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Addition of oligonucleotides to the 5'-terminus of DNA by T4 RNA ligase

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ABSTRACT

Bacteriophage T4-induced RNA ligase catalyzes the controlled template-independent addition of RNA to the 5'-phosphoryl end of large DNA molecules. Restriction enzyme-generated fragments of ColE1 DNA with completely base-paired or cohesive ends and linear single-stranded φX174 viral DNA were all good substrates. DNA molecules from 10 to 6000 nucleotides long were quantitatively joined in an hour to a number of different RNA homopolyers. The most efficient of these was $A(pA)_5$; $I(pI)_5$ and $C(pC)_5$ were also utilized while $U(pU)_5$ was not. The optimum ribohomopolymer length was six nucleotides. Joining of ribohomopolymers between 10 and 20 nucleotides long occurred at approximately 1/2 the maximal rate and a trimer was the shortest substrate. Thus RNA ligase provides a method for generating extensions of predetermined length and base composition at the 5'-end of large DNA molecules that complements the available procedures for extending the 3'-hydroxyl terminus of DNA.

INTRODUCTION

RNA ligase was first identified in bacteriophage T4-infected cells on the basis of its catalysis of the cyclization of single-stranded RNA chains in the presence of ATP.[1] Subsequently, it was found to use DNA as a substrate[2,3] and to promote intermolecular joining.[4-7] The enzyme is the product of T4 gene 63[8] and consists of a single 41,000 dalton polypeptide.[2,9] RNA ligase has been purified in large amounts from hyperproducing infected cells; it is free from contaminating nuclease activity and is homogenous as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.[10] The mechanism of RNA ligase-catalyzed reactions is analogous to that of DNA ligases[11] except for the important distinction that RNA ligase joins polynucleotides that are not held in juxtaposition by a complementary strand.[1,12] In the first stage, RNA ligase reacts with ATP to form an adenylylated enzyme intermediate and pyrophosphate is released.[12] The second step requires the presence of both a 5'-phosphoryl terminated molecule (donor) and a 3'-hydroxyl terminated nucleic acid (acceptor).[3] AMP is transferred from the enzyme to the 5'-phosphoryl of the donor, recreating a pyrophosphate

linkage.[3,4,13,14] The activated donor molecule does not necessarily join to the acceptor which stimulated its formation. The term "acceptor exchange" was coined for cases where the activated intermediate was formed in the presence of one acceptor but was ligated to a different acceptor.[3] In the last stage, enzyme condenses the activated donor and acceptor to form a phosphodiester bond with the release of AMP.[12]

Previous studies of DNA joining by RNA ligase have generally employed relatively short, single-stranded, homopolymeric substrates. RNA ligase catalyzed the cyclization of DNA and the joining of DNA to RNA and DNA acceptors.[2,3] The shortest cyclizable DNA was a hexanucleotide, and at the optimum chain length of 20 nucleotides, the rate of joining was about one-tenth that of cyclization of poly(A).[3] Intramolecular joining diminished with polymers longer than 20 nucleotides in length. Similarly, poly(A) chains 30-40 nucleotides long were much better substrates for cyclization than poly(A) chains 300 nucleotides long.[1] This report investigates RNA ligase as a tool for structuring long pieces of natural DNA. The enzyme joins ribohomopolymers in high yield to the 5'-phosphoryl terminus of DNA in a reaction we will designate as 5'-tailing. A variety of DNA donors up to 6000 base pairs long and varying in structure from completely single-stranded DNA to duplex DNA with base paired or cohesive ends have similar activity. The 5'-tailing reaction generates a single product with predetermined base composition and chain length. This procedure complements methods employing terminal deoxynucleotidyl transferase and polynucleotide phosphorylase for template-independent addition of polynucleotides to the 3'-hydroxyl end of DNA.

