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Use of the T4 Polynucleotide Ligase in The Joining of Flush-Ended DNA Segments Generated by Restriction Endonucleases

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Double-stranded DNA segments with completely base-paired ends were obtained by the action of various restriction endonucleases on phage and plasmid DNAs. These segments were joined covalently by the T4 polynucleotide ligase. The joining was monitored by the electron microscopy count of intramolecularly circularized segments. The highest extent of joining, close to 75%, was observed at 15 – 25 °C with the segments resulting from the action of the *Bacillus subtilis* (strain R) restriction endonuclease *Bsu* on the DNA of bacteriophage SPPI or of the plasmid pSC 101. The joining of double-stranded termini required about 10 times more enzyme than the short single-standed termini produced by the *Escherichia coli* restriction endonuclease *Eco*RI. A shortened purification of the T4 ligase was found to give an enzyme devoid of interfering nucleases.

The role of DNA ligases is well established in the processes of DNA replication, repair and recombination in eukaryotic and in prokaryotic cells [1]. These enzymes restore an interruption (nick) of a single strand in a double-stranded DNA helix. The distinctive activity of the T4 polynucleotide ligase on opposed fully base-paired DNAs (henceforth referred to as 'terminal joining' of 'blunt-end' DNA) was discovered using segments of the synthetic yeast alanine transfer RNA gene [2]. The nearest-neighbor analysis of the reaction products gave unequivocal evidence of the covalent joining of the DNA strands at the flush ends of the duplexes [3]. Among natural DNAs, that of *Salmonella typhimurium* phage P22 has flush ends: its oligomerization was used to study this reaction further; the reaction was found to be absent in *Escherichia coli* ligase [4].

Blunt ends in DNA duplexes can be produced by some of the type II restriction endonucleases [5]. We have developed an assay for the terminal joining of the resulting segments which can also be applied to cohesive joining of segments with complementary short single-stranded ends [6]. The assay involves intramolecular covalent circularization of appropriately diluted DNA segments, which are then visualized by electron microscopy. Using this simple assay we have been able to (a) follow the purification of T4 ligase, (b) study some of the parameters of the joining reaction, (c) obtain data to substantiate that the same enzyme mediates both the blunt-end and cohesive joining.

Abbreviations. Abbreviations for nucleotides follow IUPAC/IUB recommendations, see *Eur. J. Biochem. 15*, 203 – 208 (1970); abbreviations for restriction endonucleases follow the recommendations of Smith and Nathans, see *J. Mol. Biol. 81*, 419 – 423 (1973).

Enzymes. DNA ligase (EC 6.5.1.1); restriction endonucleases (EC 3.1.4.–).

EXPERIMENTAL PROCEDURE

DNA

Plasmid DNAs were prepared essentially according to established procedures [7]. SPP1 DNA was isolated by phenol extraction of the phage purified by CsCl density centrifugation after poly(ethylenglycol) concentration of the lysate.

Enzymes

The T4 ligase was purified following essentially the procedure of Weiss et al. [8] up to fraction V, which was concentrated by ammonium sulfate precipitation. The *E. coli* ligase was a gift of Dr I. R. Lehman. The *B. subtilis* R restriction endonuclease (endo R · *Bsu*) was purified as described by Bron et al. [9], with an additional DEAE-cellulose column to remove traces of exonucleases. *Eco*RI restriction endonuclease was purified from fraction I [10] by an ammonium sulfate precipitation, followed by a DEAE-cellulose column. Endo R · *Hin*dII was a gift of Dr G. Bernardi, endo R · *Sma*I of Drs H. Kopecka and J. Lis; endo R · *Hpa*I was a commercial product (Biolabs).

Ligations with the T4 and the *E. coli* ligases were performed as described by Sgaramella [4]. Digestion conditions were as described by Brown et al. for

endo R · *Bsu* [9], by Gromkova and Goodgal for endo R · *Hin*dII and endo R · *Hpa*I [11], by Hedgepeth et al. for endo R · *Eco*RI [12], and by Endow and Roberts for endo R · *Sma*I (personal communication).

