ENZYMATIC ADDITION OF COHESIVE ENDS TO T7 DNA

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SUMMARY

Terminal deoxynucleotidyl transferase isolated from calf thymus was used to catalyze addition of either deoxyadenylate or thymidylate residues to termini of native DNA molecules. An equimolar mixture of T7 DNA with polyadenylate termini and T7 DNA with polythymidylate termini forms fast sedimenting molecules which measure two or three times normal T7 DNA length in electron micrographs. Such molecules are apparently catenanes formed by cohesion of complementary single stranded ends on the double stranded DNA. Polynucleotide ligase does not covalently join the single strands of these synthetic catenanes.

Terminal deoxynucleotidyl transferase isolated from calf thymus has been shown to catalyze sequential addition of deoxynucleotides to the 3-prime terminus of single stranded DNA (1,3,4). The product of such addition using a single deoxynucleotide triphosphate of substrate is a single stranded DNA containing a homopolymeric 3-prime end. We considered the possibility that terminal transferase might add deoxynucleotides to double stranded DNA. Such a reaction with appropriate deoxynucleotides should provide cohesive ended DNA's whose catenanes could be covalently joined by action of a polynucleotide ligase.

Terminal transferase catalyzed addition of either deoxyadenylate or thymidylate to double stranded DNA was attempted using conditions similar to those of Yoneda and Bollum (4). Thymidylate incorporation was in a reaction mixture con-

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sisting of 0.25 micromoles $CoCl_2$, 2.0 micromoles potassium phosphate buffer, pH 7.0, 0.25 micromoles thymidine triphosphate with 1-50 microcuries of tritium or ^{14}C label, 25-50 micrograms native DNA (usually from phage T7) and about 20 micrograms of purified terminal deoxynucleotidyl transferase prepared in accord with the method of Yoneda and Bollum (4). The reaction mixture for adenylate incorporation contained: 2.0 micromoles $MgCl_2$, 2.0 micromoles potassium phosphate buffer, pH 7.0, 0.25 micromoles deoxyadenosine triphosphate with 1-50 microcuries of tritium of ^{14}C label, 25-50 micrograms DNA and about 20 micrograms of terminal transferase. Each reaction mixture contained a total volume of 0.25 ml. In general, the mixture was incubated at 37°C for 30 minutes and the reaction was terminated by adding 50 µl of 5% $Na_4P_2O_7 \cdot 10\ H_2O$.

TABLE I. Incorporation of C^{14} thymidine triphosphate into TCA insoluble material[a/]

Time at 37°C	Denatured BAP Calf thymus DNA[b/]	Native BAP Calf thymus DNA[c/]	Native T7 DNA[d/]
30 minutes	13,250	2,823	236
60 minutes	22,470	6,700	1,223
120 minutes	30,240	11,269	1,850

a/ Contents of reaction mixture are in the text.

b/ 1.5 mg calf thymus DNA (Worthington Biochemicals) in 0.01 M Tris pH 8.0, was incubated at 100°C for 10 min and quenched in ice. 1 mg of Bacterial Alkaline Phosphatase (Worthington BAPF) was added and the solution was incubated 30 min at 37°C. 1 N NaOH was added to obtain pH 10.0 and the solution was again incubated at 100°C, this time for 30 minutes. One half volume of 1.0 M Tris pH 8.0 was added and the solution dialyzed into 0.01 M Tris pH 8.0.

c/ 1.5 mg calf thymus DNA in 0.01 M Tris pH 8.0 was incubated with 1 mg of BAPF for 30 min at 37°C. After cooling to 5°C, the aqueous solution was extracted four times with freshly distilled preequilibrated phenol. Phenol was removed by ether extraction and residual ether evaporated off with dry nitrogen. The final solution was dialyzed into 0.01 M Tris pH 8.0.

