L Cell DNA Ligase Joins RNA to DNA on a DNA Template†

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ABSTRACT: L cell DNA ligase catalyzes a covalent linkage between 5′-phosphoryl oligodeoxyribonucleotides and 3′-hydroxyl oligoribonucleotides on a complementary polydeoxyribonucleotide template. This reaction occurs to a substantially lesser extent than does the sealing of DNA to DNA. The joining of [5′-^{32}P]d(pA)$_{12-18}$ to (Ap)$_{11}$A on poly[d(T)] or of [5′-^{32}P]d(pG)$_{12-18}$ to 5′-hydroxyl, 3′-hydroxyl oligo(I) on poly[d(C)] was demonstrated by the formation of alkaline phosphatase resistant radioactivity. The ^{32}P of the hybrid reaction products became sensitive to the action of alkaline phosphatase after treatment with alkali. Furthermore, hydrolysis of the products of the linkage of [5′-^{32}P]d(pG)$_{12-18}$ to 5′-hydroxyl, 3′-hydroxyl oligo(I) on poly[d(C)] with micrococcal nuclease and spleen phosphodiesterase resulted in the formation of [3′-^{32}P]IMP. Attempts to seal [5′-^{32}P]-(pA)$_{12}$ to d(Ap)$_{11-17}$A on poly[d(T)] or [5′-^{32}P]oligo(pI) to d(Gp)$_{11-17}$G on poly[d(C)] were unsuccessful.

DNA ligases catalyze the synthesis of phosphodiester bonds between adjacent pieces of DNA bearing 5′-phosphoryl and 3′-hydroxyl termini when they are in a duplex conformation (for reviews, see Lehman, 1974; Soderhall and Lindahl, 1976). Some prokaryotic DNA ligases also catalyze the joining of RNA and DNA in duplex conformations. T4 DNA ligase seals limited amounts of RNA to RNA in the presence of a complementary DNA strand, and of DNA to DNA in the presence of complementary RNA (Kleppe et al., 1970; Fareed et al., 1971). T4 and *E. coli* DNA ligases join 5′-phosphoryl DNA to 3′-hydroxyl RNA on a complementary DNA strand (Nath and Hurwitz, 1974). By extending (pA)$_3$ to (pA)$_3$d(pA)$_n$ with terminal deoxynucleotidyl transferase Nath and Hurwitz (1974), using T4 DNA ligase, joined a small portion of the 5′-phosphoryl RNA termini of this substrate to adjacent 3′-hydroxyl DNA ends in the presence of poly[d(T)]. T4 DNA ligase also catalyzes the sealing of oligoribonucleotides on a complementary RNA strand (Sano and Feix, 1974).

Whether or not eukaryotic DNA ligases are capable of catalyzing the formation of phosphodiester bonds between hybrid substrates is unclear. DNA to RNA joining on either a ribo- or deoxyribonucleotide template has not been examined, and tests of the linkage of RNA to itself on a complementary DNA strand have produced conflicting results. Calf thymus DNA ligase links 5′-phosphoryl poly(A) on a poly[d(T)] template to a limited extent (Bertazzoni et al., 1972); however, the DNA ligase of human EUE cells does not (Pedrali-Noy et al., 1973).

It is possible that RNA plays an important role in the metabolism of DNA. It has been proposed that pieces of RNA serve as primers upon which DNA synthesis is initiated and evidence of covalent linkages between RNA and newly replicated DNA has been obtained (Eliasson et al., 1974; Kurosawa et al., 1975). Covalently bound RNA has also been found in the closed circular DNA of colocinogenic factor E1 of chloramphenicol-treated *E. coli* (Blair et al., 1972) and in mitochondrial DNA (Miyaki et al., 1973; Grossman et al., 1973). The isolation of covalently closed superhelical DNA containing stretches of RNA suggests that preformed pieces of RNA and DNA can be joined to one another. Chloroplast DNA (Kolodner et al., 1975) and the DNAs of both prokaryotic (Buckley et al., 1972; Speyer et al., 1972; Marmur et al., 1972) and eukaryotic (Gordin et al., 1973; Eliasson et al., 1974) viruses and of eukaryotic cells (Sato et al., 1972) also contain stretches of RNA.

