and the fourth describes its recognition sequence. In some cases two references appear in one of these categories when two independent groups have reached similar conclusions.

Table II contains a listing of enzymes for which the reecognition sequence is known and which might be useful for preparing recombinant DNAs. They are grouped according to the nature of the fragment ends produced. Thus, fragments generated by all enzymes within any group can be joined to one another.

[3] Addition of Homopolymers to the 3'-Ends of Duplex DNA with Terminal Transferase[1]

By TIMOTHY NELSON and DOUGLAS BRUTLAG

The linkage of two DNAs *in vitro* to form recombinant molecules first became possible with the discovery of DNA ligases.[2] These enzymes, which seal nicks in DNA, can covalently join two DNAs that have complementary sticky ends such as the short, staggered ends generated by many restriction endonucleases.[3] Lobban and Kaiser[4] and Jackson *et al.*[5] showed that complementary ends could be added to DNA molecules *in vitro* with terminal transferase, thus allowing any two DNAs to be linked. These workers added complementary single-stranded homopolymers to two DNA molecules, annealed the homopolymer regions, and covalently closed the resulting hybrid *in vitro* with DNA polymerase I and DNA ligase from *Escherichia coli*. The DNA polymerase was necessary to trim any excess unpaired nucleotides at the 3'-ends or to fill in gaps generated by unequal lengths of the complementary homopolymer regions. Wensink *et al.*[6] simplified this procedure by showing that the annealed recombinant molecules were infectious and that they would be covalently closed *in vivo* during transfection.

Lobban and Kaiser[4] originally found that completely duplex molecules were inefficient primers for the terminal transferase reaction and that pretreatment of the DNA with lambda exonuclease to expose single-stranded

[1] This work was supported by a Basil O'Connor starter grant from the National Foundation-March of Dimes.

[2] I. R. Lehman, *Science* **186**, 790 (1974).

[3] J. E. Mertz and R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3370 (1972).

[4] P. E. Lobban and A. D. Kaiser, *J. Mol. Biol.* **78**, 453 (1973).

[5] D. A. Jackson, R. H. Symons, and P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2904 (1972).

[6] P. C. Wensink, D. J. Finegan, J. B. Donelson, and D. S. Hogness, *Cell* **3**, 315 (1974).

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3'-termini remarkably improved their priming ability. This degradation of the 5'-ends of duplex termini allowed terminal transferase to extend every 3'-end quantitatively, a prerequisite for the formation of biologically active recombinant molecules. Subsequently, Roychoudhury *et al.*[7,8] and Brutlag *et al.*[9] found experimental conditions that allowed terminal transferase to utilize duplex termini without prior exonuclease treatment. Both studies showed that conditions that destabilize DNA duplexes, lower ionic strength or the substitution of Co^{2+} for Mg^{2+} or both, permitted terminal transferase to extend duplex termini. Similar conditions had previously been found by Kato *et al.*[10] to be optimal for terminal transferase action upon single-stranded primers, presumably because they disrupted secondary structure in the primer.

The conditions for homopolymer addition described in this chapter are designed to unravel duplex DNA at the ends. Consequently they are of general application for addition to 3'-primer termini that are recessed, flush, or extended with respect to the 5'-end. Under these conditions addition to randomly sheared DNA is also highly efficient. Unfortunately, addition to nicks is also encouraged,[11] and thus intact DNA and terminal transferase free of endonuclease are required if homopolymers are to be added only at the ends of molecules.

The primary advantage of using homopolymer termini for forming recombinant DNA is that randomly sheared DNA segments can be cloned, eliminating the need for DNA containing specific restriction sites. A second advantage is that all successful infection events result from hybrid molecules, since the vector cannot anneal with itself hence is noninfectious. The major disadvantage of this method is that the infectivity of annealed molecules is usually much lower than that of covalently closed forms.