Gel Electrophoresis

This was performed as described [14].

Electron Microscopy

The DNA was spread by the aqueous drop technique essentially as described by Inman and Schnös [15].

RESULTS

Intramolecular Circularization of DNA Segments Generated by Restriction Endonucleases

Several type II restriction endonucleases have been shown to produce blunt termini [6]: among these we have used *Hin*dII and *Hpa*I [11], *Sma*I and *Bsu* [9]. All of the resulting DNA segments, when appropriately diluted [16], can be circularized with the T4 polynucleotide ligase: the results of our survey are given in Table 1, where the sequence recognized by the enzymes, the number of cuts introduced in the different DNAs, and the average lengths of the segments produced are also noted. The extent of circularization varied from 5 to 75%: the differences are probably due to the varying levels of purity of the enzymes used (of which only endo R · *Bsu* has been directly checked) and possibly also by the segment terminal sequence. The background level of circles in the absence of the ligase was less than 1% in all cases.

The highest extent of circularization was obtained with the segments generated by the *B. subtilis* R endonuclease cleavage of SPP1 DNA. Fig. 1 shows the results of a reaction in which these segments have been incubated with T4 ligase: close to 75% of the molecules can be scored as circles; no such structures can be seen if the T4 ligase is omitted or if the *E. coli* ligase is used.

