Five Hundredfold Overproduction of DNA Ligase After Induction of a Hybrid Lambda Lysogen Constructed in vitro

Abstract. *A lambda vector that contains the gene for* Escherichia coli *DNA ligase (λgt4-*lop*-11* lig$^+$*) has been modified to achieve overproduction of this enzyme. The third Eco RI site in the lambda chromosome has been altered by mutation, and the left-hand Eco RI fragment has been shortened. The new vector, λgt4-*lop*-11* lig$^+$*, forms a stable lysogen which, upon induction, produces a 100-fold increase in DNA ligase activity. Introduction of a phage mutation (S7) that prevents cell lysis results in an even greater increase (500-fold).*

Molecular cloning is a highly effective means to amplify the number of copies of a specific gene. It should, therefore, be expected to produce large increases in the amount of enzyme specified by that gene. In fact, cloning of genes in Col E1–type plasmids, and in bacteriophage λ, has resulted in significantly increased levels of exonuclease I and in enzymes of tryptophan and arabinose metabolism (*1*).

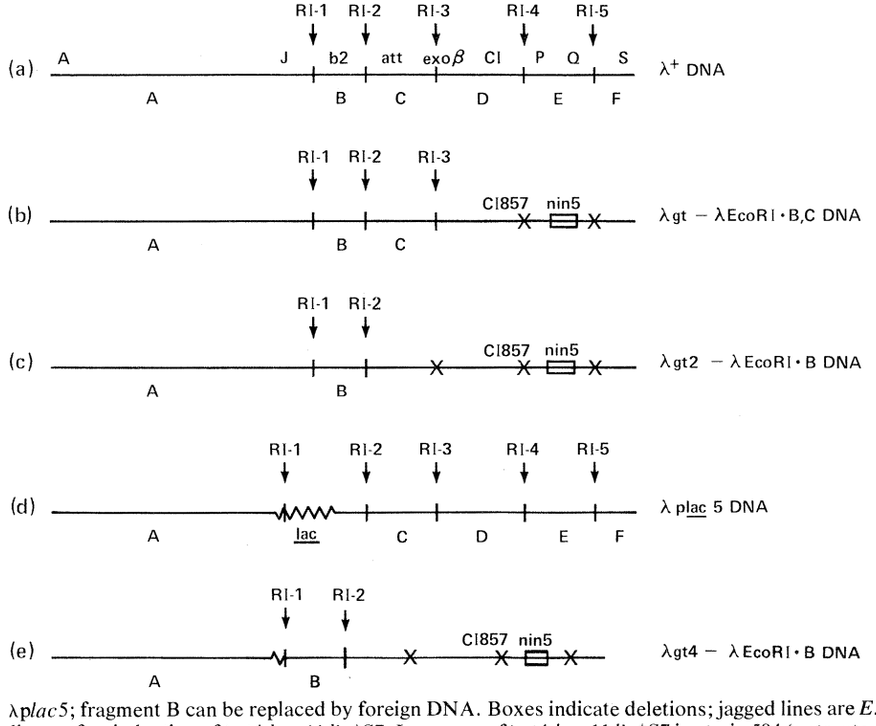
We had reported earlier the construction of a hybrid bacteriophage λ DNA containing the gene for *Escherichia coli* DNA ligase (*2*). The original vector used to construct the λ hybrid was λgt-λB, a strain that had been modified to incorporate endonuclease Eco RI–generated restriction fragments (*3*). In this vector, the inserted DNA replaces the two central Eco RI fragments of λ that are not required for growth. The fragment containing the ligase gene was derived from a mutant strain of *E. coli* carrying a ligase overproducing mutation (*lop*-11). The *lop*-11 mutation itself leads to a fivefold overproduction of ligase (*4*), and infection of *E. coli* with a λgt-*lop*-11 *lig*$^+$ hybrid resulted in a tenfold increase in DNA ligase activity over that in extracts of uninfected *lig*$^+$ cells (*2*). We now describe modification of this λ DNA molecule to produce a vector which, under the appropriate conditions, generates an amount of DNA ligase that is approximately 5 percent of the total cellular protein of *E. coli*.