In the present work we have purified the L cell DNA ligase, studied some of its enzymatic properties, and examined its ability to seal hybrid substrates. We report that L cell DNA ligase is capable of linking 5′-phosphoryl terminated DNA to 3′-hydroxyl RNA, but that it cannot catalyze the converse reaction of joining 5′-phosphoryl terminated RNA to 3′-hydroxyl DNA.

Experimental Section

Materials. Snake venom phosphodiesterase (VPD) (EC 3.1.4.1), spleen phosphodiesterase (SPD) (EC 3.1.4.18), micrococcal nuclease (MN) (EC 3.1.4.7), and bacterial alkaline phosphatase (BAP C) (EC 3.1.3.1) were purchased from Worthington Corp. BAP C was dialyzed overnight at 1 mg/mL (ca. 70 U/mL) in 0.01 M Tris-HCl (pH 8.0)–0.01 M $MgCl_2$–0.1 M NaCl. Snake venom 5′-nucleotidase (EC 3.1.3.5) and rye grass 3′-nucleotidase (EC 3.1.3.6) were obtained from Sigma Chemical Co. T4 DNA ligase (EC 6.5.1.1) was a gift of C. C. Richardson, and polynucleotide kinase (EC 2.7.1.78) was a gift of O. C. Uhlenbeck. Unlabeled nucleotides and polynucleotides were purchased from P-L Biochemicals Inc. Oligo(I) was prepared by partial hydrolysis of 4.5S poly(I) (15 s at 20 °C in 0.3 M KOH) followed by treatment with BAP to remove all 2′(3′)-phosphoryl groups. The average chain length ($\bar{n}$ = 28) was determined by labeling the 5′-termini with ^{32}P using polynucleotide kinase. Other chemicals were of re-

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[1] Abbreviations following the IUPAC-IUB Commission on Biochemical Nomenclature recommendations [(1971), *J. Mol. Biol. 55,* 299] are used throughout. TEAB, triethylammonium bicarbonate; EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin; PP_i represents pyrophosphate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; *n*, the number of nucleotide residues per chain; $\bar{n}$ for the number-average chain length.

agent grade. [γ-^{32}P]ATP was prepared by a modification of the method of Glynn and Chappell (1964) in which 1 mM dithiothreitol replaced 2 mM cysteine, using $H_3{}^{32}PO_4$ obtained from New England Nuclear Corp. Oligomers were labeled on the 5′-termini with [^{32}P]phosphate using polynucleotide kinase as described by Silber et al. (1972). The labeled products were purified by thin-layer chromatography on cellulose-coated glass plates using 1 M ammonium acetate–ethanol (1:1, v/v) as solvent. Alternatively, products were purified by chromatography on Sephadex G-50 columns with 0.05 M TEAB (pH 7.5).

Assay of DNA Ligase. The DNA ligase assay was essentially that of Olivera and Lehman (1968). Unless stated otherwise, each 50 μL of reaction mixture contained 50 mM Hepes (pH 8.0), 15 mM $MgCl_2$ (10 mM $MgCl_2$ for reactions involving T4 DNA ligase), 500 μM ATP, 5 mM dithiothreitol, 50 μg/mL BSA, 5′-^{32}P-labeled oligomer ($2–6 \times 10^4$ cpm) at a concentration of 1 μM in termini, and an equimolar amount (in nucleotide residues) of unlabeled complementary polydeoxynucleotides. Varying amounts of enzyme (10–40 units) were added, and reactions were incubated at 37 °C for 30 min and were terminated by heating at 100 °C for 2 min. For hybrid substrate sealing reactions, conditions were as described in the figure legends. Alkaline phosphatase resistance was determined as described by Walker et al. (1975), except that the DEAE-paper squares were washed with 10 mM KP_i (pH 7.0) and dried before being spotted with 50 μL of the reaction mixture. The papers were washed once with water, four times with 0.35 M ammonium formate, and once with 95% ethanol before being dried and counted. One unit of activity is defined as that amount of enzyme that seals 1 pmol of [5′-^{32}P]d(pT)$_{17}$ on poly[d(A)] in 30 min at 37 °C.