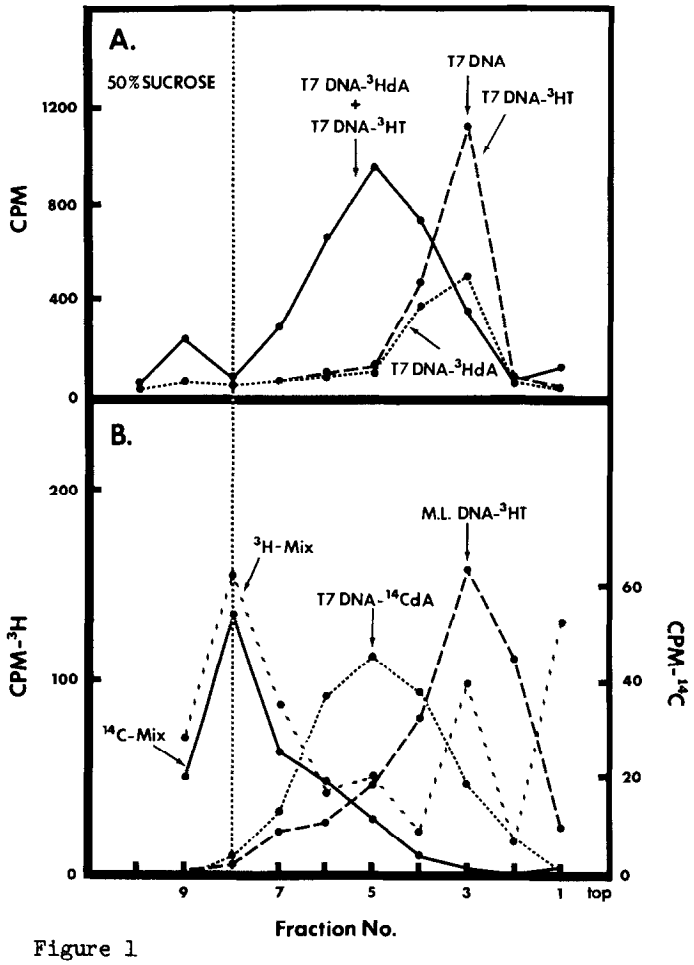
d/ T7 phage was purified by differential centrifugation and by CsCl equilibrium density gradient centrifugation. DNA was extracted by four successive phenol extractions from a suspension of phage in 0.1 M NaCl/0.01 M Tris pH 8.0. Phenol was freshly distilled and pre-equilibrated with buffer. Final traces of phenol were removed by extensive dialysis against 0.1 M NaCl in 0.01 M Tris.

The data in Table I indicate that terminal transferase is able to catalyze incorporation of nucleotides to both native and denatured DNA. DNA primers were prepared as described in the table and incubated as above. After the indicated time, the reaction was terminated by adding 5 ml of ice cold 5% trichloroacetic acid, 5% $Na_4P_2O_7 \cdot 10\ H_2O$. The precipitate was collected on Schleicher and Schuell B-6 Membrane filters, washed three times with the same solvent, dried and counted in Bray's scintillator.

Though the results are only semi-quantitative, two important conclusions can be made. First, native DNA does serve as a primer for terminal transferase even though it is poor compared to denatured DNA. Second, the rate of incorporation to T7 DNA of molecular weight 25×10^6 daltons is approximately 1/10 of the rate of incorporation to calf thymus DNA of molecular weight ca. 3×10^6 daltons. We would thus infer that the rate of addition is roughly proportional to the number of termini present.

Two reasonable explanations are possible for deoxynucleotide addition to native DNA primers. Either the enzyme preparations contain small amounts of nucleases which expose single stranded ends of the double stranded primers, or the DNA "breathes" enough at the ends to allow initiation of synthesis. The first explanation is supported by our observation that different enzyme preparations catalyzed different initial rates of addition to double stranded as compared to single stranded primers. However, our best terminal transferase preparations contained only traces of nucleolytic activity yet still retained the ability to add deoxynucleotides to native T7 DNA.