The λ Eco RI-B and -C fragments containing the attachment site and part of the gene for λ exonuclease are not required for phage growth. However, since phages lacking the λ *exo* gene (*red*$^-$) (for example, λgt-*lop*-11 *lig*$^+$) grow poorly, we have constructed a vector, λgt4, in which the λ *exo* gene has been retained. This was accomplished in the following way: A λ vector (λgt2) was prepared in which the third Eco RI site was removed by mutation. Thus, the Eco RI-C fragment containing λ *exo* and the attachment site will persist after cleavage with Eco RI (Fig. 1c). λgt2 was converted to λgt4 by removing the left Eco RI fragment and replacing it with the shorter left Eco RI fragment of λp*lac*5 (Fig. 1d), a *lac* transducing phage (*5*). Eco RI cleavage of the λgt4 DNA at the new Eco RI site (between the *E. coli* and λ DNA segments) results in a new Eco RI-A fragment which is 2.2 kilobase pairs shorter than that from λgt2 and allows insertion of a correspondingly greater length of DNA.

λgt-*lop*-11 *lig*$^+$ was cleaved with Eco RI, mixed with Eco RI–cleaved λgt4 DNA, and joined with DNA ligase. λgt4-*lop*-11 *lig*$^+$ recombinants carrying the *lig* fragment in the same orientation as λgt-

Table 1. Assay of DNA ligase activity. Cultures (250 ml) of 594 (Su$^-$) or C600 carrying the various λ prophages were grown in broth (10 g of Bacto tryptone, 5 g of Bacto yeast extract, and 5 g of NaCl per liter) to an absorbancy at 595 nm of 0.7, shifted to 43°C for 15 minutes, then incubated at 37°C. Cells carrying λgt4 and λgt4-*lop*-11 *lig*$^+$ were harvested after 20 minutes; cells carrying λgt4-*lop*-11 *lig*$^+$ S7 were harvested after 2 hours. DNA ligase assays were performed as described by Modrich and Lehman (*13*). Protein concentrations were measured by the method of Lowry *et al.* (*14*).

Phage	Activity (unit/ml)	Specific activity (unit/mg)
Uninfected	3.5	0.35
λgt4	3.5	0.45
λgt4-*lop*-11 *lig*$^+$	343	31
λgt4-*lop*-11 *lig*$^+$ S7	1950	140



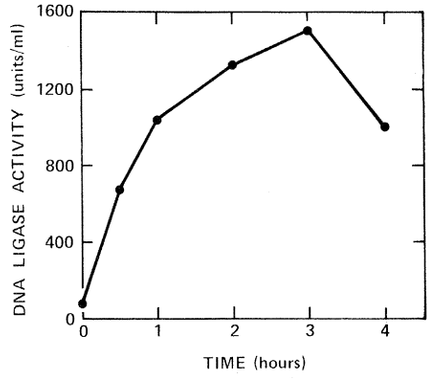


Fig. 1 (left). Construction of λgt4. (a) Eco RI cleavage sites in λ DNA (numbered RI-1 to RI-5 above the line) and cleavage fragments (lettered A to F below the line). (b) In λgt-λ Eco RI-B, and -C DNA, both right-hand Eco RI sites are altered by mutation; fragments B and C can be replaced by foreign DNA. (c) In λgt2-λ Eco RI-B DNA, the Eco RI-3 site is altered by mutation; fragment B can be replaced by foreign DNA. (d) In λp*lac*5 DNA, a new Eco RI-1 site is present in the *E. coli lac* gene substitution. (e) In λgt4-λ Eco RI-B DNA, the Eco RI-3 site is altered by mutation, and the left end is derived from λp*lac*5; fragment B can be replaced by foreign DNA. Boxes indicate deletions; jagged lines are *E. coli* DNA. Fig. 2 (right). Synthesis of DNA ligase after induction of λgt4-*lop*-11 *lig*$^+$S7. Lysogens of λgt4-*lop*-11 *lig*$^+$S7 in strain 594 (a streptomycin-resistant, nonsuppressing strain of *E. coli*) were grown in broth to an absorbancy at 595 nm of 0.7. The cultures were shifted to 43°C for 15 minutes, then maintained at 37°C for the times indicated. Harvesting of cells and assay procedures have been described (*2, 13*).