MATERIALS AND METHODS

Nucleid acids. $I(pI)_5$ was obtained from P.L. Biochemicals, Inc; all other synthetic nucleic acids were purchased from Collaborative Research, Inc. ColE1 DNA was isolated by the method of Staudenbauer[15] and φX174 viral DNA was isolated by the technique of Yamamoto et al.[16] To prepare fragments of ColE1 DNA, 40 μg of DNA was digested for 1 h with EcoRl or HaeIII restriction endonucleases at 37° in 0.1-ml reaction mixtures containing 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, and 10 mM $MgCl_2$. To label the terminal 5'-phosphoryl groups by exchange, 10 μM ADP, 10 μM [γ-^{32}P]ATP, 50 mM imidazole/HCl, pH 6.5, and 50 units of T4 polynucleotide kinase were added.[17] After 1 h at 37°, 50-90% of the termini were labeled and the reaction was stopped by heating to 70° for 10 min. The DNA was purified by filtration through a 20-ml Sepharose 4-B (Pharmacia) column equilibrated with 10 mM Tris/HCl, pH 7.5 and 1 mM EDTA

and was concentrated by extraction with 2-butanol.[18] The butanol was removed by ether extraction.

Enzymes. EcoRl and HaeIII restriction endonucleases were gifts of H. Boyer and W. Brown of the University of California, respectively. T4 induced polynucleotide kinase was purified by the method of Panet et al.[19] RNA ligase was purified to homogeneity by the procedure of Higgins et al.[10] and had a specific activity of 6500 units/mg protein. Bacterial alkaline phosphatase, type III_S, was purchased from Sigma; snake venom phosphodiesterase and deoxyribonuclease I (bovine pancreas) were from Worthington Biochemical Corp.

Enzyme assays. RNA ligase tailing reactions were performed in a 20-µl mixture containing 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), pH 7.9, 20 mM dithiothreitol, 1 mM ATP, 100 µg of bovine serum albumin per ml, 5 mM $MnCl_2$, 0.2 µM 5'-^{32}P-labeled DNA donor, 100 µM acceptor, and 0.7 unit of RNA ligase. The concentration of nucleic acids is expressed in terms of 5'-termini unless indicated otherwise. After incubation at 23°, the product was analyzed by one of two methods. The standard assay measured the conversion of the ^{32}P label to a form resistant to bacterial alkaline phosphatase.[1,2,3] Reactions stopped by heating at 100° for 2 min were treated with 1 unit of alkaline phosphatase at 65° for 20 min. Successively, 0.1 ml of 0.1 M P_i and 0.1 M PP_i, 0.1 ml of 0.4 mg/ml of salmon sperm DNA and 0.01 M EDTA, and 0.1 ml of 2.5 M perchloric acid were added, and Norit adsorbable radioactivity was measured. The second assay measured the transfer of radioactivity from the DNA donor to the RNA acceptor after alkaline hydrolysis. Reactions were made 0.3 M in NaOH and incubated at 37° for 16 h. After neutralization with HCl, a portion was spotted onto a polyethyleneiminocellulose thin-layer plate (Cel 300 PEI/UV_{254}, Brinkmann Instruments, Inc.). Salt was removed by a methanol wash, and the chromatogram was developed in 2% H_3BO_3--2 M LiCl (2:1) to resolve 2', 3' ribomononucleotides.[20] This is the more specific of the two assays since cyclization or adenylylation of the donor also confers phosphatase resistance. End-group analysis was carried out in a 20-µl reaction mixture containing 50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 0.2 µM 5'-^{32}P-labeled DNA and 200 µg/ml of DNase I. After 30 min at 37°, the pH was raised to 9.0 with glycine buffer and venom phosphodiesterase was added to 0.2 mg/ml. The incubation was continued at 37° for 30 min and a portion of the digest was analyzed by polyethyleneiminocellulose thin-layer chromatography.

RESULTS

Our previous studies with RNA ligase focused on relatively small single-

stranded synthetic substrates.[2,3] However, many applications for modifying DNA molecules dictate use of very large substrates that would commonly also be double stranded. Two types of labeled duplex DNA donors were prepared for testing as RNA ligase substrates. Duplex DNA with base-paired ends was generated by cleavage of ColE1 DNA with HaeIII endonuclease, and duplex DNA having a tetranucleotide protrusion terminated with a 5'-phosphoryl was prepared by cleavage of ColE1 DNA with EcoR1 endonuclease. The 5'-termini of the DNA were labeled with ^{32}P using the polynucleotide kinase catalyzed exchange reaction.[17] The label was shown to be specifically on the terminal phosphate of the EcoR1 nuclease-cleaved DNA in two ways. First, after digestion to 5'-mononucleotides with DNase I and snake venom phosphodiesterase, >90% of the label was in deoxyriboadenylic acid as expected from the cleavage specificity of the restriction nuclease.[21] Second, the DNA was digested with HaeIII endonuclease and the fragments were resolved by agarose gel electrophoresis (Fig. 1). All of the radioactivity was in fragments 3 and 6, the two terminal fragments.[22]

