T_4 Polynucleotide Ligase Catalyzed Joining of Short Synthetic DNA Duplexes at Base-Paired Ends†

Ken V. Deugau and Johan H. van de Sande*

ABSTRACT: The self-complementary octanucleotide dT-A-G-T-A-C-T-A has been synthesized and its sequence confirmed by two-dimensional fingerprinting. Under conditions used for the T_4 polynucleotide ligase reaction, this oligonucleotide forms a dimeric duplex which shows a T_m of 18 °C. The optimal rate of joining of the ^{32}P-labeled duplex occurs between 12 and 15 °C. The rate is highly concentration dependent, as expected for a bimolecular process. Polyacrylamide gel electrophoretic analysis of this reaction shows the presence of products up to 120 nucleotides in length. In a denaturing gel, each product appears as a double band due to the presence of its 5′-adenylylated activated intermediate. Substrates larger than eight base pairs are utilized more rapidly than the eight base pair duplex, indicating that the T_4 ligase has a higher affinity for longer substrates. The low level of nicked intermediates suggests that the joining of both strands requires two steps, the rates of which must be similar.

Polynucleotide ligases have been isolated from a variety of organisms (Lehman, 1974). The best characterized of these enzymes, from *E. coli* and from T_4 infected *E. coli*, have been purified to homogeneity and each is composed of a single polypeptide chain (Modrich and Lehman, 1973; Panet et al., 1973). Both enzymes catalyze the synthesis of phosphodiester bonds between adjacent 5′-phosphoryl and 3′-hydroxyl ends in nicked duplex DNA, the *E. coli* ligase requiring NAD^+ as cofactor (Olivera and Lehman, 1967) and the T_4 ligase requiring ATP (Weiss and Richardson, 1967). In addition, T_4 polynucleotide ligase, but not the *E. coli* enzyme, catalyzes the joining of oligoribonucleotides or oligodeoxyribonucleotides in RNA/DNA hybrid duplexes (Kleppe et al., 1970; Fareed et al., 1971) and the joining of oligoribonucleotides in RNA/RNA duplexes (Sano and Feix, 1974). Furthermore, the T_4 ligase can also carry out the joining of duplex DNA molecules at completely base-paired ends (Sgaramella et al., 1970; Sgaramella, 1972; Sgaramella and Khorana, 1972). This joining activity of T_4 polynucleotide ligase has been used to attach a synthetic *Eco*RI cleavage site to synthetic *lac* operator DNA for subsequent insertion of the operator DNA into a plasmid (Heynecker et al., 1976; Backman et al., 1976; Bahl et al., 1976) and to attach synthetic *Hin*dIII sites to cDNA complementary to rat islet mRNA (Ullrich et al., 1977). Joining of base-paired ends by T_4 polynucleotide ligase is also stimulated by T_4-induced RNA ligase (Sugino et al., 1977).

To study this unusual activity of the T_4 polynucleotide ligase in more detail, polymerization of the self-complementary octadeoxyribonucleotide dT-A-G-T-A-C-T-A has been investigated. In the present paper, we report the use of T_4 polynucleotide ligase for the polymerization of this octanucleotide duplex and describe some initial observations on the mechanism of the T_4 polynucleotide ligase catalyzed joining reaction.

Experimental Section

Materials. DEAE[1]-cellulose (DE-23) was obtained from Whatman. Cellulose (MN300) and DEAE-cellulose (MN300 DEAE) for thin-layer chromatography were purchased from Machery and Nagel Co. Cellulose acetate membrane strips were obtained from Schleicher and Schuell Inc. Urea (Baker reagent) was purified prior to use by passing a 10 M solution through mixed bed ion exchanger (Bio-Rad AG 501 X8, 20–50 mesh) and DE-23. Triethylamine (Baker reagent) was distilled from KOH prior to preparation of triethylammonium bicarbonate (TEAB) according to Smith and Khorana (1963). Pyridine (Baker reagent) was distilled from chlorosulfonic acid, then from KOH, and subsequently stored over molecular sieves (4 Å). Mesitylenesulfonyl chloride (MSCl) was recrystallized from anhydrous pentane. Acrylamide and *N,N′*-methylenebisacrylamide were recrystallized from chloroform and acetone, respectively.

[γ-^{32}P]ATP was prepared according to the published procedure (Glynn and Chappell, 1964) at a specific activity of 75–150 Ci/mmol (Chaconas et al., 1975). Some batches of [γ-^{32}P]ATP were obtained from New England Nuclear.

Enzymes. Polynucleotide ligase (final specific activity: 22 000 units/mg as defined by Kleppe et al., 1970) and kinase were isolated from T_4 *am* N82 infected *E. coli* B62 cells as described previously (Panet et al., 1973). Each enzyme displayed a single band on $NaDodSO_4$–polyacrylamide gel electrophoresis and no nuclease could be detected in either enzyme using the assay described by Weiss et al. (1968). Bacterial alkaline phosphatase (BAP) and snake venom phosphodiesterase (SVD) were purchased from Worthington Biochemicals and were used without further purification.

Synthesis of the Octanucleotide: dT-A-G-T-A-C-T-A. All phosphodiester condensations were carried out at room temperature in anhydrous pyridine. All additions of reagents during the coupling reactions were performed in a drybox. The amount of condensing agent (MSCl) was generally 0.7 equiv per phosphate charge. Selective 3′-deacylation of the protected oligonucleotide was carried out in a 25% pyridine solution containing 1 M NaOH at 0 °C for 5 min. This hydrolysis re-

† From the Division of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 1N4. *Received March 7, 1977; revised manuscript received October 28, 1977.* This work was supported by the Medical Research Council of Canada.