RNA was hydrolyzed in 0.3 N KOH at 37 °C for 16 h. Polydeoxynucleotides were hydrolyzed to 3′-nucleoside monophosphates with MN and SPD by the method of Josse et al. (1961). Polynucleotides were hydrolyzed to 5′-nucleoside monophosphates with VPD by the procedure of Wu and Kaiser (1967).

Nucleotides were separated by either high-voltage electrophoresis (Silver et al., 1970) or by chromatography on Whatman No. 1 paper with 0.7 M ammonium acetate. In the latter system, the R_f of 3′-dGMP is 0.72 and that of 2′(3′)-IMP is 0.82. Material migrating with markers was eluted with water and counted. Ribonucleotide isomers were separated by chromatography in the presence of borate (Bedows et al., 1975) and deoxyribonucleotide isomers were identified by their susceptibility to either rye grass 3′-nucleotidase or snake venom 5′-nucleotidase. Samples (0.1 mL) were adjusted to pH 7.5 with KOH and 0.06 U of 3′-nucleotidase was added. The mixture was incubated 1 h at 37 °C, another 0.06 U of enzyme was added, and the hydrolysis continued for an additional hour. To test for sensitivity to 5′-nucleotidase, the reactions (0.1 mL) were adjusted to pH 9.0 with KOH and 1.6 U of enzyme was added. The solution was incubated 1 h at 37 °C, an additional 1.6 U of enzyme was added, and the reaction continued for 1 more h. Samples were acidified and adsorbed to charcoal (Greenfield et al., 1975), and the nonadsorbed radioactivity was determined.

The presence of nuclease or phosphatase activities in the DNA ligase preparations was determined by incubating labeled substrates, both with or without complementary strands, with the enzyme under normal assay conditions and chromatographing the reaction mixtures on Whatman DE-81 paper with 0.4 M ammonium bicarbonate.

Purification of DNA Ligase. LM cells were grown to 10^6/mL in suspension culture at 37 °C in Joklik's-modified MEM (GIBCO) supplemented with 5% fetal calf serum (GIBCO) (1 L of medium per 6-L Florence flask). Cells were harvested by centrifugation at 2000g for 3 min and the pellets were suspended in isotonic buffer [140 mM NaCl–20 mM Tris-HCl (pH 7.5)–1 mM $MgCl_2$] at a concentration of ca. 4×10^7 cells/mL. The cells were washed three times in isotonic buffer, suspended at ca. 3×10^8 cells/mL in sonication buffer [100 mM KCl–50 mM Tris-HCl (pH 7.5)–1 mM 2-mercaptoethanol–1 mM EDTA], quick-frozen in acetone–dry ice, and stored at −70 °C.

Frozen cells ($6–8 \times 10^9$) were quick-thawed at 37 °C and disrupted by 20 s of sonication using a Biosonic sonifier with a macro tip at a setting of 80. Debris was removed by centrifuging 20 min at 30 000g. The supernatant fraction was adjusted to 40% saturation with ammonium sulfate (0.24 g/mL) and the pellet removed by centrifugation at 16 000g for 10 min. The resulting supernatant fraction was adjusted to 70% saturation by the addition of 0.21 g/mL of ammonium sulfate and centrifuged as before. The 40–70% ammonium sulfate pellet, which contains all the DNA ligase activity, was dissolved in 0.75 M KCl and purified by gradient sievorptive chromatography (Kirkegaard, 1973). The sample was applied to a 350-mL DEAE-Sephadex A-25 column containing a linear gradient of from 0 to 125 mM KCl in 0.1 M Tris (pH 7.5)–1 mM 2-mercaptoethanol–0.1 mM EDTA and was eluted with 1 M KCl. The tubes containing DNA ligase activity were pooled and the proteins precipitated with a final concentration of 70% ammonium sulfate.