In any case, addition of either adenylate or thymidylate termini to native T7 DNA was thus accomplished and our next task was to determine whether catenanes of terminally homologous DNA's could be formed. Such high molecular weight DNA was separated from other components by elution through an agarose gel column (1.5 x 40 cm) of BioRad Agarose (A50M) using 0.1 M NaCl, 0.05 M $Na_4P_2O_7$, 0.01 M Tris pH 7.5. Material which eluted at the void volume was pooled and dialyzed against 0.1 M NaCl, 0.01 M Tris pH 8.0. DNA isolated by the above technique is



LEGEND - Figure 1

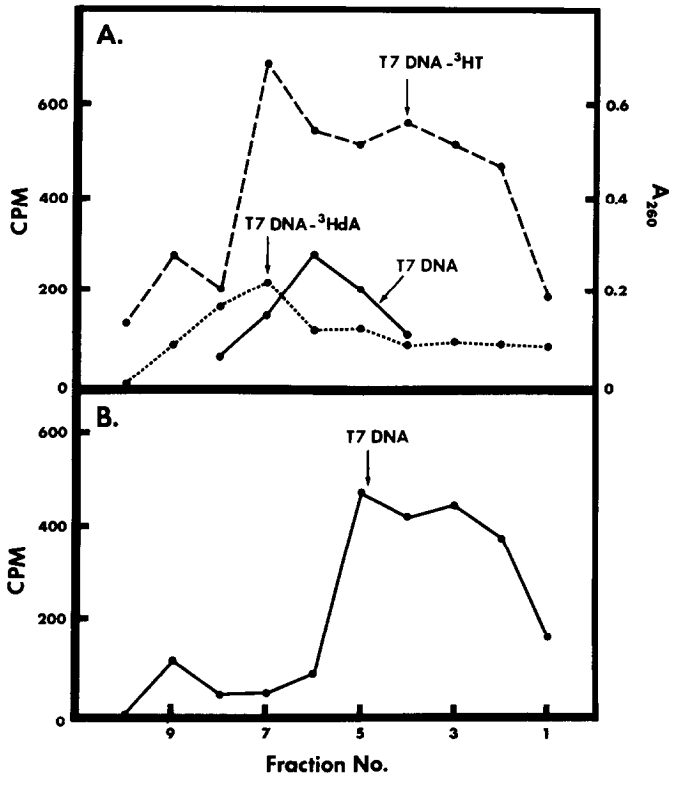
Neutral sucrose gradient sedimentation of terminal transferase treated DNA.

A. Equal volumes of T7 DNA-^{3}HdA and T7 DNA-^{3}HT isolated as described in the text were mixed in 0.1 M NaCl, 0.01 M Tris, pH 8.0, and incubated for 5 minutes at 37°C and 25 minutes at 23°C. Three separate sucrose gradients were run simultaneously. The gradient contained an 8-20% (W/V) linear sucrose gradient above a shelf of 50% sucrose. All solutions contained 1.0 M NaCl, 0.1 M Tris, and 0.001 M EDTA, pH 8.0. Aliquots of 0.20 ml of each sample were layered onto 4.8 ml gradients and centrifuged for 90 minutes at 60,000 RPM at 5°C in a Spinco SW 65 rotor. Fractions were collected from the top by pumping 75% sucrose into the bottom. Isolated fractions were precipitated with 5% TCA, filtered on Schleicher and Schuell B-6 membrane filters, and counted in Bray's scintillator.