[1] Abbreviations used: DEAE, diethylaminoethyl; TEAB, triethylammonium bicarbonate; MSCl, mesitylenesulfonyl chloride; BAP, bacterial alkaline phosphatase; SVD, snake venom phosphodiesterase; DCC, dicyclohexylcarbodiimide; TBM, Tris-borate/magnesium; Tris, tris(hydroxymethyl)aminomethane; TBE, Tris-borate/EDTA; EDTA, ethylenediaminetetraacetic acid.

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action was terminated by neutralization with excess pyridinium Dowex-50 ion-exchange resin. The condensation reaction products were fractionated on DEAE-cellulose by elution with gradients of TEAB buffer (pH 7.5) containing ethanol at 10–40%. Eluted nucleotide material was analyzed by comparison of the recorded UV spectra with calculated UV spectra for the expected products. Confirmation of peak identity was also obtained by paper chromatographic analysis of deacylated products in solvent A (1-propanol/NH_4OH/H_2O, 55/10/35, v/v/v) (Weber and Khorana, 1972). Homogeneous product fractions were pooled and concentrated by coevaporation with pyridine. The nucleotide product was obtained by amorphous precipitation in ether from an anhydrous pyridine solution. The synthetic scheme for the synthesis of dT-A-G-T-A-C-T-A is shown in Figure 1. The compounds numbered **1** through **8** in this figure are referred to by the same numbering in this paper.

Synthesis of the Trinucleotide d(MeOTr)T-bzA-ibG (**1**). d(MeOTr)T (3.0 mmol, Schaller and Khorana, 1963) was coupled with dpbzA(Ac) (4.1 mmol) in anhydrous pyridine (25 mL) using mesitylenesulfonyl chloride (MSCl) (1.1 g, 5.7 mmol). The dinucleotide d(MeOTr)T-bzA was purified by DEAE-cellulose chromatography and precipitated in ether (yield, 1.6 mmol, 53%; R_f 2.27(dpT) in solvent A).

The dinucleotide was coupled to dpibG(ib) (4.1 mmol) in anhydrous pyridine (20 mL) using MSCl (1.5 g, 6.9 mmol). The trinucleotide d(MeOTr)T-bzA-ibG (**1**) was isolated in the prescribed manner (yield, 0.62 mmol, 39%; R_f(d(MeOTr)T-A-G) = 1.63(dpT) in solvent A).

Synthesis of the Pentanucleotide d(MeOTr)T-bzA-ibG-T-bzA (**5**). The trinucleotide (**1**) (0.76 mmol) was coupled with the dinucleotide (**2**) dpT-bzA(Ac) (2.6 mmol) (Caruthers et al., 1972) with MSCl (1.43 g, 6.5 mmol) in anhydrous pyridine (10 mL). The pentanucleotide (**5**) was purified twice by DEAE-cellulose chromatography before precipitating in ether (yield, 0.15 mmol, 20%; R_f(d(MeOTr)T-A-G-T-A) = 1.37(dpT) in solvent A).

Synthesis of the Trinucleotide dpanC-T-bzA (**3**). The dinucleotide **4**, dpanC-T (1.25 mmol) (Büchi and Khorana, 1972), was treated with 5 mL of hydracrylonitrile and dicyclohexylcarbodiimide (DCC) (3.86 g) in anhydrous pyridine for 24 h. After aqueous hydrolysis of the DCC and removal of cyclohexylurea, the product, d(CNEt)panC-T, was obtained by precipitation in ether from anhydrous pyridine (yield, 1.0 mmol, 80%).

The cyanoethylated dinucleotide thus prepared was coupled with dpbzA(Ac) (2.5 mmol) with MSCl (1.04 g, 4.75 mmol) to give the trinucleotide **3**, dpanC-T-bzA (0.39 mmol, 39%; R_f(dpC-T-A) = 0.63(dpT) in solvent A).

Synthesis of the Octanucleotide d(MeOTr)T-bzA-ibG-T-bzA-anC-T-bzA (**6**). The pentanucleotide **5** (0.063 mmol) was coupled with the trinucleotide **3** (0.383 mmol) with MSCl (0.173 g, 0.791 mmol) in anhydrous pyridine (10 mL). The protected octanucleotide **6** was obtained (880 OD_{275}, 13% yield).

Isolation of the Deprotected Octanucleotide dT-A-G-T-A-C-T-A (**8**). The protected octanucleotide **6**, d(MeOTr)-T-bzA-ibG-T-bzA-anC-T-bzA (880 OD_{275}), was treated with 15 M ammonium hydroxide, the oligonucleotide was purified by preparative paper chromatography on Whatman 1 in solvent A. Octamer, d(MeOTr)T-A-G-T-A-C-T-A (**7**), was eluted from the paper and obtained 280 OD_{260}. R_f of d(MeOTr)T-A-G-T-A-C-T-A = 0.88(dpT). Removal of the 5′-methoxytrityl group was effected by treating compound **7** with 80% acetic acid for 45 min at room temperature. Preparative paper chromatography purification gave the completely deprotected octamer, dT-A-G-T-A-C-T-A, 115 OD_{260} (R_f of dT-A-G-T-A-C-T-A = 0.35(dpT), in solvent A).

An additional purification step was carried out by passing the octanucleotide through a 0.7 cm × 90 cm DEAE-cellulose column containing 7 M urea (Tomlinson and Tener, 1963). A single oligonucleotide peak was eluted from this column using a linear NaCl gradient (0–0.25 M) (500 mL) containing 7 M urea. The octanucleotide was eluted at 0.21 M NaCl and after desalting by passage over Bio-Gel P-2 it was stored at −70 °C.