The ColE1 DNA fragments were tailed with oligoribonucleotide acceptors having a hydroxyl at both the 3'- and 5'- termini in order to exclude intramolecular reaction of the acceptors and to limit the tailing product to a single addition. Substantial RNA tailing of both EcoR1- and HaeIII-generated fragments was observed under the reaction conditions used previously for RNA and DNA joining.[2,3] However, the rate of reaction was enhanced up to 20-fold by substitution of 5 mM $MnCl_2$ for 10 mM $MgCl_2$, and HEPES buffer, pH 7.9, for Tris/HCl, pH 7.5. These improved conditions were then used exclusively. The tailing of HaeIII-cleaved DNA with $A(pA)_5$ was measured as a function of donor and acceptor concentration (Fig. 2). The reaction was not linear with time and after an initial burst tailing continued at a reduced rate for several hours. The reaction was linearly dependent on donor amount so that at a given acceptor level the same percent of donor was tailed over a ten-fold range in donor concentration (Fig. 2 and data not shown). The influence of acceptor concentration was more complex. The ratio of donor to acceptor was varied from 1:80 to 1:3200; increases in acceptor concentration enhanced the initial burst of tailing but after 20 min the rate was about the same for all acceptor levels compared at any given donor concentration (Fig. 2). Saturation with donor or acceptor was not observed with any concentration of nucleic acid as has been found with other reactions catalyzed by RNA ligase.[2,12,23] The initial phase of the reaction was proportional to enzyme amount, and at a molar ratio

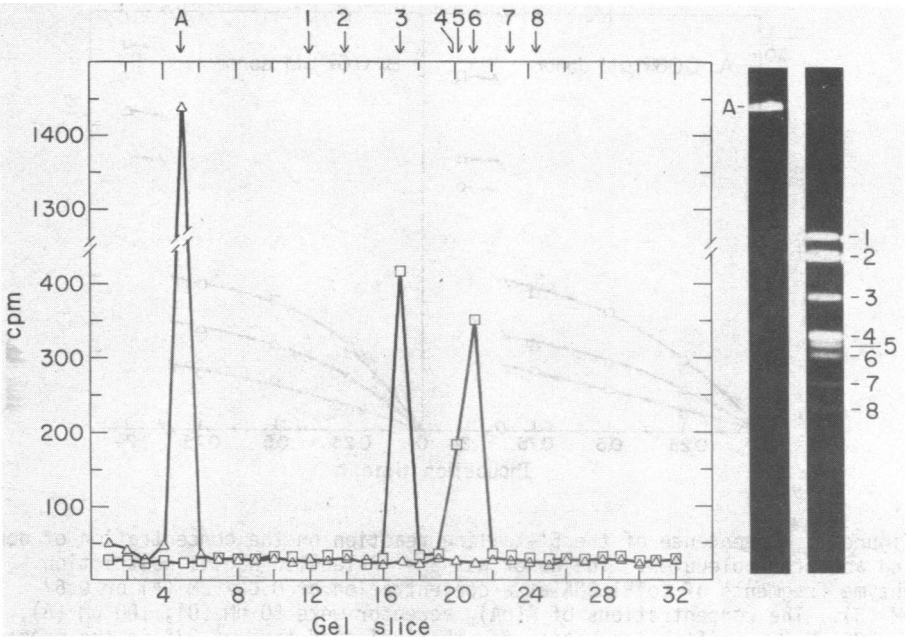

Figure 1. Specific labeling of the EcoR1 restriction ends of ColE1 DNA. Native ColE1 was digested with EcoR1 restriction endonuclease and the 5'-ends were labeled by exchange with [γ-^{32}P]ATP in a reaction catalyzed by polynucleotide kinase. The DNA was purified and displayed by electrophoresis through a 1% agarose gel with and without prior digestion with HaeIII restriction enzyme. A photograph of the ethidium bromide stained gels is shown at the right. The undigested DNA is labeled A and the restriction enzyme fragments are numbered 1 through 8. The gels with the digested (□) and undigested DNA (Δ) were cut into 1 mm slices and counted (left). At the top, the mobility of the stained DNA is shown.

of enzyme to DNA donor of 5:1 quantitative tailing was observed in 15 min (Fig. 3).