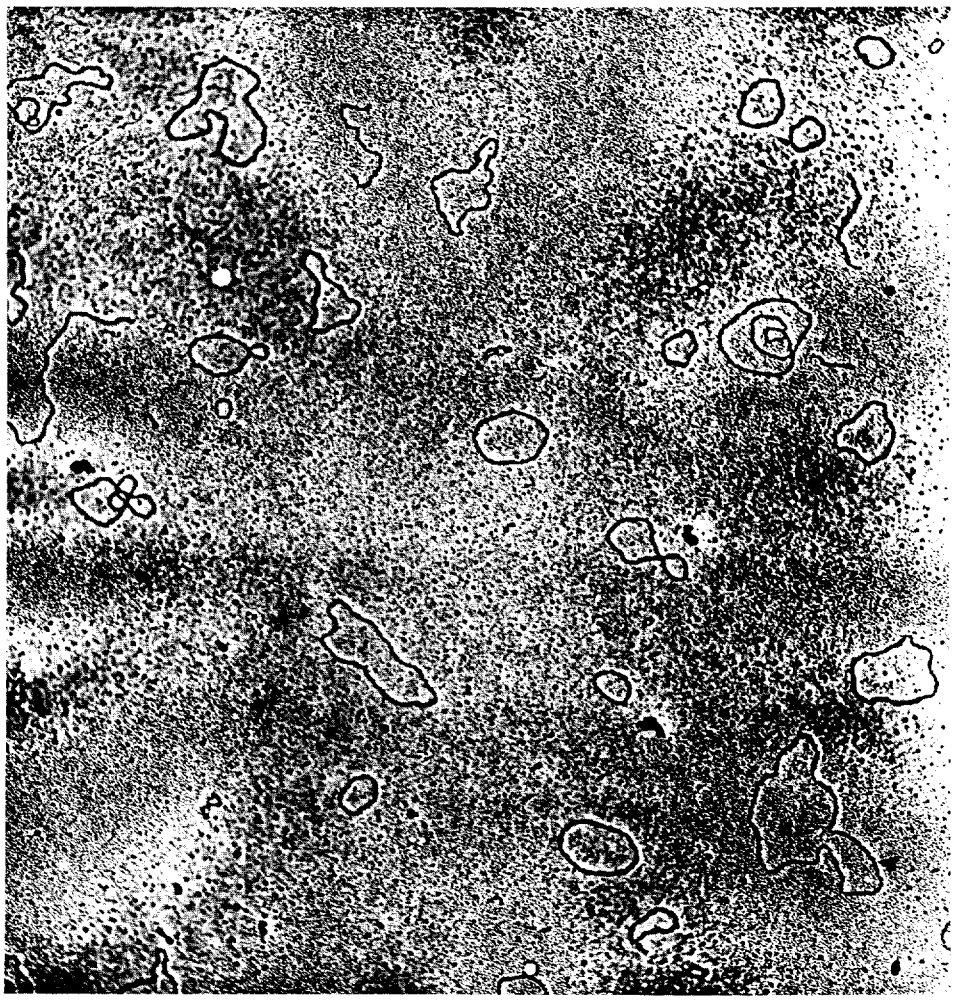

Fig. 1. *Circles formed by the T4 ligase from flush-ended segments produced by endo R* · Bsu *on SPP1 DNA.* Reaction conditions are described in legend of Fig. 3. Incubation was at 10 °C for 15 h

Most of the circles obtained with T4 ligase are covalently closed: if exposed to denaturating conditions of high temperature or alkaline pH and then returned to a non-denaturing environment, they still appeared as circles, while linear molecules (and nicked circles) collapsed into the so-called 'bushes' (Fig. 2). The ratio of circles to linear molecules in the sample not treated with alkali is essentially identical to the ratio of circles to half the number of bushes in the treated sample.

The measurement of the fraction of circles obtained after exposing *Bsu* segments of SPP1 DNA to the T4 ligase allows a quantification of the joining reaction. However, because of the heterogeneous distribution of the sizes of both the substrates and the products when

Table 1. *Circularization by T4 ligase of flush-ended DNA segments produced by restriction endonucleases*
Cleavage with endo R · *Bsu* was stopped before completion (see text). The arrows indicate the broken internucleotide bonds

Restriction endonuclease	DNA	Sequence of cleavage sites	Number of cleavage sites	Average segment length in nucleotides	Circularization
					%
*Hpa*I	pSC101	d(G-T-T$\overset{\downarrow}{-}$A-A-G)	1	9000	5
	pMB9		1	6000	5
*Sma*I	pSC101	d(C-C-C$\overset{\downarrow}{-}$G-G-G)	1	9000	20
	pMB9		1	6000	20
*Hin*dII	SPP1	d(G-T-Y$\overset{\downarrow}{-}$R-A-C)	10	4000	25
Bsu	pSC101	d(G-G$\overset{\downarrow}{-}$C-C)	≈10	≈ 900	75
	SPP1		≈60	≈ 800	75