An aliquot (0.05 OD_{260}) of the octanucleotide was analyzed by high-pressure liquid chromatography on RPC-5 using a linear gradient of ammonium acetate. A single symmetrical peak containing more than 95% of the UV absorbance was obtained. A nucleotide analysis of the octanucleotide was carried out by treating an aliquot (0.1 OD_{260}) with snake venom phosphodiesterase followed by bacterial alkaline phosphatase (BAP) and analyzing by high-pressure liquid chromatography on Aminex A-5 as described by Pike and Rottman (1974) (T:A:G:C, 2.91:3.21:1.00:0.96). Both high-pressure liquid chromatographic separations were carried out by Dr. S. Gillam, Department of Biochemistry, University of British Columbia.

Determination of T_m. The melting temperature for dT-A-G-T-A-C-T-A duplex was determined spectrophotometrically using a Gilford 2400 spectrophotometer equipped with a temperature regulated cuvette holder.

Preparation of $d^{32}pT$-A-G-T-A-C-T-A. The 5′-hydroxyl terminal of the octanucleotide was phosphorylated using [γ-^{32}P]ATP and T_4 polynucleotide kinase. A typical reaction mixture (100 μL) contained dT-A-G-T-A-C-T-A (1 nmol), [γ-^{32}P]ATP (3 nmol), and T_4 polynucleotide kinase (3 units) with the usual kinase reaction conditions (van de Sande et al., 1972). Quantitative labeling of the 5′ terminus was obtained and the d^{32}pT-A-G-T-A-C-T-A was isolated by gel filtration through Sephadex G-50.

The sequence of the labeled octanucleotide was further analyzed by partial hydrolysis with snake venom phosphodiesterase followed by two-dimensional fractionation as described by Sanger et al. (1973). Several aliquots of the partial digestion reaction were combined and applied to a cellulose acetate strip for electrophoresis (4500 V for 1 h in 7 M urea + 10% pyridinium acetate, pH 3.5) followed by thin-layer homochromatography on DEAE-cellulose in a 3% solution of 30-min hydrolyzed RNA in 7 M urea (Brownlee and Sanger, 1969). The radioactive oligonucleotides were located by autoradiography.

Ligase Catalyzed Joining of $d^{32}pT$-A-G-T-A-C-T-A Duplex. Solutions containing d^{32}pT-A-G-T-A-C-T-A (1–20 μM) in Tris-HCl (pH 7.6) (0.02 M), dithiothreitol (0.01 M), $MgCl_2$ (0.01 M), and ATP (66 or 330 μM) were prepared. Annealing by heating to 40 °C followed by slow cooling to 10 °C did not improve the extent of joining and was omitted from subsequent reactions.

After the addition of T_4 polynucleotide ligase (2 units/μL) the extent of joining was analyzed by following the development of resistance to BAP. Aliquots were treated with 2 μg of BAP at pH 8.0 and 65 °C for 60 min and then analyzed by descending chromatography on DE-81 strips in 0.35 M ammonium formate, or aliquots of the reaction were chromatographed directly on DE 81 with 0.35 M ammonium formate in 7 M urea. The joined material stayed at the origin, residual undigested octanucleotide and 5′-adenylylated octanucleotide had R_f = 0.2, and inorganic phosphate moved close to the solvent front. Background levels were generally 0.1–0.5%.

Polyacrylamide gel electrophoresis was carried out on a slab

gel apparatus similar to the one described by De Wachter and Fiers (1971). Native duplex DNA molecules were analyzed at 5 °C on 10% polyacrylamide slab gels (20 cm × 40 cm × 0.15 cm) containing Tris-borate/magnesium (TBM) buffer (0.09 M Tris-borate (pH 8.3), 5 mM $MgCl_2$). Samples were diluted with an approximately equal volume of 50% sucrose in TBM containing bromophenol blue dye at 4 °C and applied to the gel immediately. The TBM buffer was used in the electrophoresis reservoirs. Denaturing gels were carried out at room temperature on 10% polyacrylamide slab gels (20 cm × 40 cm × 0.15 cm) containing 7 M urea and Tris-borate/EDTA (TBE) buffer (0.09 M Tris-borate (pH 8.3), 2.5 mM EDTA) (Peacock and Dingman, 1969). Samples in approximately 5 μL of formamide containing bromophenol blue dye were applied after heating to 95 °C and chilling on ice. Only the anode electrophoresis reservoir contained 7 M urea in addition to the TBE buffer. Electrophoresis was carried out at constant voltage: native gels at 350 V, and denaturing gels at 800 V until the bromophenol blue dye had migrated 25 cm from the origin. Wet gels were covered with Saran Wrap (Dow Chemical Co.) and exposed to Dupont Cronex x-ray film.

Extraction of Oligonucleotides from Polyacrylamide Gels. Regions of gels containing radioactivity as indicated by autoradiography were excised, crushed, and extracted with 3 × 10 mL of 0.08 M NaCl. The supernatant was filtered through DE81 paper (2.5 cm disc) by gravity. The filter was washed briefly with deionized water and extracted with 10–20 × 50 μL of 1.0 M TEAB (pH 9–9.5) by centrifugation. Recovery was usually 85–90%.

Results and Discussion

Synthesis and Characterization of dT-A-G-T-A-C-T-A. In order to study further the joining at base-paired ends catalyzed by T_4 polynucleotide ligase (Sgaramella et al., 1970), a unique DNA duplex containing base-paired ends was designed to serve as a model substrate. A self-complementary sequence of eight nucleotides was chosen, requiring a minimum of preparative effort and simplifying its physical characterization. Since the base paired duplex of a self-complementary sequence is symmetrical, the characterization of polymerized products and kinetic analysis of the reaction itself will be simplified.