Principle

The principle of this method is extension of the 3'-end of duplex DNA with a single-stranded homopolymer by treatment with terminal transferase in the presence of a single deoxynucleoside triphosphate. The reaction conditions encourage fraying of the duplex ends and thus permit terminal transferase to act upon a duplex primer. Conditions for adding each of the four deoxynucleotide triphosphates are described to allow the use of either pair of complementary homopolymers for recombinant DNA formation. The average length of the homopolymer can be controlled by

[7] R. Roychoudhury, E. Jay, and R. Wu, *Nucleic Acids Res.* **3**, 863 (1976).
[8] R. Roychoudhury and R. Wu, this series, Vol. 0, p. 00.
[9] D. Brutlag, K. Fry, T. Nelson, and P. Hung, *Cell* **10**, 509 (1977).
[10] K. Kato, J. M. Gonçalves, G. E. Houts, and F. J. Bollum, *J. Biol. Chem.* **242**, 2780 (1967).
[11] W. Bender and N. Davidson, *Cell* **7**, 595 (1976).

varying the time or rate of the reaction, or by limiting the level of deoxynucleotide substrate. Since terminal transferase is a nonprocessive enzyme,[12,13] the reaction rate can be decreased by lowering the level of enzyme below its dissociation constant for the homopolymer being synthesized, or by decreasing the reaction temperature.

Materials and Reagents

Terminal transferase (terminal deoxynucleotidyl transferase, specific activity 34,000 units/mg) was purified according to Chang and Bollum[14] and was the gift of R. L. Ratliff. The commerical preparations of P-L Biochemicals and Bethesda Research Laboratories have also proved satisfactory. Labeled deoxynucleoside triphosphates from New England Nuclear and unlabeled triphosphates from P-L Biochemicals were analyzed for their content of nucleoside di- and monophosphates as described below. *Eco*RI endonuclease was purified according to Modrich and Zabel[15] and was the gift of Paul Modrich.

Methods

Preparation of Substrates

Linear duplex DNA samples are extracted with phenol, passed over a BioRad A-15m agarose column (in 10 m*M* Tris, pH 7.4, 1 m*M* EDTA) to remove the phenol, ethanol-precipitated twice, and resuspended in 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.4. The efficiency of homopolymer addition can be seriously reduced by failure to extract with phenol, particularly following treatment with restriction enzymes which may remain associated with the termini of DNA. For example, terminating an *Eco*RI endonuclease digestion by heating at 65° inactivates the endonuclease and results in DNA that is an efficient primer for DNA polymerase I. However, this DNA is not an effective primer for terminal transferase prior to phenol extraction.[16] Further, it is important that the DNA samples be free of nicks and short oligonucleotides, both of which can serve as efficient primers in the reactions to follow. Utilization of such primers will severely reduce the efficiency of subsequent annealing steps in the formation of hybrid molecules. The A-15m agarose chromatography helps eliminate very short primers. The number-average molecular weight of

[12] L. M. S. Chang and F. J. Bollum, *Biochemistry* **10**, 536 (1971).
[13] D. Brutlag and A. Kornberg, *J. Biol. Chem.* **247**, 241 (1972).
[14] L. M. S. Chang and F. J. Bollum, *J. Biol. Chem.* **246**, 909 (1971).
[15] P. Modrich and R. Zabel, *J. Biol. Chem.* **251**, 5866 (1976).
[16] T. Nelson, unpublished observations.

sheared preparations needed for the calculation of end concentration is most readily determined by electron microscopy.[17]

Unlabeled nucleoside triphosphates are dissolved in water and adjusted to pH 7.0. The relative amounts of nucleoside mono- and diphosphate contaminants are determined by polyethyleneimine chromatography[18] with 0.4 *M* NH_4HCO_3 as solvent. The total nucleotide concentration is determined from the extinction coefficient and then corrected for the actual amount of nucleoside triphosphate determined above. [^{3}H]Nucleoside triphosphates are similarly chromatographed to determine radiochemical purity, and the specific activity is diluted at least 10-fold with the unlabeled form.

