

# T4 Ligase Joins Flush-Ended DNA Duplexes Generated by Restriction Endonucleases

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The resealing of single-strand interruptions (nicks) in double-stranded DNA molecules is catalyzed by appropriate polynucleotide ligases (1). Prokaryotic and eukaryotic cells have been shown to contain such enzymes: so far the most extensively studied are the enzymes purified from uninfected and from T4-infected *Escherichia coli* cells. The former have received much attention, mainly thanks to the work of I. R. Lehman and co-workers (1). But T4 ligase displays additional properties of great interest, such as the ability to join DNA-RNA hybrids (2) and flush-ended DNA duplexes (3).

The interaction between the nicked DNA and the enzyme takes place after the enzyme has been activated in the form of an enzyme-adenylate intermediate: the activation is not dependent on DNA. The activated complex recognizes a nick between a 5'-phosphoryl and a 3'-hydroxyl group (4). Both of these functions are necessary for the ligation (1) and have to be kept in close register by a complementary continuous strand. The activated enzyme then links

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its adenyl moiety to the 5'-phosphate through a pyrophosphate bond. The phosphodiester bond linking the two adjacent nucleotides is eventually formed and is accompanied by the stoichiometric release of AMP. All of these events have been established for both the *E. coli* and the T4 ligase and are thoroughly reviewed by Lehman (1).

The recognition of DNA by the ligase thus takes place between an activated enzyme and a nicked double helix: the discovery that the *E. coli* ligase in the presence of AMP can catalyze the reverse reaction and convert supercoiled DNA into relaxed and nicked circles (5) is indicative of the affinity this ligase also has for continuous supercoiled DNA double helices. Data on the reversal of the joining reaction by the T4 ligase are regrettably missing, but its affinity for nicked double-helical DNA molecules is nevertheless well documented (6).

How can the T4 ligase join head-to-head flush-ended DNA duplexes? A brief review of the information available on this reaction is certainly useful in view of its theoretical and practical interest.

The so-called "terminal" joining ability of the T4 ligase was discovered using segments of the synthetic alanine transfer DNA gene prepared and characterized in Khorana's laboratory (3): the nearest-neighbor analysis of the joined products gave unequivocal evidence that the ligation had occurred at the fully base-paired termini of two opposed duplexes (7). The limited availability of such substrate allowed only to reach the conclusion that it takes place at a reasonable rate and with a yield comparable to those observed in the "cohesive" joining of short duplexes held together by single-stranded ends 4-6 nucleotides long (3,7).

Among the naturally occurring substrates with putative base-paired ends, the DNA of *Salmonella typhimurium* bacteriophage P22 was selected for practical reasons. Its native DNA cannot be terminally annealed except after critical portions of the duplex, corresponding to the terminal repetitions, have been converted into single strands by means of lambda exonuclease or *E. coli* exonuclease III (8). Intact P22 DNA could be joined by the T4 ligase, although not at very high efficiency: in a rather slow reaction, the T4 ligase converted 30-40% of the DNA molecules into linear dimers, trimers, and higher oligomers, as based on sucrose gradients and electron microscopy analysis (9). The *E. coli* ligase was unable to perform the same reaction on P22 DNA, but an exciting by-product of this investigation was the discovery of the cohesive nature of the termini produced by the restriction endonuclease *EcoRI* (9). The explosion of the research on other restriction enzymes has now made available a rich supply of substrate for the terminal joining reaction. Among the various flush-ended du-

plexes analyzed with positive results, the most useful turned out to be those produced by the *Bacillus subtilis* R *endo* (10). In the DNA of the SPP1 phage, this enzyme introduces about 100 cuts (10,11), and we have used these flush-ended molecules to develop a new assay for the terminal joining reaction. This assay is based on the intramolecular circularization of the segments in the presence of the T4 ligase and on the visualization of the circles with the electron microscope. Figure 1 gives an example of what one sees after aqueous spreading of a ligated sample of *BsuR endo*-generated segments (the details of this assay will be presented elsewhere).