The scheme for synthesis of the self complementary octanucleotide dT-A-G-T-A-C-T-A is shown in Figure 1. Products at each step of the synthesis were unambiguously characterized by three criteria: position of elution during purification by DEAE-cellulose chromatography, comparison of the observed ultraviolet spectra with spectra predicted by addition of the spectra of the component protected mononucleotides, and paper chromatography of samples from which the amino protecting groups had been removed.

The comparatively low yields reported for the protected and deprotected octanucleotides resulted from pooling only the purest fractions at each step, and from the limited recovery expected after purification by paper chromatography.

The product, dT-A-G-T-A-C-T-A, was finally purified by DEAE-cellulose chromatography in 7 M urea, eluting as a single sharp peak at 0.21 M NaCl, approximately the salt concentration expected to elute an octanucleotide. The absence of any other ultraviolet-absorbing peaks in the elution profile indicated that the product is greater than 95% pure. The purity of the oligonucleotide was confirmed on analysis of the purified material by high-pressure liquid chromatography on RPC-5. Only two minor unidentified components were seen which account for approximately 5% of the total ultraviolet absorbance.

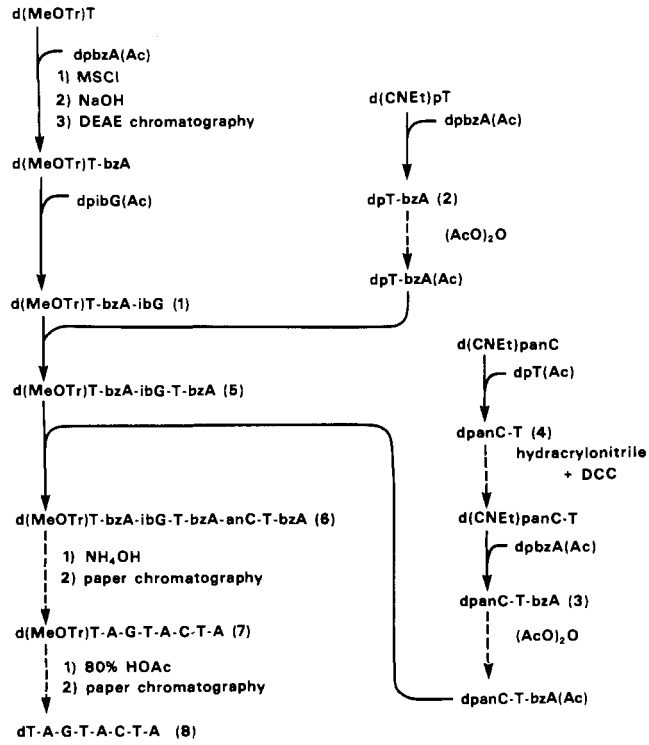

FIGURE 1: Scheme for the synthesis of the self complementary octanucleotide dT-A-G-T-A-C-T-A.

A small aliquot of the octanucleotide was completely digested with SVD and BAP. The resulting mixture of nucleosides was separated by high-pressure liquid chromatography on Aminex A-5 resin (Pike and Rottman, 1974) giving ratios of T:A:G:C of 2.91:3.21;1.00:0.96, confirming the expected base ratios.

Finally, the structure of the synthetic octanucleotide was confirmed by determination of the sequence by two dimensional fingerprinting of a 5′-^{32}P-labeled partial snake venom phosphodiesterase digest by the method of Sanger et al. (1973) as shown in Figure 2. The mobility shifts observed were those expected for the sequence d^{32}pT-A-G-T-A-C-T-A as indicated. A 5′-terminal analysis of the labeled oligonucleotide was carried out by complete digestion with SVD and subsequent separation of the resultant mononucleotides by paper electrophoresis at pH 3.5. Of the total radioactivity, 93% comigrated with dpT, confirming that dpT was the 5′-terminal nucleotide. The remaining 7% was distributed evenly over the remainder of the electropherogram, indicating that less than 1% of the radioactivity was linked to dpC, dpA, or dpG.

T_4 Polynucleotide Ligase Catalyzed Joining of d^{32}pT-A-G-T-A-C-T-A. Prior to using the synthetic, labeled octanucleotide as a substrate for joining at base-paired ends, it must be annealed to form a bimolecular duplex, and the presence of this duplex under conditions suitable for the joining reaction must be established. Using concentrations of salts (Tris-HCl, pH 7.6, 15 mM; $MgCl_2$, 5 mM) similar to those used for the polynucleotide ligase catalyzed joining at base paired ends (Sgaramella et al., 1970) a temperature absorbance profile was obtained as shown in Figure 3a. The hypochromic shift at lower temperatures shows that duplex DNA is being formed under these conditions. The T_m (18 °C) was inferred from the point of inflection of the curve because absorbances could not be determined reliably at temperatures lower than 5 °C, preventing observation of the lower end of the melting curve.