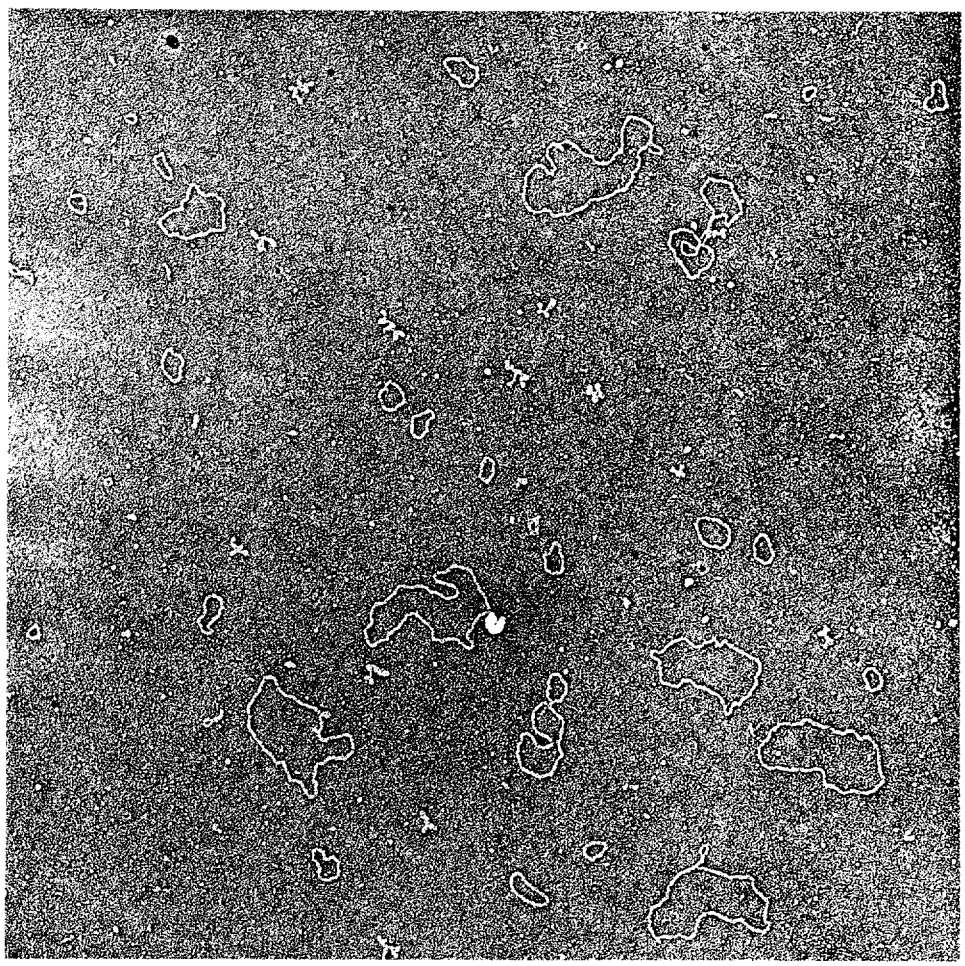

Fig. 2. *T4 ligase products of endo R* · Bsu *segments of SPP1 DNA after exposure to pH 12.5 for 10 min at room temperature and neutralization.* Linear molecules have collapsed into the 'bush'-like structures

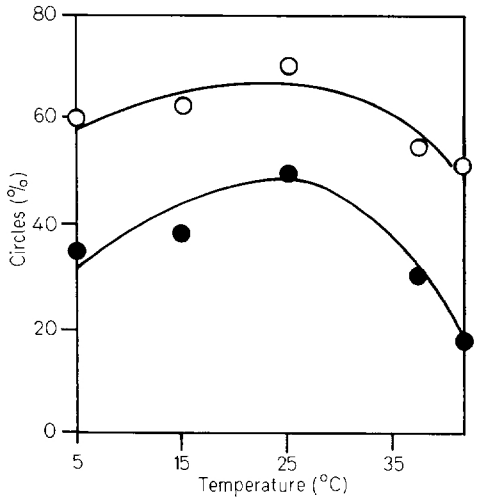

Fig. 3. *Effect of the temperature on the cohesive joining of endo R · EcoRI segments of SPP1 DNA (○) and terminal joining of endo R · Bsu segments of SPP1 DNA (●).* 720-µl mixtures containing 1 µl/ml of SPP1 DNA segments, 50 mM Tris-Cl, pH 7.6, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 50 µM ATP, 10 µM nucleotide tRNA and 0.5 or 2 units of fraction V T4 ligase for cohesive and terminal joining, respectively, were prepared at 0 °C and divided into five portions, which were incubated at the various temperatures. The reactions were terminated after 90 min (cohesive joining) or 210 min (terminal joining), transferred to chilled tubes containing 1 µl of 0.5 M EDTA and heated at 65 °C for 5 min. To determine the percentage of circles, at least 100 molecules were scored on the electron microscope for each point

these segments are used, some underestimation of the enzymatic activity is possible: in spite of the low DNA concentration, linear molecules can still be the product of one or more sealings, and similarly two or more joining events can be responsible for the appearance of a circle.

Comparison between Cohesive and Terminal Circularization of DNA Segments by the T4 Ligase

We compared the efficiency of the circularization of blunt-ended DNA duplexes produced by the action of endo R · *Bsu* on SPP1 with that displayed by segments carrying the short cohesive termini resulting from endo R · *Eco*RI cleavage of the same DNA. For brevity, the former will be called a 'terminal' and the latter a 'cohesive' mode of joining.