Synthesis of Polypyrimidine Homopolymers on Duplex DNA

Optimal conditions for the synthesis of polypurines and polypyrimidines by terminal transferase are different[9,10] and will be treated separately. Polypyrimidines [poly(dT) and poly(dC)] are added to 10–20 pmol/ml of DNA termini with 40 μg/ml terminal transferase in 100 m*M* potassium cacodylate, pH 7.0, 1 m*M* $CoCl_2$, 0.1 m*M* dithiothreitol, and 100 μM ^{3}H-dTTP (0.25 mCi/μmol). The cobalt salt must be added to the reaction last. Since EDTA is usually present in the DNA samples, it is essential that the level of cobalt be 1 m*M* in excess of the final EDTA concentration. Also, other weaker chelators of Co^{2+}, such as phosphate, which may be present in the DNA or in preparations of terminal transferase, should be avoided. Higher amounts of $CoCl_2$ (5–10 m*M*) are inhibitory to terminal transferase.The entire reaction mixture is usually warmed to 37° prior to addition of the enzyme that starts the reaction. Roychoudhury[7,8] has described similar conditions for polypyrimidine addition that use lower amounts of terminal transferase. Lower levels of terminal transferase should be used with caution when employing sheared DNA primers. Since terminal transferase is a nonprocessive enzyme,[12,13] its ability to use a primer will depend on its affinity for that terminus. Sheared DNA will contain both recessed and protruding 3'-ends, and efficient utilization of all ends is assured only if the concentration of enzyme is in excess of its dissociation constant for all types of primers. As a result of the nonprocessive action of terminal transferase, different enzyme concentrations are necessary for maximal rates of polypyrimidine and polypurine synthesis (Table I). Once the maximal rate is attained, no further increase in rate with increasing enzyme is observed. In all the reactions reported in Table I the molar concentration of enzyme is in vast excess of the molar concentration of primer termini. Higher terminal transferase

[17] R. Davis, M. Simon, and N. Davidson, this series, Vol. 12, Part D, p. 412.
[18] K. Randerath and E. Randerath, *J. Chromatogr.* 16, 111 (1964).

ADDITION OF HOMOPOLYMERS TO TERMINI GENERATED BY *Eco*RI[a]

Terminal transferase (μM)	Length of poly (dT) (dTMP/end/5 min)	Length of poly (dA) (dAMP/end/5 min)
0.42	95	19
1.05	291	17
2.11	300	42
4.22	333	130
10.5	424	140
21.1	—	124

[a] *Eco*RI-cleaved pSC101 DNA (0.03 μM in termini) was incubated with the indicated concentration of terminal transferase (13.6–683 μg/ml or 462–23,100 units/ml) under the conditions described in the methods for each reaction.

concentrations in general favor both more efficient utilization of all available primers and more uniform extension of each.

In preparative reactions the extent of addition is monitored by transferring the reaction to ice while aliquots are removed for acid precipitation. The reaction may then be returned to 37° if further chain extension is desired. The actual rate of the reaction with a particular enzyme preparation and primer sample is usually determined beforehand with a small-scale reaction at the desired concentrations of enzyme and substrate. The reactions are terminated by the addition of EDTA to 10 mM, followed by phenol extraction and A-15m chromatography in 10 mM Tris, pH 7.4, 1 mM EDTA. Since DNA containing single-stranded regions often adheres to glass surfaces, we routinely work with plastic columns and tubes. DNA in the void volume of the A-15m column is usually at a concentration in excess of that necessary for the annealing reaction and transfection without further concentration by ethanol precipitation.

Synthesis of Poly(dA) and Poly(dI) Homopolymers

Although polypurine homopolymers can be synthesized under the same conditions as described for polypyrimidine synthesis,[7,8] the use of conditions optimized for polypurine extension makes the addition reaction more rapid, more uniform, and less sensitive to contaminating enzymatic activities. Polypurine synthesis carried out in 20 mM potassium phosphate, pH 7.0, 4 mM $MgCl_2$, 0.1 mM dithiothreitol, and 100 μM deoxynucleoside triphosphate is at least 10 times faster than polypurine synthesis in the cacodylate–cobalt buffer described above.[10] Maximal rates of polypurine synthesis require the use of higher concentrations of primer ter-

mini (20–100 pmol/ml) and terminal transferase (400 μg/ml) than for polypyrimidine synthesis (Table I). This makes small reaction volumes essential. The use of phosphate buffer inhibits *de novo* poly(dA) synthesis frequently encountered during primed synthesis of poly(dA).[10]

Figure 1 compares the rate of addition of poly(dA) to a duplex DNA primer in the high-ionic-strength buffers normally used to assay the enzyme, compared with the lower ionic strengths described here. At low