lop-11 *lig*$^+$-1 were then isolated as described (*2*). Since these phages contain the attachment site and *int* and *xis* genes, they can integrate into the host chromosome and can be maintained in the prophage state (*6*). Recombinants containing *lig* can be easily detected since a conditionally lethal ligase mutant, *lig*ts 7 (*7*), will grow at 43°C when λgt4-*lop*-11 *lig*$^+$ is carried as a prophage. A double lysogen of λ$^+$ and λgt4-*lop*-11 *lig*$^+$ was used to prevent the induction of phage growth at high temperature that occurs as a result of the temperature-sensitive mutation (*c*I857) in the λ repressor carried by the λgt vector. Lysogens containing λgt4 or λgt4-*lop*-11 *lig*$^+$ were induced by a shift to 43°C, and the cell extracts were assayed for DNA ligase activity (Table 1). This modified vector system produced an increase in ligase levels to approximately 100-fold over that in uninfected *lig*$^+$ cells, a significant improvement over the tenfold increase observed with λgt-*lop*-11 *lig*$^+$ (*2*).

λgt4-*lop*-11 *lig*$^+$ was further modified by introducing an amber mutation (S7) in the S gene (which prevents cell lysis) (*8*) by genetic recombination (*9*) between λgt4-*lop*-11 *lig*$^+$ and λ*c*I857 *nin*5 S7. Lysogens containing λgt4-*lop*-11 *lig*$^+$ S7 can be incubated for up to 2.5 hours after induction without significant cell lysis. DNA ligase levels in these cells increased continuously from the time of induction until lysis finally occurred (Fig. 2). Extracts of such cells harvested 2 hours after induction showed DNA ligase levels that exceeded those in uninfected *lig*$^+$ extracts by more than 500-fold. Since there is also overproduction of λ capsid proteins, the increase in specific enzyme activity is somewhat less (400-fold). We estimate that ligase represents approximately 5 percent of the protein in these extracts.

Early work with the lactose repressor carried on a defective λ phage demonstrated the correlation between the number of copies of lactose *i* gene and the amount of repressor present (*10*). Since then, a variety of phages have been used as vehicles for gene amplification. For example, a λ transducing phage that contains the DNA ligase gene has been isolated which, during vegetative growth, produced a 30-fold increase in ligase activity (*11*). The λ vector system that we have constructed in vitro has a number of very attractive features. Cells containing the vector as a prophage are stable, and λ carries no known translocons capable of the *rec*A-independent transfer of DNA segments known to occur with some plasmids (*12*). Since the hybrid DNA is present in only a single copy in the prophage, it should be possible to clone genes in λ which would be lethal to the host in multiple copies. Furthermore, the presence of the cloned gene in a single, unique sequence within the cell lessens the chance of sequence variants arising among a pool of gene copies. Amplification of the gene and, hence, the amount of enzyme coded for by the gene, is achieved, when desired, by induction of the prophage. The amber mutation in the S gene allows a long period of gene amplification and expression before extracts are prepared and, thus, even further enhances overproduction. It is obvious that the enzyme overproduction attained with this system significantly reduces the amount of purification necessary to obtain homogeneous protein and thus should make it substantially easier to prepare the large quantities of pure DNA ligase required for the detailed analysis of its structure and mechanism.