The resulting ammonium sulfate pellet was dissolved in 1 mL of 50 mM Hepes (pH 8.0)–100 mM KCl–1 mM DTT (buffer A) and applied to a 0.9 cm diameter × 60 cm column of Sephadex G-200 which had been equilibrated with the same buffer. Fractions of 2.0 mL were collected at a flow rate of 10 mL/h. The fractions containing ligase activity were pooled and the enzyme was adenylylated by incubation in buffer A containing 10 mM $MgCl_2$ and 500 μM ATP at 37 °C for 5 min. The reaction was terminated by the addition of EDTA to 25 mM. The DNA ligase–AMP complex was precipitated at 70% ammonium sulfate, collected by centrifugation, dissolved in buffer A, and applied to a 0.9 cm diameter × 30 cm Sephadex G-100 column which was equilibrated and eluted with buffer A containing 1 mM EDTA. Adenylylated DNA ligase at this step was stable at 4 °C for at least 2 months.

Results

Enzyme Purity. The purified L cell DNA ligase (10 to 40 units per 50 μL of reaction mixture) was incubated with each labeled substrate, both in the presence and absence of a complementary strand, and the reaction products were characterized by chromatography on DEAE paper. Since no radioactive material migrating faster than the labeled substrates (indicative of shorter oligomers and of orthophosphate) was detected, we conclude that, under these assay conditions, there were no interfering phosphatase, ribo- and deoxyribonuclease activities. It should be mentioned that, although [5′-^{32}P]-d(pT)$_{17}$ and [5′-^{32}P]d(pG)$_{12–18}$ migrate slightly from the origin in this system, oligoribo- and deoxyriboadenylate (n = 12–18) and oligo(I) ($\bar{n}$ = 28) do not. Thus, an exonuclease activity removing a few bases from the 3′ termini of the latter substrates might not have been detected. When the protein concentration was increased sixfold over that used in the joining experiments, some degradation of [5′-^{32}P]d(pA)$_{12–18}$ was observed after 1 h at 37 °C.

It is difficult to determine the degree of purification of the

TABLE I: Requirements of L Cell DNA Ligase.[a]

Conditions	Alkaline phosphatase resistance (pmol/15 min)
1. Complete	20.7
2. Omit Mg^{2+}	<0.05
3. Omit Mg^{2+}, add 0.5 mM Mn^{2+}	5.8
4. Omit BSA	15.9
5. Omit dithiothreitol	5.5
6. Omit ATP	<0.05
7. Omit poly[d(A)]	<0.05
8. Add 0.5 mM PP_i	<0.05

[a] Assays were run for 15 min as described in Experimental Section using [5′-^{32}P]d(T)$_{17}$ and poly[d(A)] as the substrate.

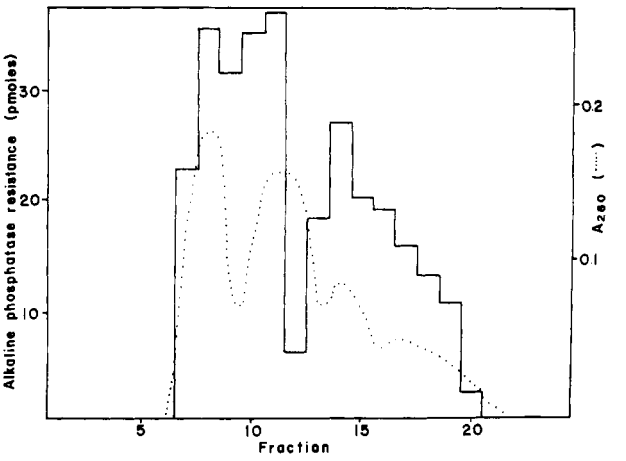

FIGURE 1: Gel filtration of adenylylated L cell DNA ligase on Sephadex G-100. The dotted line (···) represents A_{280} and the solid line (—) DNA ligase activity.

L cell ligase because it exhibits low and highly variable activity in crude extracts and purification yields large increases in total activity. The gradient sievorptive chromatography step consistently produced 6- to 12-fold increases in the total units of recoverable L cell DNA ligase activity. Adding purified enzyme to crude extracts resulted in only 50% inhibition of the added activity, indicating that the low endogenous activity was not simply due to the presence of nucleases or proteases. It is possible that some nondiffusable inhibitor associated with the DNA ligase is removed during the gradient sievorptive chromatography step resulting in the large apparent increase in total activity.