Ligation was measured in the experiments described thus far by conversion of the 5'-^{32}P-label to a form which is resistant to hydrolysis by bacterial alkaline phosphatase. However, large amounts of the adenylylated donor intermediate accumulated in some RNA ligase reactions.[3] Since adenylylation as well as cyclization would protect the 5'-phosphoryl group from phosphatase digestion, proof that the donor had been joined to the acceptor was required. Alkaline digestion of a portion of DNA tailing reaction mixtures resulted in production of [^{32}P]2'-3'AMP that was equal in amount to the phosphatase-resistant ^{32}P present in the sample. This demonstrated that the product was a block copolymer of DNA and RNA joined by a phosphodiester bond. We found no significant ac-

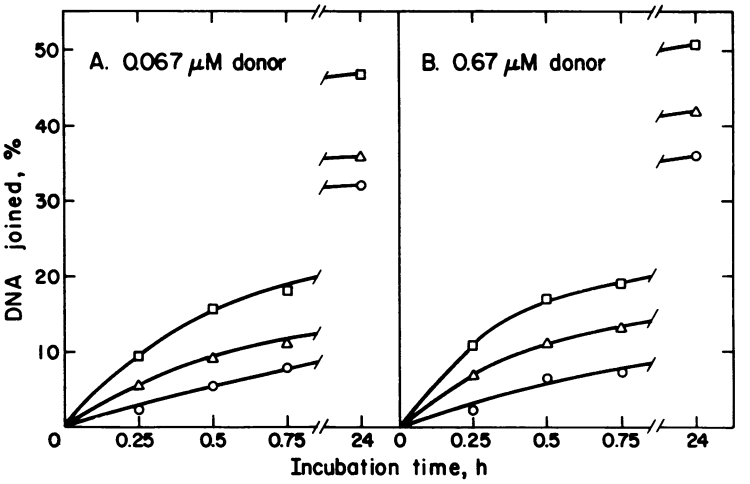

Figure 2. Dependence of the 5'-tailing reaction on the concentration of donor and acceptor molecules. The donor was 5'-^{32}P-labeled HaeIII restriction enzyme fragments of ColE1 DNA at a concentration of 0.067 μM (A) or 0.67 μM (B). The concentrations of A(pA)$_5$ acceptor were 50 μM (O), 100 μM (Δ), or 200 μM (□). After incubation for the indicated time at 23° in the presence of 0.7 unit of RNA ligase the phosphatase-resistant ^{32}P was measured.

cumulation of the adenylylated donor in any of the tailing reactions in this report.

The acceptor specificity of 5'-tailing was markedly influenced by chain length and base composition (Fig. 4). Both the initial rate and the final reaction yield rose with increasing acceptor chain length up to an optimum at 5-6 nucleotides and fell off gradually with increasing chain length. The efficiency of various donors expressed as the fraction of the rate observed with a hexanucleotide was <0.05 for ApA, 0.3 for A(pA)$_2$, 1.0 for A(pA)$_4$, 1.0 for A(pA)$_5$, 0.8 for A(pA)$_9$, 0.5 for A(pA)$_{11-21}$, and <0.05 for A(pA)$_{200}$. For homopolymers containing inosine, I(pI)$_9$ was used only 25% as well as I(pI)$_5$, and in the C series, C(pC)$_9$ was only slightly less effective than C(pC)$_5$ (Fig. 4B). There was a notable effect of base composition for all lengths of polymers tested. I(pI)$_5$ and C(pC)$_5$ were 40% and 20% as effective acceptors as A(pA)$_5$ (Fig. 4), and U(pU)$_5$ was inactive under all conditions tested (data not shown).

We had anticipated that the terminal structure and conformation of the DNA donor might exert a strong influence on the 5'-tailing reaction. Fortunately, this was not the case. The HaeIII-generated fragments containing

