bicarbonate, lyophilized, and the number average chain length determined by end-group labeling with polynucleotide kinase.

Enzymes. Bacterial alkaline phosphatase (BALP), types III S and III R, and 5'-nucleotidase were obtained from Sigma Chemical Co. Micrococcal nuclease and spleen phosphodiesterase were purchased from Worthington Biochemical Corp. and the latter enzyme was further purified (8). Phage T4 DNA ligase and polynucleotide kinase were purified by the method of Panet et al. (9). Bacillus subtilis DNA polymerase III was prepared according to Low et al. (6).

Enzyme Assays. The standard RNA ligase reaction mixture (20 µl) contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM dithiothreitol, 1 mM ATP, 10 µM $[5'-^{32}P]$ribo- or deoxyribooligomer, and 0.001 to 0.1 unit of enzyme. The assay for joining DNA to RNA contained 50 µM 3',5'-OH ribooligomer in addition to labeled DNA. After 15 min at 37°, the reaction mixture was heated at 100° for 2 min and treated with 0.9 unit of BALP for 20 min at 65°. Phosphatase-resistant ^{32}P was measured by adsorption to either Norit or DEAE paper (6). One unit of enzyme converts 1 nmole of ^{32}P in $[5'-^{32}P]r(A)_{20}$ to a BALP-resistant form in 30 min. DNA ligase was assayed similarly except the substrate was $[5'-^{32}P]d(T)_{12-18}$:poly(dA). DNase and RNase assays used the same reaction conditions except the substrate was either $[^{3}H]d(T)_{50}$ or $[^{3}H]$poly(rU) at 20 µM in total nucleotide, and the uranyl acetate-perchloric acid soluble material was measured. A unit of nuclease catalyzes the hydrolysis of 1 nmole of nucleotide in 30 min.

Characterization of Products. A. Ligation of $d(T)_{12-18}$--After a 2-hr reaction in which 10% of the $[5'-^{32}P]d(T)_{12-18}$ substrate was ligated, the reaction mixture was treated with BALP and passed through Sephadex G-25 to remove $^{32}P_i$. To determine the nearest neighbor of the 5'-terminal substrate residue (10), one-half of the product was degraded to 3'-mononucleotides by successive treatment with micrococcal nuclease and spleen phosphodiesterase. The digestion products were separated by chromatography in n-propanol:conc. NH_4OH:H_2O; 6:3:1 (solvent I). Labeled material which migrated as Tp was eluted, mixed with unlabeled pT as an internal standard, and treated with 5'-nucleotidase (11). The other half of the reaction products was analyzed by homochromatography (7) along with the $[5'-^{32}P]d(T)_{12-18}$ substrate to determine the chain lengths of the products. The substrate and products were eluted from the thin-layer plate and treated with 0.1 unit of B. subtilis DNA polymerase III exonuclease (6) to monitor circularity. B. Joining of $d(T)_{12-18}$ to $r(A)_{20}$ --After RNA ligase treatment in which 90% of the $[5'-^{32}P]d(T)_{12-18}$ was converted to a BALP-resistant form, the reaction mixture was subjected to homochromatography to measure chain length. The labeled material was eluted from the plate and a portion was hydrolyzed with 10% aqueous piperidine for 18 hours at 45°. After removal of the piperidine in vacuo, the digestion products and a non-hydrolyzed control were electrophoresed in 50 mM sodium citrate, pH 3.5. The labeled compound migrating as 2'(3')-AMP was eluted and developed by ascending chromatography in isopropanol:conc. NH_4OH : 0.1 M sodium borate; 6:1:3 (solvent II) which resolved 2'(3')-AMP from 5'-AMP.