Although the polynucleotide ligase is active down to 0 °C

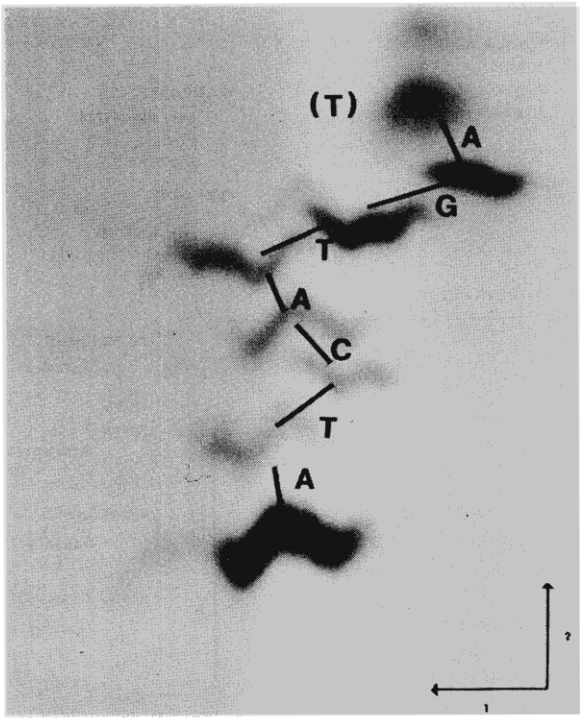

FIGURE 2: Autoradiograph of a two-dimensional fractionation of the products from a partial snake venom phosphodiesterase digestion of $d^{32}pT$-A-G-T-A-C-T-A. A sample of labeled octanucleotide in 10 μL of 0.01 M Tris-HCl (pH 8), 0.005 M $MgCl_2$ was incubated at 37 °C with 2 μg of snake venom phosphodiesterase. Aliquots (2 μL) were removed at times 0, 20, 40, 75, and 120 min. The combined aliquots were lyophilized and subjected to two-dimensional analysis as described in the Experimental Section.

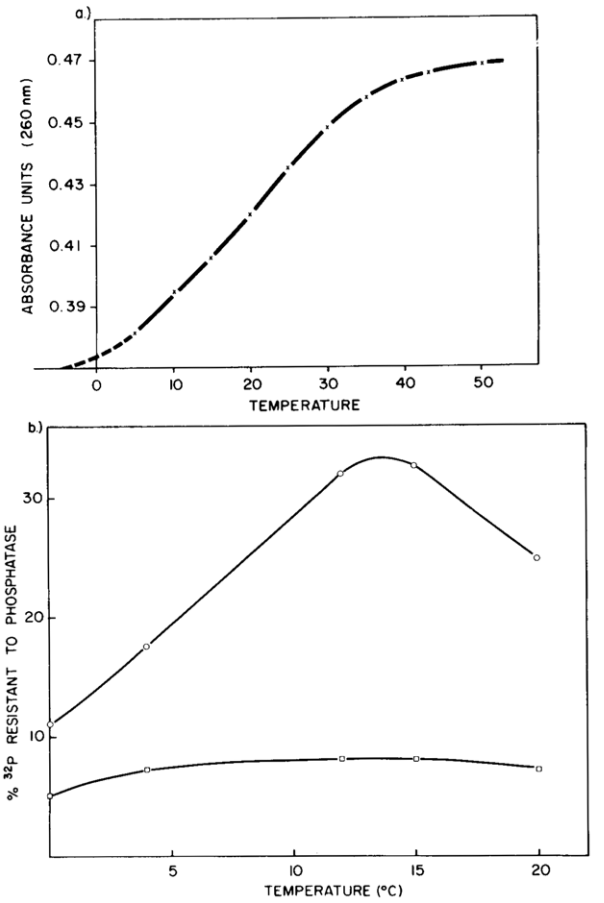

FIGURE 3: (a) Temperature–absorbance profile of the octanucleotide in 0.015 M Tris-HCl (pH 7.6) and 0.005 M $MgCl_2$. Prior to hyperchromicity measurements, the sample was heated to 40 °C and slowly (4 h) cooled to 5 °C. Temperature was increased at 0.5 °C/min. (b) Temperature profile of ligation at base-paired ends. One complete reaction mixture was divided into five portions and each was incubated at a different temperature between 0 and 20 °C. Aliquots of each reaction were analyzed after 20 h to obtain the fraction of radioactivity in the joined products and activated octanucleotide. Similar data obtained after 3 and 6 h of reaction showed similar profiles. (Joined (○); activated (□)).

(Gupta et al., 1968), the activity does decrease with temperature. For this reason, the joining reactions should be carried out at the highest temperature at which the duplex concentration is high. If the melting curve is approximately symmetrical about the inflection point (T_m), then, at temperatures between 5 and 10 °C, 80–90% of the octanucleotide should be present in the duplex form required for joining to occur. The temperature profile of joining at base-paired ends (Figure 3b) shows that the temperature optimum lies between 12 and 15 °C. However, all experiments were conducted at 4–7° C to ensure maximum utilization of the eight base pair duplex.

In the preliminary experiments, other potentially significant conditions such as the concentration of octanucleotide (4 μM) and the concentration of ATP (66 μM) were the same as those used previously (Sgaramella et al., 1970). Under these conditions only 1–2% joining (as measured by the increase in radioactivity resistant to release by BAP) was observed.

Since the joining reaction must be bimolecular in duplex substrate, increasing the concentration of octamer should increase the rate of reaction. Raising the concentration of octanucleotide to 20 μM without increasing the amount of ATP may also limit the reaction. Therefore the ATP concentration was also raised fivefold to 330 μM. At these concentrations, up to 25% joining was observed.

The increase in extent of joining at the higher substrate concentration indicated the need to determine the relationship between concentration of octanucleotide and rate of reaction. Rates of reaction were determined at concentrations of octanucleotide between 1 and 20 μM. The reactions were linear for up to one hour at higher substrate concentrations, and up to 6 h at the lowest substrate concentrations. These initial velocities, plotted as $1/v$ vs. $1/s$, did not give a straight line (in contrast to the results obtained by Sugino et al. (1977) for polymerization of decanucleotide duplex), while the corresponding plot for reactions bimolecular in substrate, $1/\sqrt{v}$ vs. $1/s$ (Figure 4) is linear. This clearly indicates that the rate-determining step is bimolecular, as expected.