Because of the different numbers of targets in SPP1 for restriction endonucleases *Bsu* and *Eco*RI (about 100 and 14 respectively [17], the sizes of the limit digestion products could affect the results of the comparison. We therefore resorted to the use of partial digestion of SPP1 DNA with endo R · *Bsu:* in this way we expected the segments to be of a similar average size as those produced by endo R · *Eco*RI. Electrophoretic analysis of the digestion and of the ligation products (not shown) confirmed this expectation.

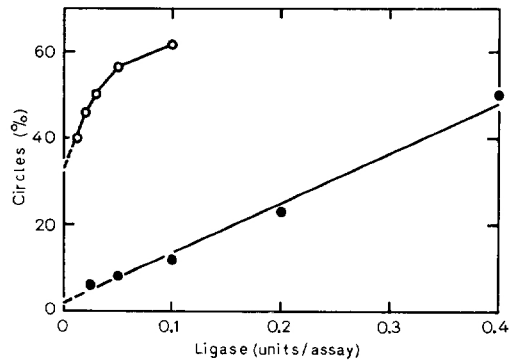

Fig. 4. *Comparison of the extents of the cohesive (○) and terminal (●) ligation as a function of amount of enzyme.* Reactions mixtures were as described in the legend of Fig. 3. Incubation with varying amounts of enzyme was performed at 25 °C

Temperature dependence of cohesive and terminal joining is shown in Fig. 3. The optimum appears to be close to 25 °C, although little variation was observed between 5 and 42 °C in the former case.

On the basis of these results, we then investigated the effects of varying amounts of enzyme on both types of joining. The data shown in Fig. 4 indicate that about 10 times more ligase is needed for terminal joining to reach the levels observed for cohesive joining.

In the case of the cohesive joining, it is apparent that the extrapolated line intercepts the ordinate axis above 30%: this could be a fraction of segments capable of undergoing efficient circularization, possibly because the hydrogen-bonded circles they form are stable enough to be joined even in the presence of low amounts of enzyme. If no enzyme was added less than 1% of circles could be seen.

As for the terminal joining, the intercept of the ordinate is close to the origin, as if the fraction of segments capable of efficient circularization at low enzyme levels was negligible.

Purification of the Terminal and Cohesive Joining Activities of T4 Ligase

The ability of the T4 ligase to circularize segments generated by restriction endonucleases *Eco*RI and *Bsu* was exploited to monitor the purification of the cohesive and the terminal joining activities, respectively, associated with this enzyme. The purification procedure detailed by Weiss et al. was essentially followed until the second DEAE-cellulose column chromatography [8]. The fractions were assayed for (a) ATP-[^{32}P]PP_i exchange, (b) circularization of *Eco*RI segments, and (c) circularization of *Bsu* segments. Both substrates were obtained from SPP1 DNA, the former as a limit digest and the latter after partial digestion as outlined above. Fig. 5 gives the elution of the various activities following DEAE-cellulose chromatography.