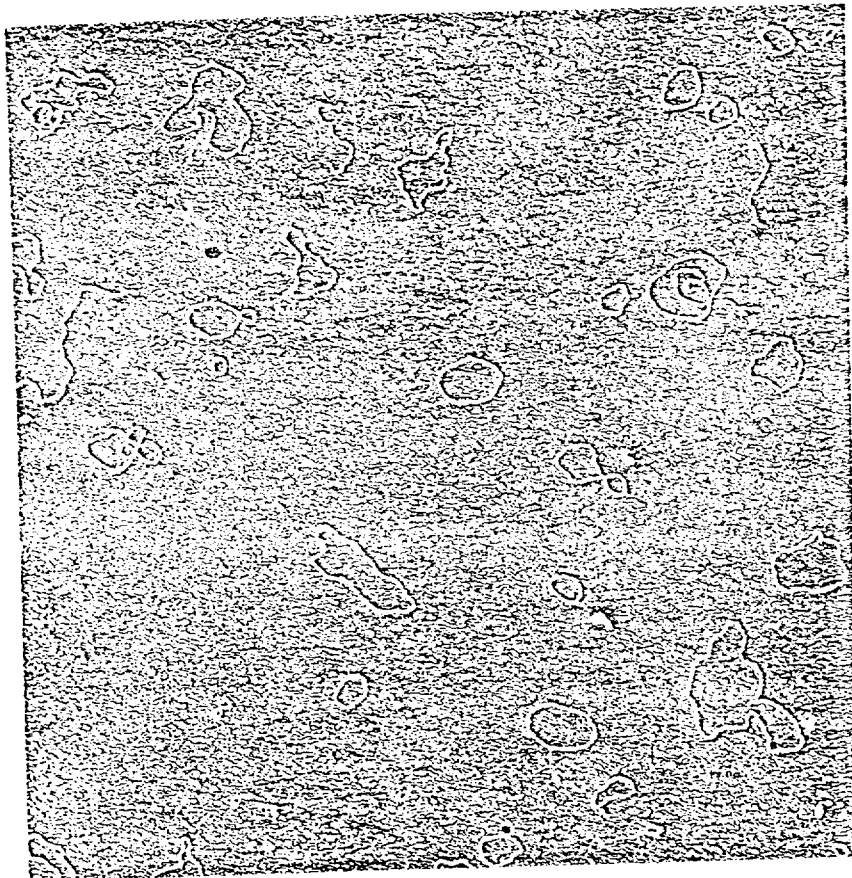


Fig. 1. Electron microscopic visualization of the circles formed by the T4 ligase on flush-ended segments produced by *BsuR endo* on SPP1 DNA. Purification of *BsuR endo* and its use were essentially according to Bron *et al.* (10). For the purification of the T4 ligase, the protocol of Weiss *et al.* was followed until fraction V (13).

It seemed interesting to compare the efficiency of joining DNA duplexes through flush-ended termini to that taking place through the short cohesive termini introduced by *EcoRI* into SPP1 DNA (10). It is important that the average size of the linear molecules produced by limited digestion with *EcoRI* is about 3 times longer than that of the limit digest products of *BsuR* on the same substrate (10). We therefore resorted to the use of limited digestion of SPP1 DNA with *BsuR* *endo*, so that the segments could be of similar average size as those generated by *EcoRI* on SPP1 DNA.

In Fig. 2 are given the kinetics of circularization of *EcoRI*-generated segments: at all the temperatures tested, ranging from 5° to 42°C, the reaction proceeds at comparable rates. The final extents are also very