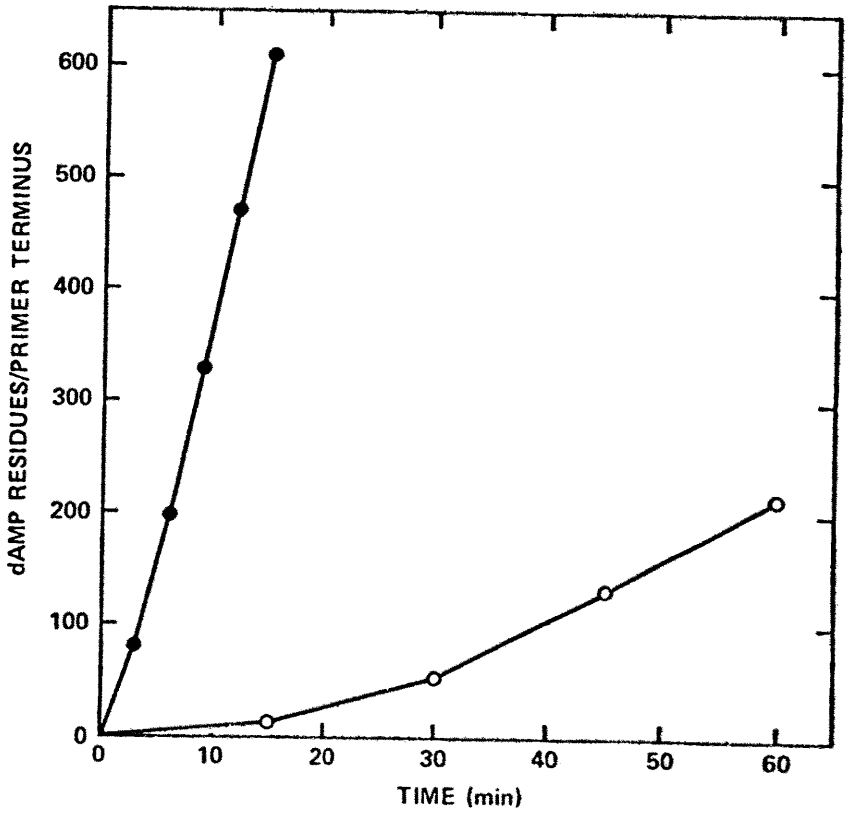


FIG. 1. Addition of poly(dA) to sheared duplex DNA at low and high ionic strength. The solid circles show the average length of poly(dA) added to hydrodynamically sheared satellite DNA (1.705 g/cm³ DNA from *Drosophila melanogaster*,[9] 0.006 μ*M* termini) which was incubated with terminal transferase (4 μ*M*) under the low-ionic-strength conditions (20 m*M* potassium phosphate, pH 7.0, 4 m*M* $MgCl_2$) described in the text. The open circles show an identical reaction performed in 100 m*M* potassium cacodylate, 15 m*M* potassium phosphate, pH 7.0, 8 m*M* $MgCl_2$, 0.1 m*M* dithiothreitol. These higher ionic strength conditions are those originally used by Lobban and Kaiser[4] for terminal addition to duplex DNA. Kinetics with exclusively phosphate buffers gives similar results.

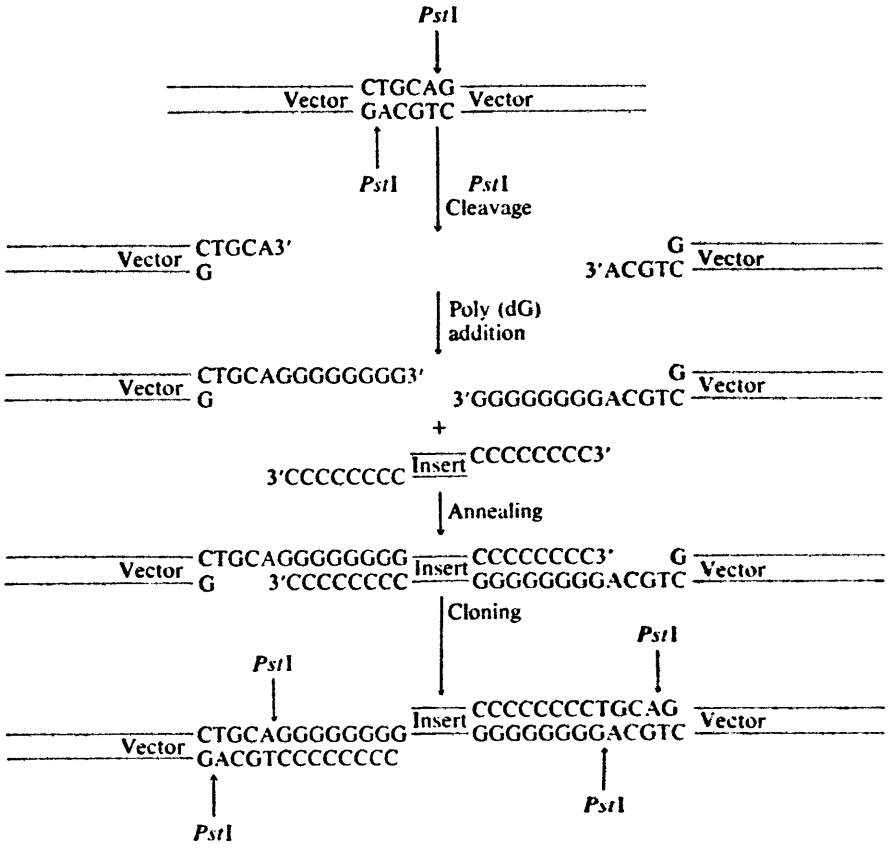
ionic strength, the addition of poly(dA) is nearly linear for the first 600 nucleotides, while at higher ionic strength the rate progressively increases as more and more primers become available, either through extension of less favorable recessed 3'-ends or by endonuclease cleavage of growing chains to generate new primer termini. These autocatalytic reaction kinetics were first observed with duplex primers by Lobban and Kaiser[4] who obtained uniform primer extension only after digestion of the 5'-ends with lambda exonuclease.