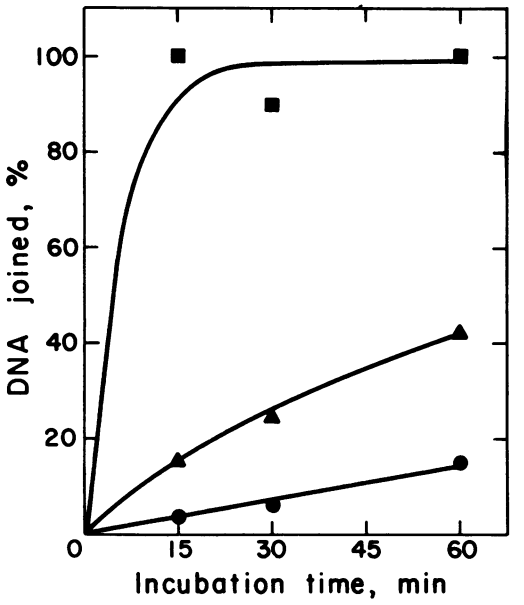

Figure 3. Dependence of the tailing reaction on enzyme concentration. Tailing reactions incubated at 23° for the indicated times contained 0.67 μM 5'-^{32}P-labeled HaeIII restriction endonuclease fragments as donor, 100 μM $A(pA)_5$ acceptor, and 3.5 (■), 0.7 (▲), or 0.14 (●) units of RNA ligase. The production of phosphatase resistant radioactive material was measured.

fully base-paired ends and the EcoRl-cleaved DNA with a protruding 5'-end were equally effective for $A(pA)_5$ tailing and nearly so for $I(pI)_5$ tailing (Fig. 5). Large single-stranded DNA donors are also good substrates in the tailing reaction. Heat denaturation of ColEl DNA restricted with HaeIII nuclease did not appreciably alter the rate or extent of joining to $A(pA)_5$ (Fig. 5B). The intermolecular nature of the joining was confirmed by a nearest neighbor analysis in which the amount of ^{32}P transferred by alkali to 2'-3'AMP was, within experimental error, equal to the amount of the phosphatase-resistant radioactivity. We also tested the donor activity of φX174 viral DNA because it is much larger than the HaeIII fragments of ColEl DNA and because it does not contain a complementary strand and therefore can not renature during the reaction. DNA was prepared containing about 30% broken rings and 5'-labeled with ^{32}P by polynucleotide kinase catalyzed exchange with [γ-^{32}P]ATP; the DNA was also uniformly ^{3}H-labeled to provide an internal marker for size analysis. After incubation of this substrate for 2 h in the presence of 0.7 unit of RNA ligase and 100 μM $A(pA)_5$, a portion of

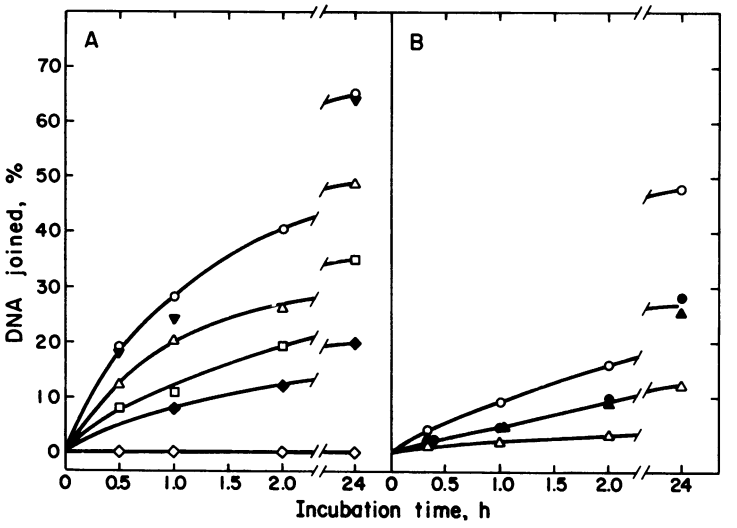

Figure 4. Effects of acceptor chain length and base composition on the 5'-tailing reaction. 5'-^{32}P-labeled HaeIII restriction enzyme fragments of ColE1 DNA (0.21 μM) were incubated at 23° with 0.7 unit of RNA ligase and 100 μM of the following acceptors: in A, ApA (◇), A(pA)$_2$ (◆), A(pA)$_4$ (○), A(pA)$_5$ (▼), A(pA)$_9$ (△), A(pA)$_{11-21}$ (□), or A(pA)$_{200}$ (◇); in B, I(pI)$_5$ (○), I(pI)$_9$ (△), C(pC)$_5$ (●), or C(pC)$_9$ (▲). After the indicated times, the phosphatase-resistant ^{32}P was measured.

the reaction mixture was digested with alkali. 50% of the ^{32}P radioactivity was transferred to 2'-3'AMP; this proves that substantial joining had occurred. To ensure that contaminating small pieces of DNA had not preferentially joined to the acceptor, the product was displayed by sedimentation through a neutral sucrose gradient (Fig. 6). The ^{3}H and the phosphatase-resistant ^{32}P were coincident, and most of the counts were in 12-13 S material proving that joining had occurred on full length molecules. Some ^{3}H and ^{32}P was in contaminating small molecules near the top of the gradient. The reaction rate with single-stranded DNA seemed independent of donor chain length since dentaured HaeIII-cut ColE1 DNA and ϕX174 DNA at equivalent concentrations of ends were tailed at nearly the same rate (Fig. 5).