SHARON M. PANASENKO
JOHN R. CAMERON
RONALD W. DAVIS, I. R. LEHMAN
Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

References and Notes

1. D. Vapnek, N. K. Alton, C. L. Bassett, S. R. Kushner, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3492 (1976); V. Hershfield, H. W. Boyer, C. Yanofsky, M. A. Lovett, D. R. Helinski, *ibid.* **71**, 3455 (1974); A. S. Hopkins, N. E. Murray, W. J. Brammar, *J. Mol. Biol.* **107**, 549 (1976); L. Clarke and J. Carbon, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4361 (1975).
2. J. R. Cameron, S. M. Panasenko, I. R. Lehman, R. W. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3416 (1975).
3. M. Thomas, J. R. Cameron, R. W. Davis, *ibid.* **71**, 4579 (1974).
4. M. M. Gottesman, M. L. Hicks, M. Gellert, *J. Mol. Biol.* **77**, 531 (1973).
5. J. Shapiro, L. MacHattie, L. Eron, G. Ihler, K. Ippen, J. Beckwith, *Nature (London)* **224**, 768 (1969); A. Rambach and P. Tiollais, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3927 (1974).
6. M. E. Gottesman and M. B. Yarmolinsky, *Cold Spring Harbor Symp. Quant. Biol.* **33**, 735 (1968).
7. C. Pauling and L. Hamm, *Proc. Natl. Acad. Sci. U.S.A.* **60**, 1495 (1968).
8. A. R. Goldberg and M. Howe, *Virology* **38**, 200 (1969).
9. J. S. Parkinson, *Genetics* **59**, 311 (1968).
10. W. Gilbert and B. Müller-Hill, in *The Lactose Operon*, J. R. Beckwith and D. Zipser, Eds. (Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y., 1970), p. 93.
11. M. M. Gottesman, *Virology* **72**, 33 (1976).
12. S. Cohen, *Nature (London)* **263**, 731 (1976).
13. P. Modrich and I. R. Lehman, *J. Biol. Chem.* **245**, 3626 (1970).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *ibid.* **193**, 265 (1951).
15. Supported by National Cancer Institute contract NO1 CP61026 and NCI postdoctoral fellowship CA 09151 (to J.R.C.).

31 January 1977

Interspersion of Short Repetitive Sequences Studied in Cloned Sea Urchin DNA Fragments

Abstract. *The length and spacing of repetitive sequence elements were studied in several cloned sea urchin DNA fragments. Sequence organization in these fragments was found to be of the short interspersed form expected for sea urchin DNA, and for one clone the evidence shows that nearby repeats do not belong to the same repetitive sequence family.*

Interspersion of short repetitive DNA sequences with single copy sequences appears to be a widespread feature of higher animal genomes. DNA sequence organization in many animal genomes, representing a wide range of phylogenetic groups, has now been examined in some detail (*1–8*). In the typical case a majority of the genome consists of short repetitive sequences about 300 nucleotide pairs long interspersed with single copy sequences about 1000 to 3000 nucleotide pairs long. Exceptions to this general pattern of DNA sequence organization have so far been found only in the DNA's of several insects, including *Drosophila melanogaster* (*5, 6*), the honeybee *Apis mellifera* (*7*), and the midge *Chironomus tentans* (*8*). The genomes of these organisms are relatively small, and they do not contain detectable quantities of short repetitive DNA sequences interspersed with single copy DNA.

Current knowledge of DNA sequence organization derives largely from measurements of binding to hydroxyapatite of partially renatured DNA fragments of various lengths which contain repetitive duplex. The fraction of these fragments included in repetitive duplex regions and in the flanking single-strand nonrepetitive "tails" has been assayed optically, and by measuring the amount of renatured DNA that is resistant to digestion with single-strand specific nuclease. Additional evidence has been obtained by electron microscopy of the renatured DNA structures (*3–5*). These methods have yielded a quantitative insight into the average patterns of sequence organization in animal DNA's. We now describe recent progress in analyzing the specific features of interspersed sequence organization in several cloned fragments of sea urchin DNA. In such fragments, it is possible to determine the spacing and the exact length of the individual repetitive sequence elements, as well as their repetition frequency in the genome. An additional question of significant theoretical interest (*9*), which can be investigated in cloned DNA fragments, is whether neighboring