B. Equal volumes of T7 DNA-^{14}CdA and M.l.DNA-^{3}HT (M. luteus DNA) isolated as described in the test, were mixed and incubated for 30 minutes at 37°C. Three separate sucrose gradients were run simultaneously. Gradients were as in A. 0.01 ml of the separated components or 0.20 ml of the mixture were layered onto the gradients and centrifuged for 120 minutes at 60,000 RPM at 5°C in a Spinco SW 65 rotor. Fractions were collected, precipitated with TCA and counted as in A. Correction for backgrounds of 33 CPM (^{3}H) 20 CPM (^{14}C) and 16.5% crosstalk in the ^{3}H window were made before the results were plotted.

termed DNA-dA or DNA-T depending on which deoxynucleotide was incorporated.

Sucrose gradient analysis of the reaction products was consistent with the hypothesis that such DNA is largely double stranded with at least one single stranded homopolymeric terminus. Figure 1 shows profiles of DNA-dA and DNA-T after sedimentation through neutral sucrose gradients either separately or after mixing. The radioactivity in each individual sample co-sedimented with untreated DNA, whereas the label in the mixture sedimented approximately twice as fast as



LEGEND - Figure 2

Alkaline sucrose gradient sedimentation of terminal transferase treated T7 DNA.

A. T7 DNA-^{3}HdA or T7 DNA-^{3}HT isolated as described in the text were layered onto separate 5-20% (W/V) sucrose gradients containing 0.1 M NaOH, 0.05 M $Na_4P_2O_7$ and sedimented in a Spinco SW 65 rotor for 4 hours at 60,000 RPM at 5°C. After centrifugation, fractions were collected and analyzed as in Fig. 1. Also plotted is the absorbance profile of a sample of untreated T7 DNA.

B. Equal volumes of T7 DNA-^{3}HdA and T7 DNA-^{3}HT were mixed as described in Fig. 1A. Alkaline sucrose gradient sedimentation carried out as in A for 2.5 hours. Fractions were collected and analyzed as in Fig. 1A. Untreated T7 DNA sedimented to the region where the arrow appears.

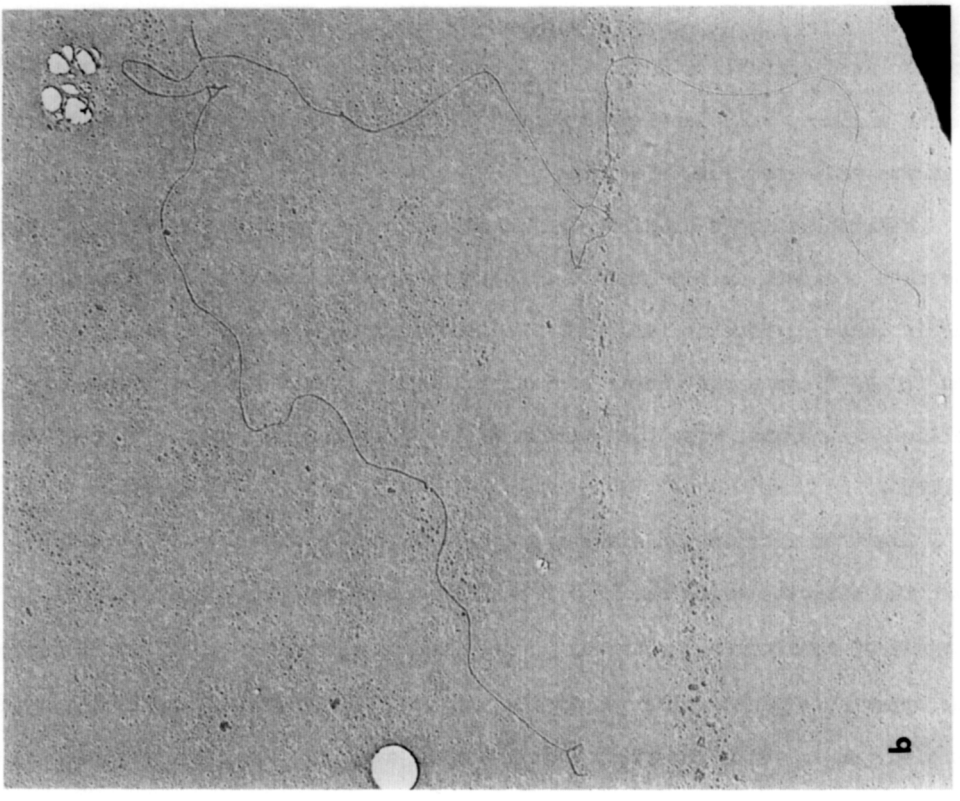
the isolated components. A double label experiment (Fig. 1b) demonstrated that in the mixture, both labels sedimented together at a rate higher than their separate sedimentation velocities.