Purification of RNA ligase. RNA ligase was purified from 50 g of E. coli B infected with either T4 amN82 (gene 44) or amE13 (gene 30) at a multiplicity of infection of 3.5 by the procedure of Silber et al. (1) except the cells were lysed with a French Pressure Cell Press and Sephadex G-100 replaced Sephadex G-75. This preparation was free of RNase activity but further purification was required to remove the considerable DNase contamination. The enzyme was dialyzed overnight against 20 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 50 µM ATP. 180 units of enzyme were diluted 4-fold with 20 mM potassium phosphate, pH 6.8, 1 mM 2-mercaptoethanol, 50 µM ATP, and 10% glycerol and applied to a 0.95 x 3.4 cm hydroxylapatite column. The column was washed with 8 ml of the dilution buffer and the enzyme eluted with a 0.02 to 0.25 M potas-

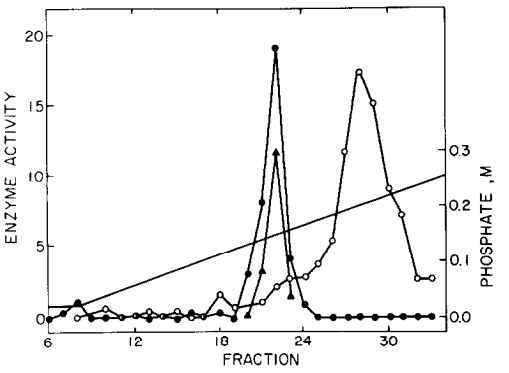

Fig. 1. Co-purification of DNA and RNA joining activities during hydroxylapatite chromatography. Enzyme (180 units) was applied to an hydroxylapatite column and eluted with a 0.02 to 0.25 M, pH 6.8, potassium phosphate gradient. One-ml fractions were assayed for DNase (o) and ligation of [5'-^{32}P]r(A)$_{20}$ (●) and of [5'-^{32}P]d(T)$_{12-18}$ (Δ). Enzyme activity is plotted as units per fraction (●), units x 10 per fraction (Δ), or units x 0.25 per fraction (o).

sium phosphate, pH 6.8, gradient containing 1 mM 2-mercaptoethanol, 50 μM ATP, and 10% glycerol (Fig. 1). The remaining low level 3'→5' deoxyriboexonuclease contamination could be almost completely removed by passage through single-stranded DNA-agarose (12) equilibrated with 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 50 μM ATP, 0.1 M KCl, 10% glycerol. The final ligase preparation had a specific activity of 1800 units/mg. Electrophoresis through 5% polyacrylamide gels containing 0.2% sodium dodecyl sulfate (13) showed one major band, representing 82% of the total protein, which had the identical mobility as RNA ligase provided by Dr. J. Last (Fig. 2). Under these conditions the enzyme moved slightly faster than ovalbumin and had a subunit molecular weight of 41,000 rather than 47,000-48,000 as reported previously (14).

RESULTS AND DISCUSSION

The standard assay of RNA ligase measures the conversion of [5'-^{32}P]-oligonucleotides to a form resistant to BALP. By this criterion, RNA ligase catalyzes the joining of d(T)$_{12-18}$ at about 1/12 the rate with r(A)$_{20}$; the DNA joining activity varied from 5-14% of RNA joining activity with various polymer preparations perhaps reflecting the marked effect of chain length on reaction rate (2,4). The velocities of the reactions were proportional to the concentration of 5'-termini; the highest DNA concentration tested was 66 μM (Fig. 3). Under the standard assay conditions, the rate of d(T)$_{12-18}$ joining was linear with time up to two hours and with the amount of added ligase. RNA ligase