Subsequently, reactions were carried out at 330 μM ATP and 10 μM octanucleotide except as noted.

Product Distribution from Polymerization of $d^{32}pT$-A-G-T-A-C-T-A. Random polymerization of the eight base pair duplex is expected to produce a distribution of products of various lengths in multiples of eight base pairs. Investigation of the joining of short oligonucleotides by the nick-sealing reaction (Gupta et al., 1968) showed that the efficiency of joining decreased as the length of the substrate decreased. If the T_4 polynucleotide ligase shows this preference for larger duplexes as substrates for joining at base-paired ends also, then the distribution of products will be shifted to larger products. The presence of 5′-adenylylated sequences which are the activated intermediates (Sgaramella et al., 1970) may also be expected.

Polyacrylamide slab gel electrophoresis as described by Maniatis et al. (1975) for sizing of DNA molecules offered a direct method for visualization of the various products. Aliquots were withdrawn from a polymerization reaction at times from 0.5 to 24 h and analyzed by electrophoresis on 10% polyacrylamide gels under native and denaturing conditions.

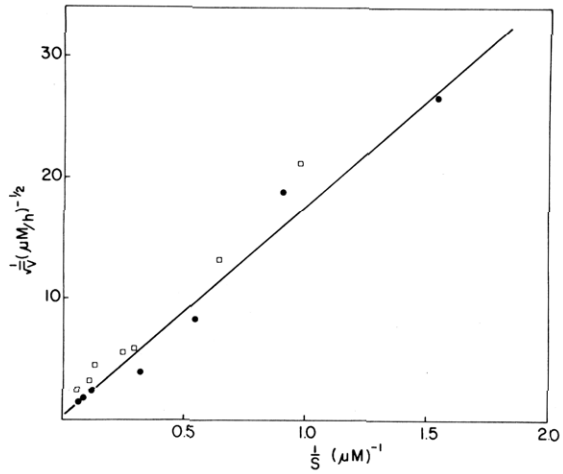

FIGURE 4: Double-reciprocal plot for reaction bimolecular in substrate $1/\sqrt{v}$ v. $1/s$. In one experiment, initial velocities from the linear phase of the reaction were plotted (●) and in another, 3-h time points were used to calculate the velocities (□). The graph drawn represents only the first data set.

Figure 5a shows an autoradiogram of the denaturing gel, containing $[(dpT)_{10}]_n$ markers as an internal calibration for lengths of products. Products up to 120 nucleotides at intervals of eight units are resolved. The expected progressive increase in the amount of higher molecular weight species with longer reaction times is apparent.

The appearance of products as double bands suggested the presence of 5′-adenylylated intermediates in this reaction. Upper and lower bands corresponding to 8, 16, and 24 nucleotide products were excised and extracted and the extracts treated with bacterial alkaline phosphatase. In each case, the expected fraction of the total radioactivity was released from the lower band, while about half that fraction was released from the upper band (data not shown). Contamination of the upper band by the lower, and possible hydrolysis of the activated species during the extraction procedure could not be quantitatively assessed and may explain why the activated material is not 100% phosphatase resistant.

Figure 5b shows an autoradiogram of the native gel. Again, products above 100 base pairs are resolved, and progressive increase in higher molecular weight species with longer reaction times is observed.

In order to estimate the relative efficiency of joining with respect to the length of the substrate duplex, the amount of each duplex product in each size class, including both activated and unactivated forms, was calculated from the areas under the peaks of a microdensitometer scan of each slot of the autoradiogram of Figure 5b. These data are shown in Figure 6. The rapid synthesis of 16 base pair product in the earliest stage of the reaction is observed as expected. However, after the first 0.5–1 h, the fraction of total radioactivity present in this product changes very little, indicating that it is being used as rapidly as it is being synthesized. Since the 16 base pair duplex contains only 2% of the radioactivity, and its concentration is therefore only about 1% that of the octanucleotide duplex, the T_4 polynucleotide ligase must use the 16 base pair duplex more efficiently than the 8 base pair duplex by a factor of almost 100.

Two other relationships in these data also support this conclusion. Net synthesis of the 24 base pair product from 8 and 16 base pair duplexes also proceeds at a relatively high rate and continues up to 4 to 8 h. Net synthesis of the 32 base pair product is even greater than that of the 24 between 2 and 4 h.