Alkaline sucrose gradient sedimentation showed that denaturation dissociates the fast sedimenting material such that the mixture sedimented identically with the isolated components (Fig. 2). The plots in Fig. 2 also show that T7 DNA-dA and T7 DNA-T contained single strand nicks in a significant fraction of the molecules such that when the strands are separated, short single strands are evident.

Electron microscopic examination of terminal transferase treated DNA's revealed that the material which sedimented rapidly in neutral solution consisted largely of double length threads. The electron micrographs in Plate I indicate the appearance of the material in the mixture. Although many molecules appeared tangled, those which were well spread are of a length which corresponded in length to linear dimers or linear trimers. No confirmed circular molecules of any length could be detected. Imperfections at the ends of terminal-labeled T7 DNA threads were seen fairly often (Plate Ib).

The catenanes formed by mixing T7 DNA which contained synthetic homologous ends were apparently not as well formed as those which occur in nature (e.g. phage lambda DNA). We have been unable to catalyze covalent joining of our T7 DNA catenanes using E. coli polynucleotide ligase, even though numerous experimental conditions were used including variations in temperature, salt concentration and enzyme-substrate level. Alkaline sucrose gradient sedimentation profiles of such catenanes were always identical with those obtained for untreated mixtures (Fig. 2b).

Catenanes of T7 DNA molecules with various lengths of homopolymeric ends were also not joinable. T7 DNA was incubated either under thymidylate addition conditions or adenylate addition conditions for 10, 20, 30 or 60 minutes. Sixteen different mixtures of the complementary DNA's were made, and joining with polynucleotide ligase was attempted. Alkaline sucrose gradient sedimentation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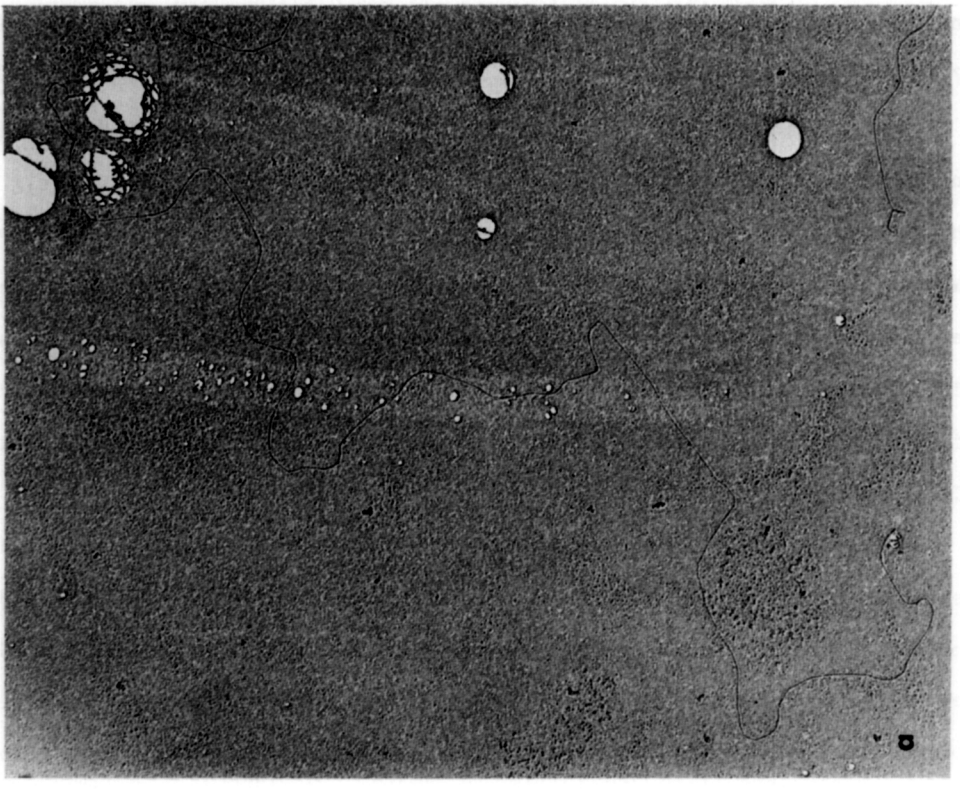
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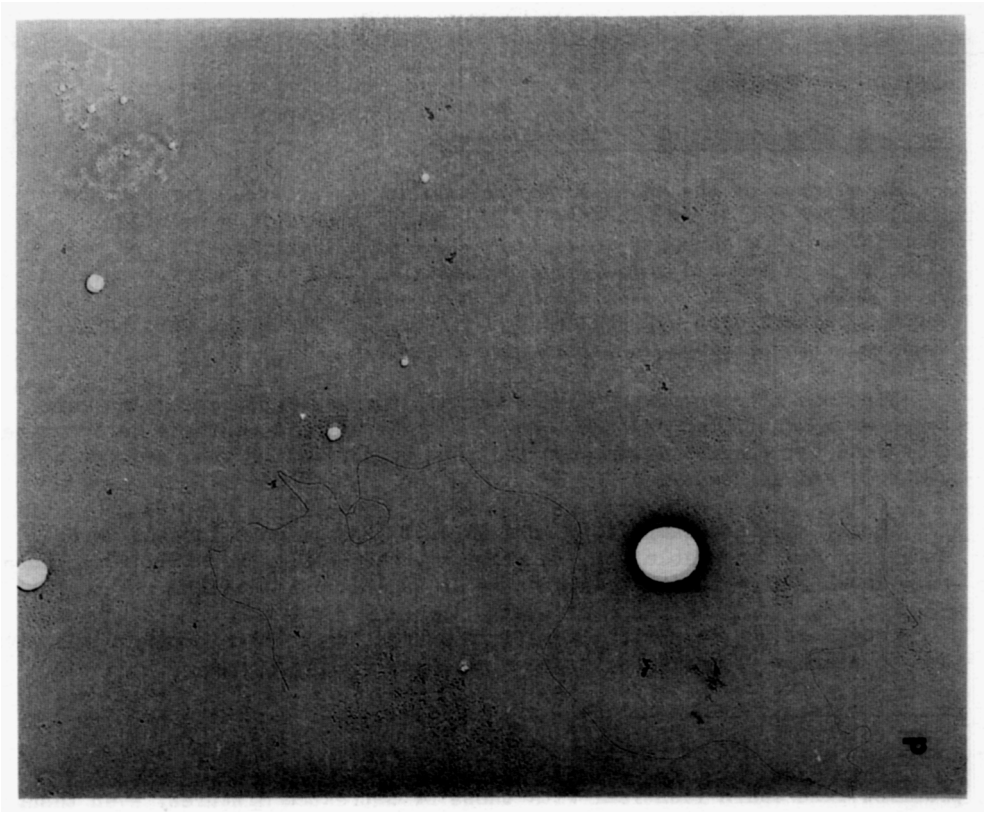