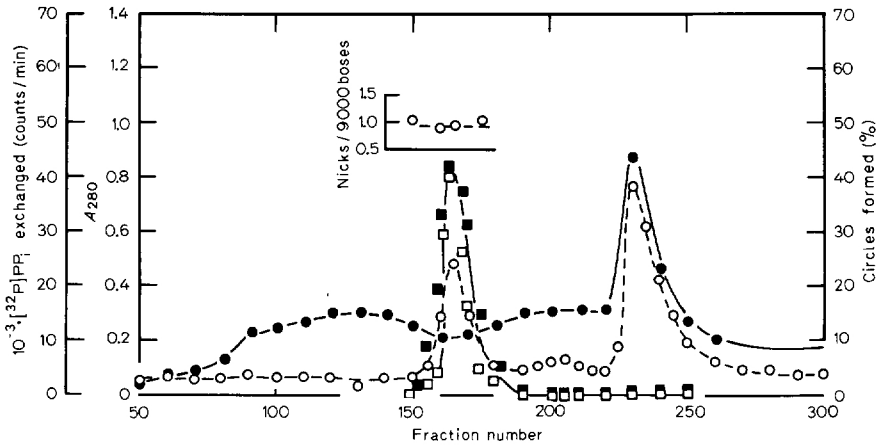

Fig. 5. *DEAE-cellulose chromatography of the T4 ligase.* For the details of the purification, see Weiss et al. [8]. The various profiles refer to: (○) ATP-[^{32}P]PP$_i$ exchange; (□) cohesive joining of endo R · *Eco*RI segments of SPP1 DNA; (■) terminal joining of endo R · *Bsu* segments of SPP1 DNA; (●) A_{280}. The insert gives an estimation of the contaminating endonuclease, based on the conversion of supercoiled pSC101 into relaxed circles, as determined by electron microscopy

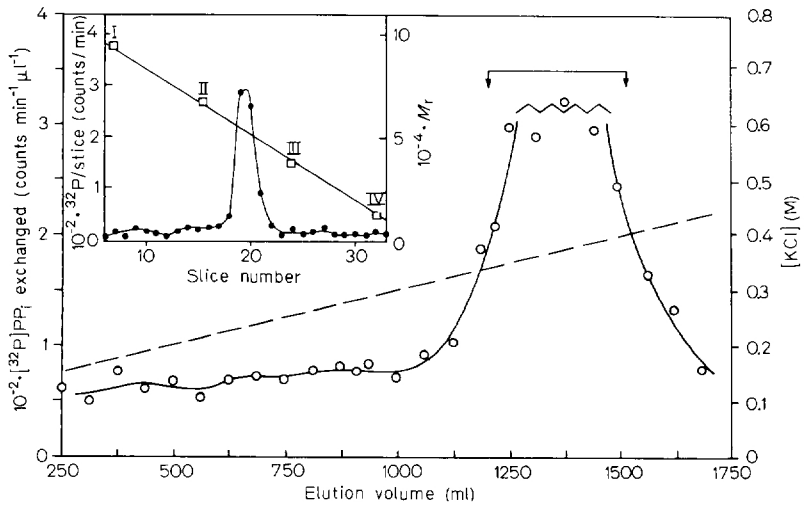

Fig. 6. *Phosphocellulose chromatography of T4 ligase.* The DEAE-cellulose fraction of T4 ligase (fraction V) was loaded on a phosphocellulose P11 column (1.5 × 15 cm) as described [8]. A 2-l linear gradient (– – –) between 0.1 and 0.5 M KCl in 0.01 M potassium phosphate buffer pH 7.6, 0.01 M 2-mercaptoethanol was used to elute the enzyme: the assay was the ATP-[^{32}P]PP$_i$ exchange reaction (○——○). The fractions were pooled as indicated and concentrated [8] with an overall recovery of 40%. Insert displays a molecular weight determination of the ligase-AMP complex, which was formed by incubating 1 µl (0.3 unit) of the concentrated phosphocellulose fraction, with [α-^{32}P]ATP (specific activity 6×10^4 counts min^{-1} µmol^{-1} [20]). Electrophoresis was performed on a dodecylsulfate/polyacrylamide (10%) gel for 7 h at 3 mA/tube [21]. The gel was stained, then sliced and counted [22]. M_r standards: I, phosphorylase *a*; II, bovine serum albumin; III, yeast aldolase; IV, lysozyme. (●——●) Radioactivity; (□) the position of M_r standards

The ATP-[^{32}P]PP$_i$ exchange activity is eluted in two large peaks and an intermediate minor one. The terminal and cohesive joining activities elute essentially together, coinciding with the first ATP-[^{32}P]PP$_i$ exchange peak (1 µg of protein, estimated by A_{280} measurement, exchanges close to 6 nmol of ATP under standard assay conditions [8]). The high levels of joining activities associated with this peak provide indirect evidence that already at this point in the purification there is little, if any, interference by contaminating phosphatases, endonucleases or exonucleases. Nevertheless, the endonucleolytic contaminations were estimated by electron microscopic analysis of the fraction of superhelical pSC101 DNA [18] converted into relaxed circles [19] by an amount of enzyme 10 times higher than that necessary for optimal joining. Only traces of endonucleases were present, as shown by the finding that about one nick per 9000 nucleotides of pSC101 DNA was introduced during a 3-h incubation (Fig. 5, insert).