The product of the polypurine synthesis is purified as described for the polypurine products. The A-15m chromatography is particularly important here to eliminate the phosphate buffer which strongly inhibits Ca^{2+}-mediated transfection.

Synthesis of Poly(dG) Homopolymers

The joining of vectors and inserts with poly(dG·dC) homopolymers can permit the reconstruction of certain restriction sites (such as *Pst*I, *Hae*III, *Kpn*I, etc.), resulting in two such sites on either side of the insert in the hybrid plasmid.[19] This restriction site reconstruction permits easy removal of the inserted DNA from the vector after cloning:
Direct synthesis of long poly(dG) homopolymers by the polypurine procedure described above is not possible because of the secondary structure formed by poly(dG).[20] While it is possible to circumvent this problem,[21] Röwekamp and Firtel[22] have found that very short poly(dG) and poly(dC) homopolymers (8 to 10 bp average length) can be used for the formation of highly infective hybrid DNA molecules. These workers use the cacodylate–$CoCl_2$ reaction conditions described by Roychoudhury *et al.*[7] (140 m*M* potassium cacodylate, 30 m*M* Tris-OH, 1 m*M* $CoCl_2$, 0.1 m*M* dithiothreitol, pH 6.9) but with a limiting level of dGTP or dCTP (only a 50- to 100-fold molar excess of nucleoside triphosphate over the available termini) and high concentrations of terminal transferase (1000 units/ml) and ends (140 pmol/ml). Under these conditions incubation for only 45 sec at 37° is reported to result in homopolymers of 10 nucleotides average length. Again we have found that it is wise to chill the reaction to 0° and check the extent of reaction before termination. Since the nucleoside triphosphates are well below the K_m for terminal transferase, the rate of the reaction is very sensitive to the absolute amount of nucleoside triphos-

[19] A. Otsuka, personal communication.
[20] F. J. Bollum, *in* "The Enzymes" (P. D. Boyer, ed.), 3rd ed., Vol. 10, p. 145. Academic Press, New York, 1974.
[21] C. F. Lefler and F. J. Bollum, *J. Biol. Chem.* **244**, 594 (1969).
[22] W. Röwekamp and R. Firtel, unpublished results.



Hybrid DNA

phate and to the presence of other contaminating nucleotide species. Röwekamp and Firtel[22] report very high infectivities (100–10,000 colonies/μg of pBR322 vector DNA) even with such short homopolymers. We have confirmed this and find the infectivity with short poly(dG)-poly(dC) homopolymers to be reproducibly 10–100 times higher than with the longer poly(dA)-poly(dT) homopolymers. The success of this method for regenerating restriction sites on either site of the inserted DNA depends strongly on the elimination of all 3'-exonuclease from the restriction endonuclease. The removal of even a single nucleotide from the 3'-end of the vector prior to the terminal transferase reaction will prevent resynthesis of the site. Commercial preparations of *Pst*I from Bethesda Research Laboratories have been satisfactory in this respect. Our current results indicate that 80% of all hybrids have both sites reconstructed, indicating that at least 90% of all the 3'-ends are intact after *Pst*I cleavage.