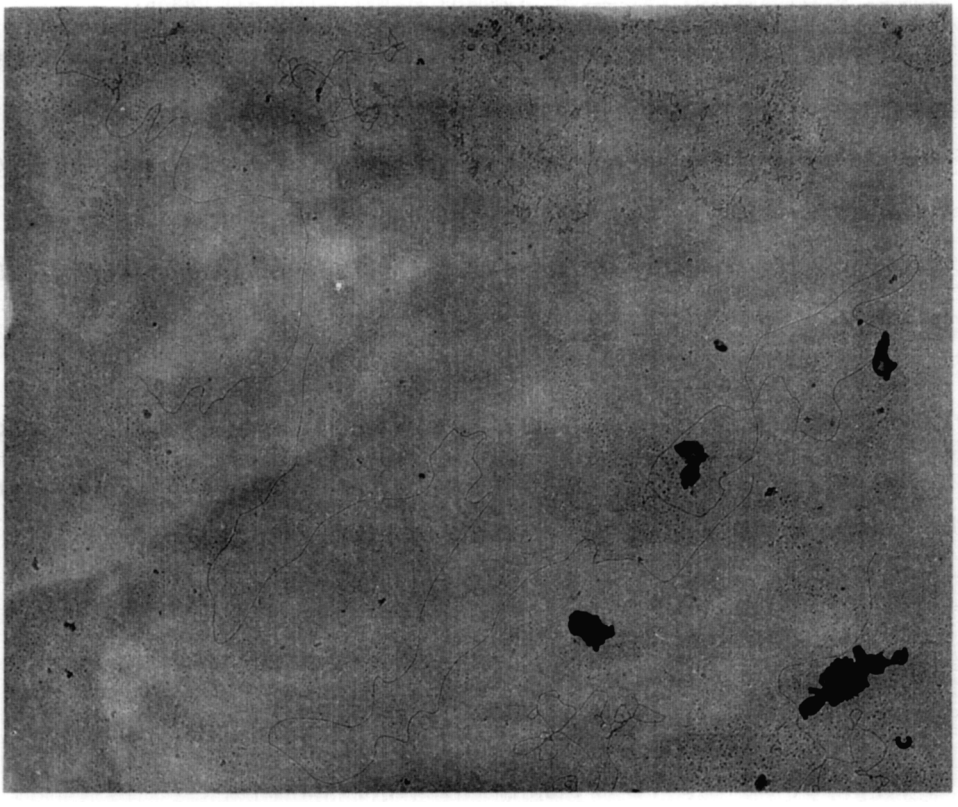

LEGEND - Plate I

Electron micrographs of T7 DNA catenanes.

An aliquot of the annealed mixture which was prepared for sucrose gradient sedimentation (as in Fig. 1A) was diluted into a solution containing 3.3 M ammonium acetate and 0.21 mg/ml cytochrome C, pH 8.0. Aliquots were layered onto 0.15 M ammonium acetate in a parafin covered petri dish. Formvar coated electron microscope grids were touched to the surface, washed with 95% ethanol and air dried. The grids were rotary shadowed with platinum at a 10 degree angle.

Electron micrographs of fields containing measurable molecules were taken at a magnification of 10,000 times. No attempt was made to accurately calibrate the magnification, but the same magnification was used for both controls and unknowns.

In the separate samples (T7 DNA-dA or T7 DNA-T) most threads were untangled and measurable (ca. 12 microns), whereas in the annealed mixture only about 30% of the threads were in an untangled condition.

Length of the measurable threads pictured is: a) 25 microns, b) 33 microns, c) 36 microns (12 microns, upper right) and d) 23 microns.

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profiles were again identical with those of untreated mixtures, even though neutral sedimentation indicated catenane formation in all cases.

Ligase joining during concurrent repair DNA synthesis was also attempted under conditions identical with those described by Goulian and Kornberg (2). Alkaline sucrose gradient sedimentation of the product after 30 minutes at 37°C again was identical to that seen in Fig. 2b. We are uncertain as to which of the several requirements for ligase action is lacking in our synthetic T7 DNA catenanes.

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