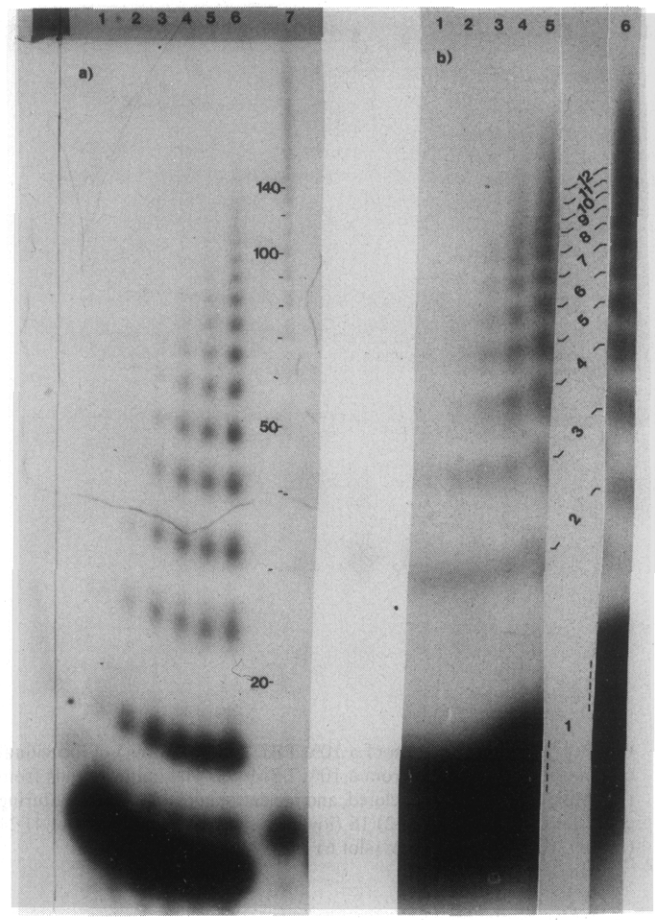

FIGURE 5: Autoradiograms of 10% polyacrylamide gels after electrophoresis of the reaction products at various time intervals. The reaction mixture (20 μL) contained 200 pmol of d^{32}pT-A-G-T-A-C-T-A (27 000 cpm/pmol), and aliquots of 2 μL were used for each slot except the 24-h points which were 5 μL. (a) Denaturing (TBE, 7 M urea) gel: (slot 1) 0.5 h; (2) 1 h; (3) 2 h; (4) 4 h; (5) 8 h; (6) 24 h; (7) $(pT_{10})_n$ markers. (b) Native (TBM) gel: (slot 1) 0.5 h; (2) 1 h; (3) 2 h; (4) 4 h; (5) 8 h; (6) 24 h. Between sample applications, the TBM gel was run at 50 V to prevent further reaction. This accounts entirely for the nonalignment of the observed bands.

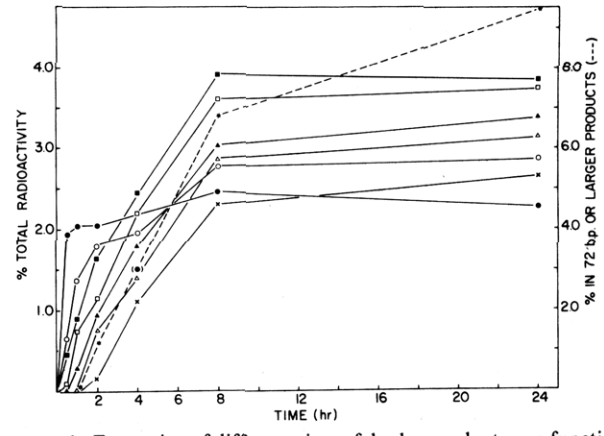

FIGURE 6: Formation of different sizes of duplex products as a function of time. Microdensitometer scans of each slot from two autoradiograms (Figure 5b) (2-day and 8-day exposures) were recorded, and two independent determinations of the areas under the peaks were made. These values were corrected for the extent of radioactive decay and times of exposure, and the average values were normalized by comparison with the percentage of radioactivity in joined products determined by DE-81 paper chromatography for each time point. The error of measurement is = 10%. Symbols: (●) 16, (○) 24, (■) 32, (□) 40, (▲) 48, (△) 56, and (X) 64 base pair products; the broken line represents the remaining larger products not resolved in the gel and is calibrated on the right-hand axis.

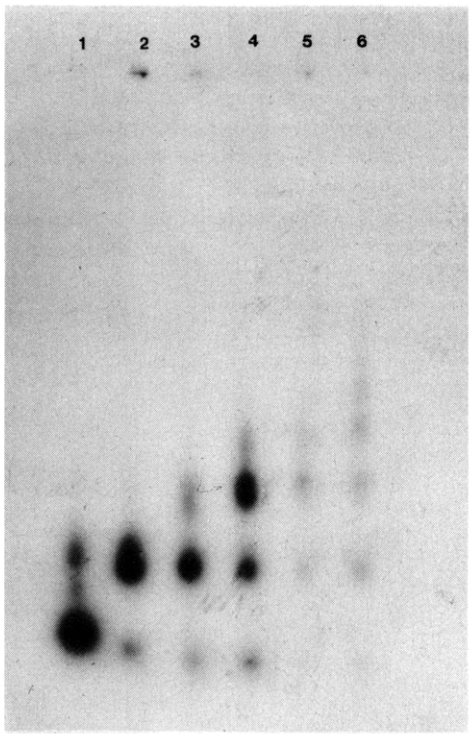

FIGURE 7: Autoradiogram of a 10% TBE gel (7 M urea) of individual oligomer bands obtained from a 10% TBM gel. The duplex bands from the native gel were excised, eluted, and reelectrophoresed under denaturing conditions: (slot 1) 8; (slot 2) 16 (lower); (slot 3) 16 (upper); (slot 4) 24 (lower); (slot 5) 24 (upper); (slot 6) 32.

Thus the rate of reaction between two 16 base pair duplexes is comparable to or greater than the rate of reaction of 16 base pair duplexes with 8 base pair duplexes even though the reaction is second order in the substrate which is present in relatively low concentration.

The preferential joining of larger substrates is also reflected in the continuing accumulation of larger products.

Thermal stability of the duplex substrates, as indicated by the temperature optimum (Figure 3b) relative to the T_m of the octanucleotide duplex, is clearly not sufficient to explain entirely this preferential joining. This indicates that at least two factors are responsible for the preferential joining of larger duplexes: their increased thermal stability and their greater size.