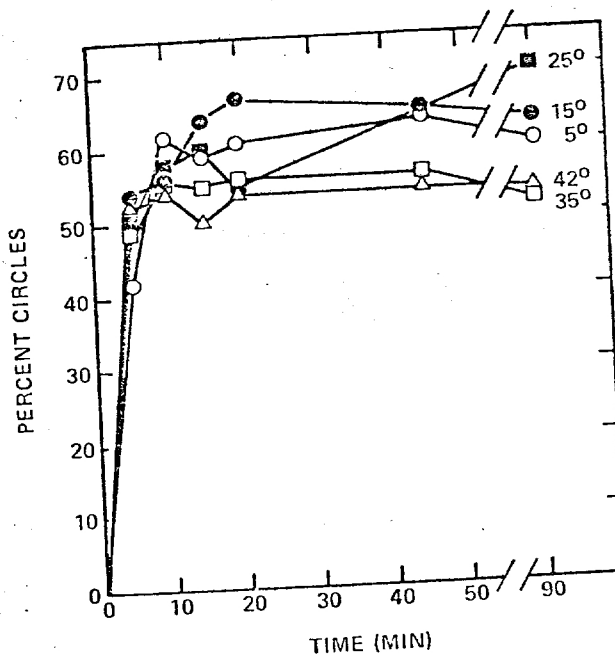


Fig. 2. Effect of the temperature on the T4 ligase-catalyzed joining of cohesive-ended segments generated by *EcoRI* on SPP1 DNA. A reaction mixture of 720  $\mu$ l containing 1  $\mu$ g of *EcoRI* cut DNA per milliliter, 50 mM Tris-Cl, pH 7.6, 5 mM Mg Cl<sub>2</sub>, 1 mM 2-mercaptoethanol, 50  $\mu$ M ATP, 10  $\mu$ M nucleotide tRNA, and 0.6 unit of fraction V T4 ligase was prepared at 0°C and divided into five portions, which were incubated at the various temperatures. Aliquots of 20  $\mu$ l were withdrawn at the indicated times, transferred to chilled tubes containing 1  $\mu$ l of 0.5 M EDTA, and heated at 65°C for 5 min. For electron microscope analysis, the samples were spread according to Inman and Schnös (12) and observed in a Philips 200. For each point, at least 100 molecules were scored to determine the percentage of circles.

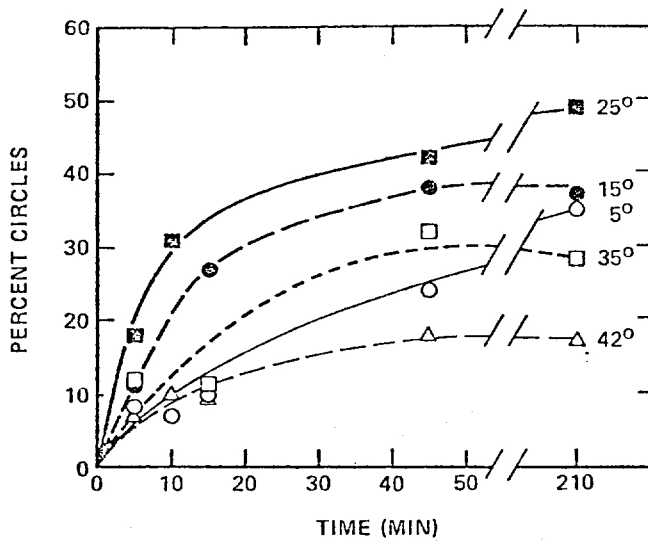


Fig. 3. Effect of the temperature on the T4 ligase joining of flush-ended DNA segments. A reaction mixture was set up as described in Fig. 2, except that it contained per milliliter 1  $\mu$ g of flush-ended DNA segments generated by *BsuR endo* on SPP1 DNA and 2 units of ligase.

close, the highest level being probably attained between 15° and 25°C. Figure 3 shows the results of an analogous experiment on *Bsu*-generated segments. It is apparent that here the highest rate and extent are obtained at 25°C, and the lowest at 42°C. On the basis of these results, we then investigated the effects of various amounts of enzymes on both types of joining. The enzyme activity was determined by the ATP-<sup>32</sup>PP<sub>i</sub> exchange reaction (13) as shown in the inset to Fig. 4. Figure 4 gives the results obtained with *EcoRI*-generated segments. The initial rates seem to be slightly affected by different amounts of enzyme, whereas the final extents tend to level at different values, in spite of prolonged incubations. In the reaction with flush-ended substrates, the differences are even more pronounced (Fig. 5). Both initial rates and final extents are approximately proportional to the amount of enzyme present. As compared to the cohesive joining, the initial rates are close to fiftyfold lower. This difference can be explained in several ways. One explanation is that the presence of cohesive single-stranded termini allows the interaction between two duplexes to last long enough for the enzyme, even when present at relatively low concentrations, to bring forth a rapid ligation. Flush-ended termini, on the other hand, could interact by means of stacking forces,