Requirements of the Reaction. The requirements of L cell DNA ligase are summarized in Table I. It is apparent that the reaction requires a divalent cation. Optimal activity occurred with 15 mM $MgCl_2$ or with 0.5 mM $MnCl_2$ which was 25–50% as effective as $MgCl_2$ in supporting the reaction. Ca^{2+} could not satisfy the divalent cation requirement. The partial loss of activity upon omission of dithiothreitol suggests a mercaptan requirement for the enzyme.

L cell DNA ligase, like other eukaryotic DNA ligases, requires ATP. Under standard assay conditions, the apparent K_m for ATP was 70 μM and NAD did not substitute for ATP. Pyrophosphate (0.5 mM) abolished the activity (Table I), presumably by inhibiting the adenylylation of the enzyme (Soderhall and Lindahl, 1973; Lehman, 1974).

The pH optimum for L cell DNA ligase is approximately 8.0 and the enzyme exhibits more activity when assayed in Hepes than it does in Tris at all pH values tested. The enhancement of activity observed by substituting Hepes for Tris in the presence of mercaptoethanol is approximately fivefold at pH 8.0, whereas it is only twofold in the presence of dithiothreitol (data not shown).

When assayed at 37, 30, 20, or 0 °C, the relative rates of L cell DNA ligase activity were 1.0, 0.78, 0.22, and 0.01, respectively. At those temperatures at which activity could be detected, the formation of product remained linear with respect to time and enzyme concentration until about 50% of the substrate became sealed.

Gel Filtration Chromatography. DNA ligase elutes from Sephadex G-200 at a position characteristic of a globular protein with a molecular weight of approximately 150 000 as estimated by the method of Andrews (1965). If the fractions of the G-200 column containing DNA ligase activity are pooled, adenylylated, and passed over a Sephadex G-100 column, the profile shown in Figure 1 is observed. Two peaks of enzyme activity are obtained. One is excluded from the column and the other, which contains less total activity, elutes at a position characteristic of a globular protein with a molecular weight of approximately 75 000. In the particular profile shown in Figure 1, the relative areas of the two peaks do not represent the true proportions of the activities since greater than 60% of the substrate was consumed in the assays of several of the fractions of the peak in the excluded volume. The first peak usually contained four to five times more activity than did the second peak. The enzymes from both peaks demonstrated an identical apparent K_m for ATP (70 μM) and the same concentration optima for both Mg^{2+} (15 mM) and Mn^{2+} (0.5 mM). All the tests of hybrid sealing were performed with the activity from the major peak.

Ability to Catalyze Sealing of Hybrid Substrates. To determine if L cell DNA ligase could catalyze the formation of phosphodiester bonds between short pieces of DNA and RNA on a complementary DNA template, homopolymeric substrates were employed as models. The ability of the ligase to join [5′-^{32}P]d(pA)$_{12-18}$ to (Ap)$_{11}$A or [5′-^{32}P](pA)$_{12}$ to d(Ap)$_{11-17}$A on poly[d(T)] was examined. The rates and extents of these reactions were compared with the DNA to DNA sealing of [5′-^{32}P]d(pA)$_{12-18}$ to itself on poly[d(T)] and to the linkage of [5′-^{32}P]d(pT)$_{17}$ to itself on poly[d(A)]. We also examined the ability of the enzyme to covalently join [5′-^{32}P]d(pG)$_{12-18}$ to 5′-hydroxyl, 3′-hydroxyl oligo(I), [5′-^{32}P]oligo(pI) to d(Gp)$_{11-17}$G, and [5′-^{32}P]d(pG)$_{12-18}$ to itself on poly[d(C)]. In each experiment, an amount of purified T4 DNA ligase which gave approximately the equivalent DNA to DNA sealing was assayed as a control.