Mechanism of T_4 Polynucleotide Ligase Catalyzed Joining at Base-Paired Ends. This mode of joining requires joining of both strands of the DNA duplex. If both strands are joined simultaneously (or in rapid succession), then only duplexes containing no internal nicks will be observed as products. A comparison of the native and denaturing gels shown in Figures 5a and 5b reveals a larger proportion of smaller products (16, 24 nucleotide products) in the denaturing gel. This suggests that some of the larger duplexes are nicked, having 16, 24, etc. (and possibly 8) nucleotide fragments in one or both strands. The presence of such nicked products may be demonstrated more directly by electrophoresis of the products under nondenaturing (native) conditions, followed by electrophoresis of each duplex band under denaturing conditions. In the denaturing gel, nicked products would dissociate to show bands of lower molecular weight in addition to the band corresponding to the original duplex length.

Electrophoresis under native conditions of an aliquot from a polymerization reaction gave bands, the mobilities of which were proportional to the logarithm of their molecular weights (data not shown) suggesting that these products may be used as convenient markers for polyacrylamide gel electrophoresis of duplex DNAs under native conditions. The 8 base pair band, upper and lower portions at the 16 and 24 base pair bands, and the 32 base pair band were eluted from the native gel and analyzed by electrophoresis under denaturing conditions, and the resulting autoradiogram is shown in Figure 7.

The fainter spots appearing above (at lower mobility than) the designated sizes of products applied indicate the extent of contamination from adjacent bands in this procedure.

The remaining nonadjacent bands must therefore arise from the postulated nicked products. The sensitivity of this method is limited by the amount of radioactivity initially available in the octanucleotide. The presence of nicked products at low levels indicates that the joining at base-paired ends must proceed by sealing of the nicked duplex after the rate-determining joining of the first strand in the initial bimolecular reaction, and that the rates must be similar.

Conclusion

Synthesis of the self-complementary octanucleotide d^{32}pT-A-G-T-A-C-T-A has provided a model duplex substrate for investigation of the joining of duplex DNA at base-paired ends catalyzed by T_4 polynucleotide ligase. The polymerized products may be used as convenient double-stranded markers for polyacrylamide gel electrophoresis under native conditions. A 100-fold faster rate of reaction of 16 base pair duplexes was demonstrated, and the greater efficiency of utilization of larger substrates was established.

Kinetic data and the presence of nicked intermediates indicated that the rate-determining step was a bimolecular reaction in which two duplexes are joined in one strand, prior to sealing the remaining nick in the opposite strand.

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Gaps in DNA Induced by Neocarzinostatin Bear 3′- and 5′-Phosphoryl Termini†

Lizzy S. Kappen* and Irving H. Goldberg

ABSTRACT: Neocarzinostatin (NCS)-induced strand breakage of DNA generates nonfunctional binding sites for the *E. coli* DNA polymerase I. Treatment of the NCS-nicked DNA with alkaline phosphatase at 65 °C prior to the polymerase reaction results in 60–100-fold stimulation of dTMP incorporation, whereas in a control not treated with the drug there is only a 2-fold increase. Sites of strand scission on the NCS-treated DNA bear phosphate at the 3′ termini. This conclusion is supported by the kinetics of release of inorganic phosphate from NCS-cut DNA by exonuclease III. Since our earlier work has shown that virtually all the 5′ ends of the nicks caused by NCS bear phosphomonoester groupings, the 3′- and 5′-phosphoryl termini could be quantitated using alkaline phosphatase and exonuclease III. Over a wide range of drug levels the amount of inorganic phosphate released by alkaline phosphatase is approximately twice as much as that removed by exonuclease III, indicating the presence of equal amounts of 3′- and 5′-phosphoryl termini. This, taken together with other previously demonstrated effects of NCS on DNA, such as the introduction of nicks not sealable by polynucleotide ligase, the release of thymine, and the formation of a malonaldehyde type compound, suggests that NCS-induced strand breakage involves base release accompanied by opening of the sugar ring with the destruction of one or more nucleosides and results in a gap bounded by 3′- and 5′-phosphoryl termini.

Neocarzinostatin (NCS[1]), an acidic protein antibiotic of molecular weight 10 700, is cytotoxic to gram positive organisms (Ishida et al., 1965) and a variety of tumor cells (Ishida et al., 1965; Ono et al., 1966; Kumagai et al., 1966; Bradner & Hutchison, 1966). Studies on the molecular mechanism of action of NCS revealed DNA to be a principal target of action. Thus, NCS primarily inhibits DNA synthesis in certain gram positive bacteria (Ono et al., 1966) and in a variety of mammalian cell lines (Ono et al., 1966; Homma et al., 1970; Sawada et al., 1974; Beerman & Goldberg, 1977). It induces degradation of DNA in sensitive bacteria (Ohtsuki & Ishida, 1975a) and causes DNA single-strand scissions in mammalian cell lines (Beerman & Goldberg, 1974; Sawada et al., 1974; Tasumi et al., 1974; Ohtsuki & Ishida, 1975b; Beerman & Goldberg, 1977). Strong support for DNA damage being involved in NCS action comes from the recent finding that the drug is a mutagen for *E.* coli and from genetic studies in *E. coli* suggesting that a nonexcisable misrepair type of damage to the DNA is produced (Tatsumi & Nishioka, 1977). Further, we

† From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115. *Received August 29, 1977.* This work was supported by U.S. Public Health Service Research Grant GM 12573 from the National Institute of General Medical Sciences.

[1] Abbreviations used: NCS, neocarzinostatin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; P_i, inorganic phosphate.

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