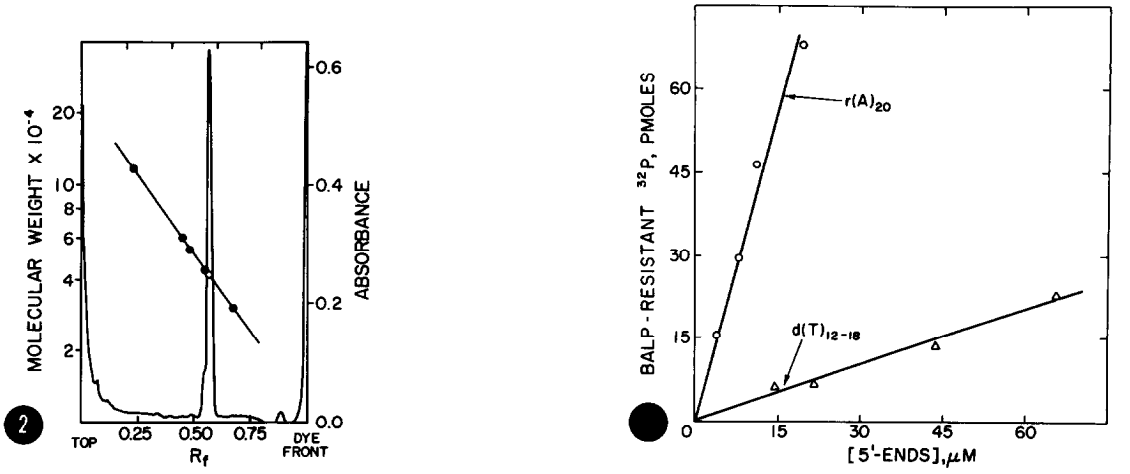

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of RNA ligase. The scan of 3 μg RNA ligase is shown; the absorbance at the top is an artifact arising from the edge of the gel and the absorbance at the dye front is from India ink marking this position. The standards (•) were β-galactosidase, catalase, ovalbumin, glutamic dehydrogenase, and carbonic anhydrase. The position of RNA ligase provided by Dr. J. Last is also shown (o).

Fig. 3. Dependence of reaction rate on substrate concentration. The standard RNA ligase reaction mixture contained the indicated concentrations of either [5'-^{32}P]r(A)$_{20}$ or [5'-^{32}P]d(T)$_{12-18}$ and 0.07 unit of enzyme.

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similarly catalyzed the joining of d(T)$_{10}$, d(T)$_8$, and d(T)$_6$ but the rate diminished with decreasing chain length. The enzyme can also form RNA-DNA copolymers. The addition of 50 μM 3',5'-OH r(A)$_{20}$ to 10 μM [5'-^{32}P]d(T)$_{12-18}$ stimulated the rate of joining 3-5 fold. 3',5'-OH oligo(rU) and oligo(rC) also augmented the conversion of [5'-^{32}P]d(T)$_{12-18}$ to a BALP-resistant form but much less efficiently than oligo(rA). The favored reaction in the presence of oligo(rA) is not the result of joining adjacent oligothymidylates aligned by formation of a duplex with the oligo(rA), since the d(T)$_{12-18}$ was joined to the r(A)$_{20}$ acceptor rather than to oligo(dT) (see below). Also, the addition of long polymers of riboadenylate and deoxyriboadenylate at a concentration sufficient to convert all the [5'-^{32}P]d(T)$_{12-18}$ substrate into a duplex did not stimulate joining. Joining of [5'-^{32}P]pG-C-T-T-C-C-C-G-A to 3',5'-OH r(A)$_{20}$ was observed and these polymers can not form a duplex.