Linkage of DNA to DNA. The experiment shown in Figure 2A demonstrates the ability of both the L cell and T4 DNA ligases to seal [5′-^{32}P]d(pT)$_{17}$ on poly[d(A)] at 20 °C. Figure 2B shows the ability of both enzymes to seal [5′-^{32}P]d(pA)$_{12-18}$ on poly[d(T)] at 20 °C after preincubation of the substrate at 0 °C for 6 days. When oligodeoxyadenylates are incubated in the presence of poly[d(T)], a triple-stranded structure is formed initially and only after prolonged incubation at 0 °C in the absence of high salt is this kinetically favored structure converted to the more stable double helical form (Olivera and Lehman, 1968). Virtually no joining occurred without preincubation of the polynucleotides for at least 48 h or after boiling and cooling of the preincubated material prior to the addition of the enzyme. Similar effects have been reported by Olivera and Lehman (1968) with *E. coli* DNA ligase. This figure also compares the rates of joining at 30 °C of [5′-^{32}P]d(pT)$_{17}$ on poly[d(A)] (Figure 2D) to those of [5′-^{32}P]d(pG)$_{12-18}$ on

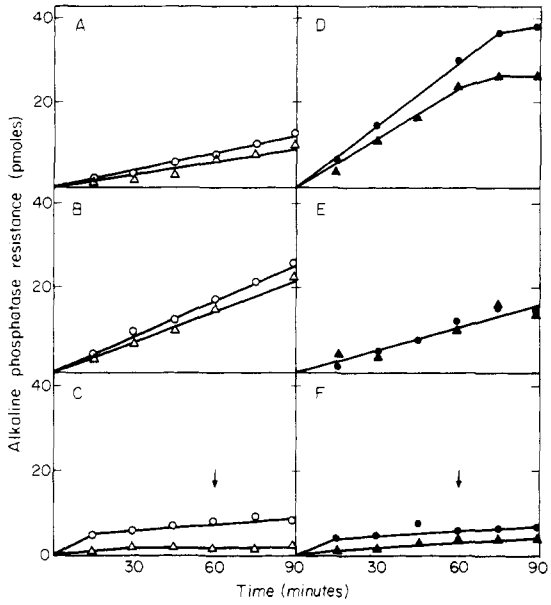

FIGURE 2: The kinetics of DNA ligase sealing. All the experiments were performed with either L cell DNA ligase at 20 (○—○) or 30 °C (●—●) or with T4 DNA ligase at 20 (△—△) or 30 °C (▲–▲) as described in Experimental Section, except that the following substrates were used. A and D represent the sealing of [5′-^{32}P]d(pT)$_{17}$ on poly[d(A)]. B represents the linkage of [5′-^{32}P]d(pA)$_{12-18}$ on poly[d(T)]. C represents the joining of [5′-^{32}P]d(pA)$_{12-18}$ to (Ap)$_{11}$A on poly[d(T)]. E represents the linkage of [5′-^{32}P]d(pG)$_{12-18}$ on poly[d(C)]. F represents the linkage of [5′-^{32}P]d(pG)$_{12-18}$ to oligo(I) on poly[d(C)]. For each of the above, the final concentration in oligomers was 1 μM, and for C and F the [5′-^{32}P]oligomer was present at 0.5 μM. In each reaction, an equimolar amount (in total nucleotide residues) of the complementary polydeoxyribonucleotide was present. Each 50-μL reaction sample contained the following number of counts/min: (A) 20 000; (B) 36 000; (C) 16 000; (D) 42 000; (E) 28 000; (F) 32 000. Twenty units of L cell DNA ligase, or an amount of T4 DNA ligase which gave roughly the same level of DNA to DNA joining at 20 or 30 °C, was added per assay tube. An equal additional amount of enzyme was added after 1 h of incubation in C and F as indicated by the arrows.

poly[d(C)] (Figure 2E). Because r(I)·d(C) (Chamberlin and Patterson, 1965) and d(G)·d(C) (Inman and Baldwin, 1964) form only duplex structures, these substrates did not require preincubation.