DISCUSSION

RNA ligase is a versatile reagent for the construction of nucleic acids. Intermolecular reactions leading to high yield synthesis of oligoribonucleotides with a defined sequence have been described by several groups.[6,13,23-26] With

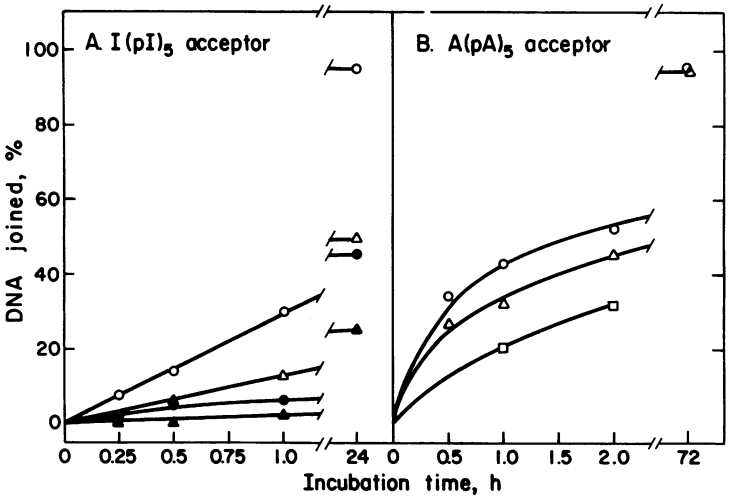

Figure 5. Comparison of DNA donors in the 5'-tailing reaction. In A, 5'-^{32}P-labeled EcoRI restriction enzyme fragments (open symbols) or HaeIII restriction enzyme fragments (closed symbols) of ColE1 DNA were incubated with a 100 μM $I(pI)_5$ acceptor and 0.7 unit of RNA ligase (Δ, ▲) or 2.1 units of RNA ligase (O, ●). In B, the reaction contained 2.1 units of RNA ligase, 100 μM $A(pA)_5$ as acceptor, and 0.21 μM of 5'-^{32}P-labeled native (O) or heat-denatured (Δ) HaeIII restriction enzyme fragments of ColE1 DNA or 0.15 μM φX174 single-stranded linear DNA (□). Tailed product was measured by phosphatase-resistant ^{32}P.

natural RNA substrates, Kaufmann and Littauer joined phenylalanine tRNA half-molecules[4] and Meyhack et al. constructed substrates for the enzyme that processes ribosomal RNA.[27] Bruce and Uhlenbeck and England and Uhlenbeck demonstrated 3'-labeling of several molecules including tRNA, mRNA, and double- and single-stranded viral RNA with mononucleoside 3', 5'-bisphosphates.[28,29] As first shown in this laboratory, DNA is also a substrate for RNA ligase. RNA ligase catalyzes the intermolecular joining and circularization of a number of small DNA homopolymers,[2] and efficient construction of RNA-DNA chimeras using small single-stranded synthetic substrates has been demonstrated.[2,3] Several oligodeoxyribonucleotide acceptors have been joined to 2'-deoxyribonucleoside 3', 5'-bisphosphates.[30] The joining of DNA fragments at fully base-paired ends by T4 DNA ligase,[31] a useful genetic engineering procedure, is stimulated up to 20-fold by RNA ligase.[32]

In this report, we have shown that RNA ligase can efficiently generate single-strand extensions at the 5'-termini of large DNA molecules derived from cells. There is a striking discrepancy, however, between donor and