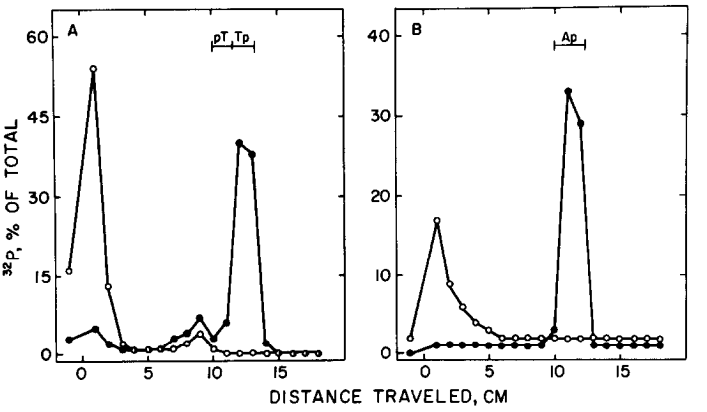

Fig. 4. Proof of joining by nearest neighbor analyses. Products of enzyme activity on [5'-^{32}P]d(T)$_{12-18}$ alone (A) and plus 3',5'-OH r(A)$_{20}$ (B) were purified as described in Materials and Methods. A. Enzymatically hydrolyzed (•) and undigested (o) reaction products were chromatographed in solvent I. The material just behind [^{32}P]Tp was shown to be $^{32}P_i$ by its inability to adsorb to Norit. B. Alkali hydrolyzed (•) and undigested (o) reaction products were subjected to paper electrophoresis at pH 3.5.

We proved that a true phosphodiester link was formed by RNA ligase by nearest neighbor analyses (10). After ligation of [5'-^{32}P]d(T)$_{12-18}$, the polymers were treated with BALP and separated from $^{32}P_i$ by filtration through Sephadex G-25. After successive digestion with micrococcal nuclease and spleen phosphodiesterase the labeled material was displayed by chromatography in solvent I (Fig. 4A). 80% of the labeled material migrated with internal Tp which was resolved from pT, and the remainder was $^{32}P_i$ and undigested polymer. Without nuclease treatment, the label remained near the origin. The identity of the [^{32}P]Tp was confirmed by resistance to digestion by 5'-nucleotidase which degraded only 6% of the labeled material eluted from the chromatogram while quantitatively converting internal pT to thymidine plus inorganic phosphate. Analogously, the products of ligation of [5'-^{32}P]d(T)$_{12-18}$ and 3',5'-OH r(A)$_{20}$ were treated with alkali; prior removal of unreacted ^{32}P by BALP treatment and purification was unnecessary since 90% of the donor ^{32}P was rendered BALP resistant. After electrophoresis at pH 3.5, 90% of the label migrated with internal 2'(3')-AMP, whereas without alkali, the material remained near

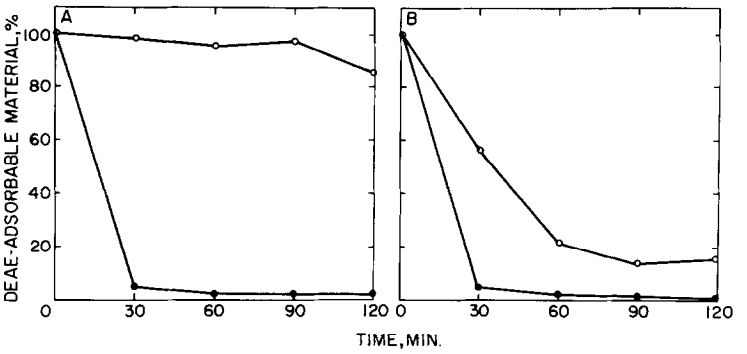

Fig. 5. Resistance of $d(T)_{12-18}$ joined products to digestion by exonuclease. (A) BALP-resistant ligase reaction products (o) and (B) [5'-^{32}P]$d(T)_{12-18}$ substrate (o) were purified successively by Sephadex G-25 and by homochromatography, mixed with [^{3}H]$d(T)_{50}$ (•), and incubated with 0.1 unit of B. subtilis DNA polymerase III. At the indicated times, samples were assayed for retention by DEAE paper.

the origin (Fig. 4B). The identity of the reaction product was further confirmed by ascending chromatography in solvent II where 95% of the labeled material eluted from the electropherogram migrated identically with 2'(3')-AMP. With a [5'-^{32}P]pG-C-T-T-C-C-C-G-A donor and a 3',5'-OH $r(A)_{20}$ acceptor, the BALP-resistant product was similarly alkali-sensitive.