An enzyme preparation obtained as described was further purified by a phosphocellulose column chromatography (Fig. 6), shown to retain terminal joining activity, and then converted into the [^{32}P]AMP inter-

mediate. Dodecylsulfate/polyacrylamide gel electrophoresis analysis revealed a single peak of radioactivity (Fig. 6, insert).

These combined results lend support to the hypothesis that the terminal joining reaction is carried on by the same enzyme that catalyzes the cohesive reaction.

DISCUSSION

In this communication we report on: (a) a novel assay for the DNA ligase, based on the electron microscopy visualization of covalently closed circles; (b) the varying efficiency of the T4 ligase to join flush-ended DNA segments (generated by restriction endonucleases) at different temperatures: (c) the lower efficiency of the terminal joining as compared to the cohesive one mediated by the short (dA+dT)-rich sticky ends generated by endo R · *Eco*RI; (d) the possibility of obtaining T4 ligase essentially free of interfering contamination through the shortening of a published procedure; (e) the probable identity of the polypeptide responsible in T4-infected *E. coli* cells for both the terminal and the cohesive joining activities.

The electron microscopy assay described here could be made more quantitative by the use of a homogeneous substrate of optimal size. Experiments are in progress to establish a convenient source of such material.

We have shown here that the assay can be used as a measure of terminal and cohesive joining activities. A slight modification should allow investigation of the time sequence of the formation of two phosphodiester bonds at a joined site: spreading of denatured ligation product in the presence of formamide could reveal upon electron microscopy inspection the possible existence of single-stranded circles.

The optimal temperature for both the cohesive and terminal joining is about 25 °C. This presents strong evidence in favor of the stabilizing effect of the enzyme in the interaction of the ends to be joined: the melting temperature of the hydrogen-bonded sticky ends produced by endo R · *Eco*RI is close to 4 °C. The stacking forces, which probably play a role in the apposition of both flush and single-stranded ends, tend also to be destabilized with the increased temperature. Terminal ligation occurs also at 42 °C; it should thus be possible to study the effect of high temperatures on the terminal activity of the temperature-sensitive mutant T4 ligase [23].

A higher enzyme concentration is required for the terminal reaction as compared to be cohesive one. The explanation might be that the interaction between the flush ends to be joined is relatively weak and thus rare; the substrate-enzyme complex could be destabilized at any of the various steps required for the covalent joining [1]. This could result in the accumulation of DNA-AMP intermediate [3] which could hinder the subsequent joining because at the ATP concentration used for the reaction all the enzyme might be converted into the ligase-AMP intermediate [20]. Another explanation is that the enzyme is less firmly associated with DNA in the presence of flush ends than at nicks.

The shortening of the procedure outlined by Weiss et al. [8] for the preparation of the T4 ligase or other available procedures [24,25], coupled by an assay specific for either mode of joining should make it easier to investigate the physiological role, if any, of the terminal ligase in *E. coli* cells infected with T4 phage, as well as in other cells, and also provide a larger supply of this enzyme.

A firm conclusion on whether the two joining reactions are performed by the same polypeptide needs more direct evidence. Investigation of the temperature-sensitive enzyme and purification to physical homogeneity of the ligase preparation should help. At present, the identical chromatographic and electrophoretic mobilities of the two activities strongly suggest that they are both carried by the same enzyme.

Recently, Sugino et al. [26] have performed experiments analogous to some reported here and have confirmed several of our findings reported already in preliminary form [27,28], e.g. the lack of linear dependence on enzyme concentration, the inability of the *E. coli* ligase to join blunt-ended segments, and the presence of both blunt-ended and cohesive joining in the same polypeptide. In addition, they have noticed a strong stimulating effect of RNA ligase on the terminal joining, an interesting observation which still awaits an explanation in molecular terms.

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