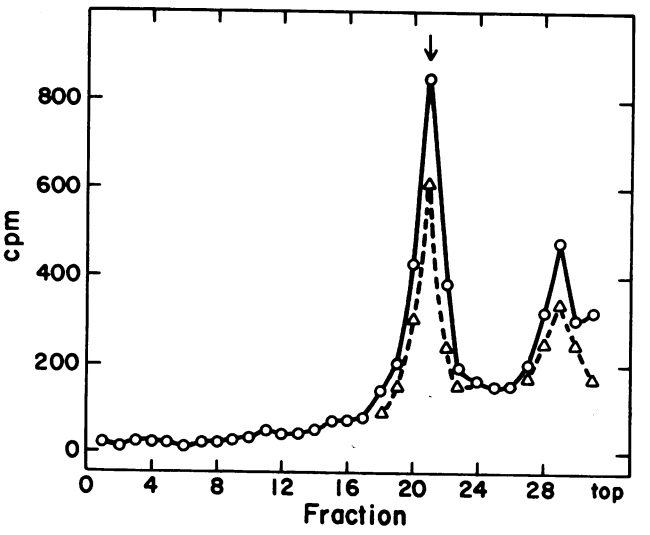

Figure 6. Sedimentation of ϕX174 DNA tailed with $A(pA)_5$. Linear, single-stranded ϕX174 DNA labeled uniformly with [^{3}H]thymidine and with ^{32}P at its 5'-terminus was tailed with $A(pA)_5$; 50% of the donor reacted as judged by the transfer of ^{32}P to Ap after alkaline hydrolysis. After sedimentation through a 3.8-ml 5 to 20% linear sucrose gradient, fractions were first counted by Cherenkov radiation (O) and then treated with bacterial alkaline phosphatase and the acid-precipitable ^{32}P (Δ) determined. The arrow indicates the position of the ^{3}H-labeled ϕX174 viral DNA peak.

acceptor molecules in the RNA ligase tailing reaction. Recognition of acceptor involves the sugar, base, and length of the chain. Ribose is much better than deoxyribose, and the base preference is adenine>inosine>cytosine>> uracil; intermolecular joining of short oligoribonucleotides showed similar base effects in that acceptors containing U at the 3'-hydroxyl end were much poorer than molecules containing A, I, or C, and $I(pI)_5$ was 5-fold poorer than $A(pA)_5$.[33] The optimum chain length for acceptors is very small, approximately 5-6 nucleotides, although molecules 10-20 nucleotides in length are utilized at about half of the maximum rate. The minimum acceptor chain length for both 5'-tailing and intermolecular joining of oligoribonucleotides is the same--a trinucleoside diphosphate.[29] For the donor in 5'-tailing, the base composition, chain length, and secondary structure are largely irrelevant. Double- or single-stranded molecules up to 6000 base pairs long can be tailed quantitatively and at about the same rate. The synthetic decanucleotide duplex, pC-C-G-A-A-T-T-C-G-G / G-G-C-T-T-A-A-G-C-Cp, can be tailed quantitatively with $A(pA)_2$ in 1 h (A. Sugino and N.R. Cozzarelli, unpublished data).

RNA ligase complements polynucleotide phosphorylase and terminal deoxynucleotidyl transferase, which sequentially add deoxyribonucleotides or ribonucleotides to the 3'-hydroxyl ends of DNA.[34,35] RNA ligase 5'-tailing occurs in a single condensation reaction so that the size and base composition of the extension product is predetermined. The product of transferase and phosphorylase reactions, on the other hand, has a distribution of different lengths that can be varied somewhat by altering the extent of incubation or the ratio of nucleotide precursors to primer termini.

The permissive utilization of donors suggests a number of applications for the RNA ligase 5'-tailing technology. These include: 1) Synthesis of highly selective substrates for nucleases such as RNase H. 2) Isolation of the 5'-terminal fragments of DNA molecules by tailing the DNA with $A(pA)_n$, digesting the product with a restriction endonuclease, and subjecting the fragments to oligo(dT)-cellulose chromatography. 3) Generation of complementary ribonucleotide termini on two separate DNA fragments by tailing one with $C(pC)_n$ and the other with $I(pI)_n$. 4) Creation of a single-stranded DNA circle held together with a terminal duplex by tailing the single-stranded DNA at the 5'-end with RNA ligase, adding the complementary sequence to the 3'-end with terminal deoxynucleotidyl transferase, and annealing the extensions. 5) Generation of defined duplex extensions of variable chain length on duplex DNA fragments by first tailing with RNA ligase and then copying the tail into DNA with reverse transcriptase. This procedure could prove useful for placing a cloned DNA fragment into all possible reading frames near a bacterial or viral promoter.

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