The size of the ligase reaction products was measured by homochromatography. With 5'-^{32}P-labeled $d(T)_{12-18}$ or pG-C-T-T-C-C-C-G-A as donor and 3',5'-OH $r(A)_{20}$ as acceptor, at least one-half of the ligated material migrated slower than the substrate signifying an increased chain length for the product. The retarded ^{32}P was BALP-resistant but alkali-sensitive as expected for an RNA-DNA copolymer. Reaction in the absence of riboacceptor, however, led to BALP-resistant ^{32}P in molecules the same size as the [5'-^{32}P]$d(T)_{12-18}$ substrate; all seven length components of the $d(T)_{12-18}$ mixture were resolved. This suggested that the enzyme had catalyzed an intramolecular joining, analogous to its activity on some RNA substrates (1,4). Proof of the circularity of the $d(T)_{12-18}$ self-joined product was obtained by subjecting BALP-treated, Sephadex G-25 purified product to digestion with the single-strand specific

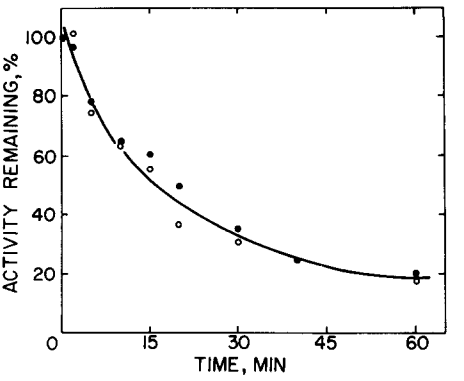

Fig. 6. Thermal inactivation of RNA ligase. DNA-agarose purified enzyme was diluted 5-fold in 50% glycerol, 20 mM Tris-HCl, pH 7.5, 50 μM ATP, 5 mM dithiothreitol, and incubated at 46°. At the times indicated, 2-μl samples were assayed under standard conditions for [5'-^{32}P]d(T)$_{12-18}$ (o) and [5'-^{32}P]r(A)$_{20}$ (●) joining.

exonuclease of B. subtilis DNA polymerase III. This enzyme, which is devoid of endonuclease activity, digested less than 10% of the product, whereas the [5'-^{32}P]d(T)$_{12-18}$ substrate as well as internal [^{3}H]d(T)$_{50}$ were quickly degraded (Fig. 5). Preliminary experiments with smaller oligo(dT) substrates indicated that intermolecular ligation can also occur, as has been found with RNA substrates (5).

These novel DNA joining activities are intrinsic to RNA ligase and not the result of a contaminating enzyme. Under the RNA ligase assay conditions, purified T4 DNA ligase catalyzed the joining of [5'-^{32}P]d(T)$_{12-18}$ to itself or to 3',5'-OH r(A)$_{20}$ acceptor at a level at most 0.5-1.5% of the activity with [5'-^{32}P]d(T)$_{12-18}$:poly(dA). Even if all the joining of this duplex by our preparation of RNA ligase were catalyzed by contaminating DNA ligase, the DNA ligase would account for only 1/100 of the activity with d(T)$_{12-18}$ alone. In addition, RNA ligase was purified through the DNA-agarose step from cells infected by a T4 mutant (amE13) devoid of DNA ligase. The substrate specificity was the same as for enzyme purified from amN82-infected cells. RNA ligase which was purified by Dr. J. Last using a procedure different from ours also catalyzed DNA joining at a rate about 1/10 of RNA joining. Three lines of evidence

establish directly that the DNA joining is catalyzed by RNA ligase. First, the DNA and RNA joining activities co-purified (Fig. 1) and the ratio of the activities remained constant during purification. Second, DNA joining required ATP and divalent metal ions and was inhibited more than 90% by 1 mM PP_i and 0.5 mM p-chloromercurisulfonic acid as has been reported (1) for RNA joining. Third, the two activities showed the same thermolability (Fig. 6).

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