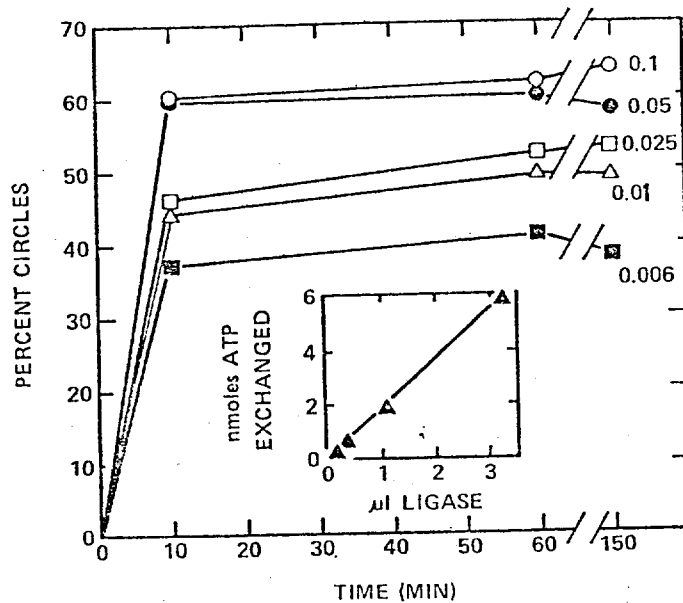


Fig. 4. Effect of the amount of enzyme on the joining of *Eco*RI-generated segments. A 500- $\mu$ l mixture was set up as in Fig. 2 except for the absence of the enzyme. The mixture was divided into five 100- $\mu$ l aliquots, and then appropriate dilutions of the enzyme were added corresponding to the units indicated next to the curves. The inset gives the linear response of the T4 ligase in the ATP- $^{32}$ P<sub>i</sub> exchange reaction (13). The joining reactions were run at 20°C.

but the apposition would conceivably be very transient. Temperatures higher than the optimal one could destabilize the stacking interaction, and lower ones could affect the frequency of productive collisions.

The multistage nature of the joining reaction has already been discussed: in the presence of flush-ended termini, some of the intermediate steps could be slowed down. For example, in the ligation of the small duplexes intermediate in the assembly of the synthetic alanine-tRNA gene (14), small changes in the parameters of the reaction (temperature, Mg<sup>2+</sup> or ATP concentrations) have been found to cause drastic differences in the extent of ligation: in two cases (14,15) of cohesive joining it has been possible to isolate the adenylated oligonucleotides responsible for the low level of ligation. In both cases the interruption to be sealed was six base pairs away from the flush terminus of the duplex under investigation. In the other cases, where different plateaus were obtained, the reasons have not been found, except for a possible not better defined "freezing" of the sub-

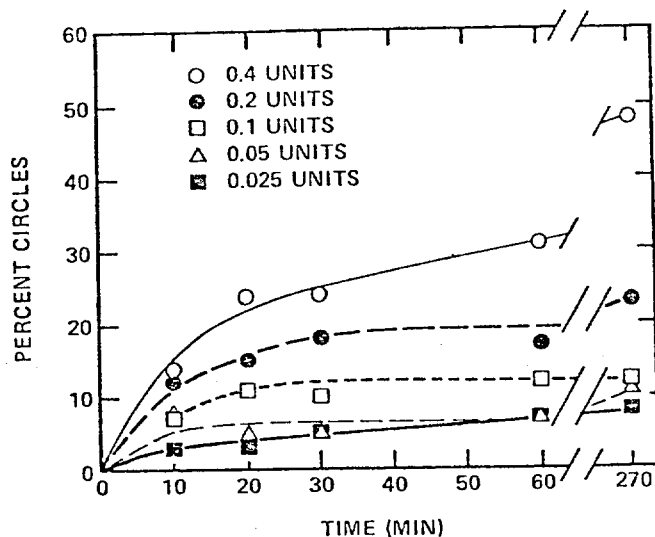


Fig. 5. Effect of the amount of T4 ligase on the joining of *BsuR*-generated flush-ended DNA segments. The mixture was set up as described for Fig. 4 except for the presence of flush-ended DNA segments.

strate in some unreactive structure. Experiments are in progress aimed at establishing whether the substantial amount of uncircularized substrate left after incubation at suboptimal temperatures or in the presence of lower levels of enzyme has been converted into the adenylated form. An additional explanation for the low efficiency of the terminal ligation is the possibility that the enzyme is inactivated faster in the presence of flush-ended substrates than of cohesive termini.

In order to shed light on these possible explanations, experiments are planned in which the efficiency of joining flush-ended duplexes is compared to that in which the cohesion is mediated by different single-stranded termini of decreasing length.

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