In addition to the formation of alkaline phosphatase resistant material as a function of time, sealing was confirmed by hydrolyzing the reaction products with micrococcal nuclease and spleen phosphodiesterase and identifying the nucleotides produced. The hydrolysis of the products of the joining of [5′-^{32}P]oligodeoxythymidylate shown in Figures 2A and 2D yielded material which co-migrated with TMP during high voltage electrophoresis and which was sensitive to the action of 3′-nucleotidase but was not hydrolyzed by 5′-nucleotidase (data not shown). No such material was observed in hydrolysates of reactions incubated in the absence of DNA ligase.

Linkage of RNA to [5′-^{32}P]DNA. Figures 2C and 2F show the rates and extents of formation of alkaline phosphatase resistant material for the joining of [5′-^{32}P]d(pA)$_{12-18}$ to (Ap)$_{11}$A and [5′-^{32}P]d(pG)$_{12-18}$ to 3′-hydroxyl,5′-hydroxyl oligo(I), respectively. In both instances the amount of product formed was (1) substantially lower than for the corresponding DNA to DNA sealing reactions, (2) essentially complete within 30 min, and (3) not stimulated by the addition of more enzyme after 1 h of incubation.

To determine if the alkaline phosphatase resistant material represented the linkage of hybrid substrates or the sealing of DNA to itself, the products were treated with alkali and reincubated with BAP. If a 5′-phosphoryl-DNA to 3′-hydroxyl-RNA linkage had occurred, the products, which were resistant to alkaline phosphatase, should be rendered sensitive to the enzyme by alkaline hydrolysis. If an aliquot from the sample shown in Figure 2F was hydrolyzed with KOH, neutralized, and retreated with BAP, 63% of the counts originally resistant to the alkaline phosphatase were rendered sensitive, whereas greater than 90% of those originally resistant became sensitive to the action of BAP in material from the experiment shown in Figure 2C. The amounts of BAP resistant material formed by the T4 DNA ligase which were rendered sensitive following KOH treatment were 69% in samples from the experiment shown in Figure 2F, and 81% in material from the experiment shown in Figure 2C. When the products of the L cell DNA ligase catalyzed [5′-^{32}P]d(pG)$_{12-18}$ to oligo(I) linkage (Figure 2F) were hydrolyzed with micrococcal nuclease and spleen phosphodiesterase to give 3′-nucleotides, 67% of the counts appearing as nucleoside monophosphates were recovered as IMP, and the rest as dGMP. Greater than 95% of the isolated IMP chromatographed as 2′(3′)-IMP and 72% of the dGMP proved sensitive to treatment with 3′-nucleotidase. Thus it appears that, although some of the [5′-^{32}P] oligodeoxynucleotide joins to itself, approximately two-thirds or more become linked to oligoribonucleotides. Under our assay conditions of equimolar concentrations of ribo- and deoxyribooligomers, approximately equal amounts of RNA and DNA would be expected to become sealed to the 5′-phosphoryl-DNA, assuming random alignment on the template. Since the preferential joining of RNA to the 5′-phosphoryl-DNA occurs with both the L cell and T4 DNA ligases, it is likely a property of the substrates rather than the enzymes. Polyriboadenylates form a duplex when incubated with an equivalent concentration of poly[d(T)] (Riley et al., 1966), whereas oligodeoxyadenylates initially form triple helical structures which shift to a double helical conformation (Olivera and Lehamn, 1968). It is possible that oligo(A),oligo-[d(A)]·poly[d(T)] form unusual structures which lead to the preferred joining of RNA to DNA. We have no explanation for the increased RNA to DNA joining observed with the oligo-[d(pG)],oligo(I)·poly[d(C)] substrate.

Linkage of DNA to [5′-^{32}P]RNA. Little, if any, alkaline phosphatase resistant material was observed when we attempted to join [5′-^{32}P]RNA to a 3′-hydroxyl-DNA. About 1% of the L cell DNA ligase catalyzed products of the [5′-^{32}P](pA)$_{12}$ to d(Ap)$_{11-17}$A reaction consistently appeared as alkaline phosphatase resistant material. This product remained BAP resistant following treatment with KOH as would be expected for a 3′-hydroxyl-DNA joined to a 5′-phosphoryl-RNA. When the alkali treated BAP resistant material was chromatographed on DEAE paper by elution with ammonium bicarbonate, it remained at the origin indicating that it was still an oligomer. Because the yields were low, not enough material could be obtained to perform enzymatic hydrolyses in order to verify the product. No indication of sealing was observed when we attempted to join [5′-^{32}P]oligo(pI) to d(Gp)$_{11-17}$G. Thus, we were unable to convincingly demonstrate the joining of 5′-phosphoryl-RNA to 3′-hydroxyl-DNA with either L cell DNA ligase or T4 DNA ligase.