This method could also be used to clone *Pst*I-generated DNA fragments at any site in a vector by adding poly(dG) to the *Pst*I fragment to be inserted and poly(dC) to any linear vector DNA.

Comments

The efficiency of primer extension by these procedures has been measured in several ways including adsorption of DNA with homopolymer termini to poly(dT) or poly(dA) cellulose;[5] the ability of two DNAs with complementary homopolymers to form circles after annealing,[9] and the nearest-neighbor transfer of ^{32}P from the homopolymer to the terminal nucleotide of the DNA segment.[7] Although these methods are no longer employed routinely, they are useful when one first attempts to add homopolymers with a new preparation of terminal transferase or when a very low infectivity results from transfection. Similarly, new preparations of terminal transferase should be checked for endonuclease activity under the conditions used for homopolymer synthesis. The simplest tests include the conversion of supercoiled DNA to nicked form, monitored either by electron microscopy or by agarose gel electrophoresis. If endonuclease is detected, it may be advisable to try the Co^{2+} conditions rather than the Mg^{2+} conditions described as optimal for polypurine synthesis, since many endonucleases are less active in the presence of $Co.^{2+}$

One of the major disadvantages of using homopolymers to link hybrid DNAs had been the inability to excise them readily from the recombinant molecule. This disadvantage is largely overcome by the ability to resynthesize restriction sites as described above. Even if reconstruction of a restriction site is not feasible, at least two methods are available for excising fragments joined by homopolymer linkers. Hofstetter *et al.*[23] showed that the low thermal stability of poly(dA·dT) regions made possible their specific cleavage by S1 nuclease under conditions of partial denaturation (45–50% formamide, w/v). This technique has been used analytically to estimate the length of the linker regions and preparatively to isolate the cloned segment free of vector DNA. Goff and Berg[24] have described a technique for excision of cloned segments, which takes advantage of the opposite polarity of the homopolymers at either end of the insert. This procedure can be used to isolate segments flanked by either poly(dA·dT) or by poly(dG·dC).

It should also be cautioned that heterogeneity in the lengths of homopolymer joins has been observed both in cloned and subcloned recom-

[23] H. Hofstetter, A. Schambock, J. van den Berg, and C. Weissmann, *Biochim. Biophys. Acta* 454, 587 (1976).

[24] S. Goff and P. Berg, *Proc. Natl. Acad. Sci. U.S.A.* 75, 1763 (1978).

binant plasmids.[9,25] This length heterogeneity may be related to the instability observed for other simple repeating sequences within cloned DNA.[9,26] Such length variation should be considered in any sequencing strategy that involves sequencing through the homopolymer joins or that relies on a restriction site near such a join. The use of very short homopolymers as described above should largely eliminate this problem.

[25] T. Maniatis, G. K. Sim, A. Efstratiadis, and F. C. Kafatos, *Cell* **8**, 163 (1976).
[26] D. Brutlag, K. Fry, T. Nelson, and P. Hung, *Cell* **10**, 509 (1977).

[4] DNA-Joining Enzymes: A Review

By N. PATRICK HIGGINS and NICHOLAS R. COZZARELLI

The first DNA-joining enzymes identified were the DNA ligases. They join together DNA chains by transmuting the high-energy pyrophosphate linkage of a nucleotide cofactor into a phosphoester bond between 5'-phosphoryl and 3'-hydroxyl termini. The impetus for the nearly simultaneous discovery of DNA ligase in bacteria[1-3] and in bacteriophage-infected cells[4,5] was the recognition that DNA fragments were joined together during genetic recombination.[6,7] Shortly thereafter, Okazaki and co-workers[8] showed that ligase played the key role in DNA replication by joining the nascent small pieces of DNA at the replication fork. Ligase has since been found to participate in the synthesis and repair of cellular DNA in a variety of organisms.[9-11]

The termini of DNA strands to be joined by ligase must in general be abutted by base pairing to a complementary strand. This ensures proper alignment and preservation of the nucleotide sequence of DNA; thus, the reaction can be considered *conservative*. Template-dependent synthesis and faithful conservation of DNA coding information is also the hallmark

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[9] I. R. Lehman, *Science* **186**, 790 (1974).
[10] K. A. Nasmyth, *Cell* **12**, 1109 (1977).
[11] L. H. Johnston, and K. A. Nasmyth, *Nature (London)* **274**, 891 (1978).

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