Discussion

The fact that some of the ribonucleotides which appear in covalently closed circles of mitochondrial DNA are present as oligomers rather than as individual nucleotides (Miyaki et al., 1973; Grossman et al., 1973) suggests that they are sealed in as preformed pieces rather than being randomly misincor-

porated. If the RNA found covalently sealed into mitochondrial DNA were to arise from the extension of an RNA primer with DNA to form a circular molecule, then the 3′ terminus of the DNA would have to be joined to the 5′ terminus of the RNA. It is conceivable that DNA ligase catalyzes this hybrid sealing reaction. However, the homopolymeric models used to test for the joining of 3′-hydroxyl-DNA to 5′-phosphoryl-RNA were inactive with the L cell DNA ligase. These substrates were also inactive with the T4 DNA ligase. Similarly, Nath and Hurwitz (1974) were unable to join a variety of homopolymeric 5′-phosphoryl polyribonucleotides to 3′-hydroxyl polydeoxynucleotides in the presence of a complementary DNA strand with either T4 or *E. coli* DNA ligases, although a limited amount of linkage of $(pA)_3d(pA)_n$ was observed with the T4 DNA ligase in the presence of poly[d(T)]. On the other hand, L cell DNA ligase joins 5′-phosphoryl-DNA to 3′-hydroxyl-RNA, as do the T4 and *E. coli* DNA ligases (Nath and Hurwitz, 1974). Since the DNA ligases studied thus far are incapable of sealing 5′-phosphoryl ribohomopolymers to 3′-hydroxyl deoxyhomopolymers on complementary DNA strands, the question of how RNA becomes sealed into circular DNA remains. It is possible that a 5′-triphosphate terminated RNA is required for sealing. Such 5′-ribonucleoside triphosphate terminated RNA stretches have been observed in replicating polyoma virus DNA (Eliasson et al., 1974). Since T4 DNA ligase cannot catalyze the linkage of 5′-phosphoryl homopolymeric RNA to 3′-hydroxyl homopolymeric DNA in duplex conformation, but can catalyze such sealing utilizing RNA "primed" DNA molecules (Westergaard et al., 1973), it is possible that our inability to detect joining of 5′-phosphoryl-RNA to 3-hydroxyl-DNA with the L cell DNA ligase was due to differences between our homopolymeric model substrates and heteropolymeric nucleic acids. Alternatively, another enzyme may be responsible for the sealing of RNA into DNA.

Multiple forms of DNA ligases have been isolated from eukaryotic cells (for a review, see Soderhall and Lindahl, 1976). In addition, a DNA ligase activity has recently been isolated from purified rat liver mitochondria (Levin and Zimmerman, 1976). It is identical with the rat liver nuclear ligase by all of the criteria examined. In the present study we have investigated the properties of the DNA ligase activity of L cells which corresponds to the high-molecular-weight species (DNA ligase I) isolated from other eukaryotic cells. Whole cell lysates of L cells contain lower levels of a second DNA ligase activity. This activity and that of the larger form share identical pH optima, apparent K_m for ATP, and concentration optima for divalent cations. The low-molecular-weight DNA ligase activity could not be detected in the presence of Tris, but was observed when assays were buffered with Hepes. Exclusive of the present work, no eukaryotic enzymes have been examined for their ability to seal RNA to DNA.

Whether there is a biological function for the observed sealing of a 5′-phosphoryl-DNA to a 3′-hydroxyl-RNA is unknown. However, the demonstration that a eukaryotic DNA ligase can join RNA to DNA to form a copolymer with the RNA at its 5′ terminus cautions against concluding, from the mere isolation from cells of such a copolymer, that an RNA primer was involved in the genesis of the DNA segment.

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