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"IN VIVTRO" METHOD OF ASSEMBLING A SYNTHETIC GENE

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Without prior *in vitro* enzymatic ligation a DNA duplex was assembled successfully by directly transforming competent cells with a mixture containing six synthetic complimentary oligodeoxyribonucleotides and a linearized plasmid. One out of 100 transformants was positive in colony hybridization with one of the synthetic fragment probe. The sequence of the DNA duplex inserted into the plasmid was confirmed by dideoxy sequencing method.

While tremendous progress has been made in developing a rapid and efficient chemical method of oligonucleotide synthesis (1,2), the procedure of gene assembly remained unchanged, i.e. stepwise enzymatic joining of the complimentary fragments. This strategy (3), although slow, has been extensively used in almost all cases. Recently, an improvement of this approach involving one step ligation *in vitro* has been reported from various laboratories (4-6). In this communication, we wish to report a simple and new approach of gene assembly which is termed as '*in vivtro*' method. It involves the transformation of competent cells with a mixture containing synthetic complimentary fragments and linearized plasmid having the same cohesive ends. The effectiveness of this approach is demonstrated in the assembly of DNA from six synthetic complimentary oligodeoxyribonucleotides.

MATERIAL AND METHOD

Deoxyribooligonucleotides were synthesized using DNA synthesizer Model 380A (Applied Biosystem). The unprotected

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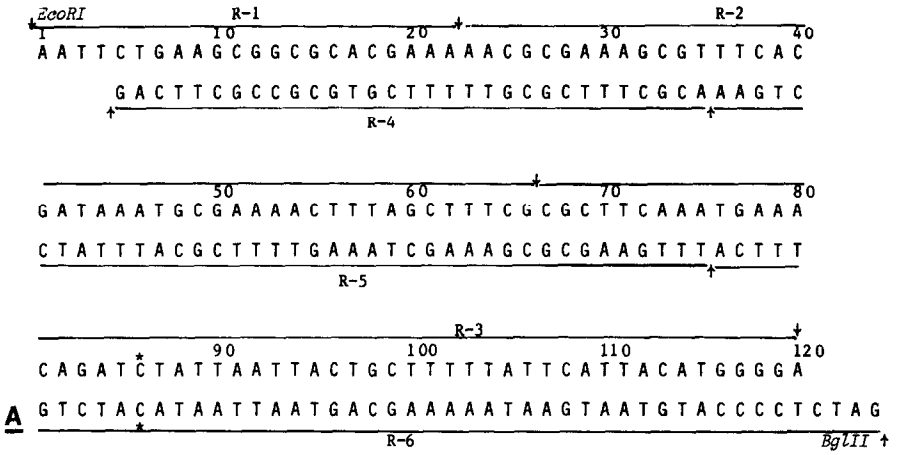
oligonucleotides were purified on 12% polyacrylamide gel containing 7M urea. Plasmid pUC8 (BRL) was purchased commercially. Competent cells HB101 was used for transformation.

In vivtro assembly of Synthetic DNA

Each of the six oligonucleotides R-1 to R-6 (1.5 pmoles) was phosphorylated individually in 5 µl kinase buffer (50 mM Tris HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 5 mM DDT and 0.1 mm ATP) containing 0.5 units of T_4-polynucleotide kinase enzyme. After incubation at 37°C for 2 hr, they were heated to 90°C for 5 min., mixed, heated to 90°C for 5 min, and annealed slowly in water bath to room temperature in a 3 hr time period. Plasmid pUC8 (100 ng) cut with EcoRI-BamHI and dephosphorylated with calf intestine phosphatase, was added to the annealed fragments along with 3.5 µl (10 × ligase buffer), 3.5 µl (4 mM ATP) and 150 µl (H_2O). This reaction mixture was used for transformating E. coli strain HB101. About one hundred colonies were screened by colony hybridization with a ^{31}P-labelled oligonucleotide probe (R-2) and one clone out of one hundred was found to be positive. The DNA sequence analysis of its plasmid by the dideoxy method (7) confirmed the complete sequence as shown in Figure 2.

RESULTS AND DISCUSSION

The traditional approach of gene assembly requires in vitro enzymatic ligation of synthetic complimentary fragments with a plasmid followed by transformation of the competent cells. In our recent studies on gene assembly, we observed various unexpected ligation products which could be rationalized if the enzymatic phosphorylation and joining steps occurred inside the cells (manuscript submitted). Can the enzymatic systems inside the cell be used for gene assembly or for other purposes? To test in vivo DNA-ligase activity, we mixed six complimentary phosphorylated synthetic fragments with a linearized plasmid pUC8 and transformed competent cells HB101 as outlined in Figure 1B. One hundred transformations were probed with a ^{32}P-labelled oligonucleotide fragment (R-2). One of the positive clones, on DNA sequence analysis of its plasmid, confirmed the presence of a completely assembled DNA (Figure 2). A similar



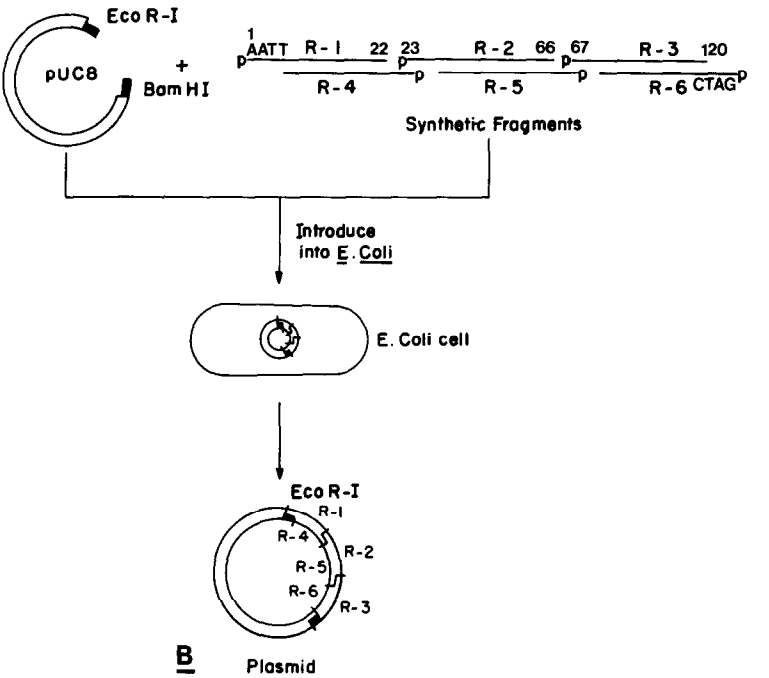


Figure 1. A: Nucleotide sequence of assembled gene beginning with EcoR-I and ending with BglII cohesive sites. The sequence was divided into six fragments, Upper-strand constitutes three fragments; R-I (nucleotide 01-22); R-2 (nucleotide 23-66); R-3 (nucleotide 67-120); lower-strand constitutes three synthetic fragments; R-3, R-4, R-5; Arrow (↓) indicate the junction of synthetic fragments. Star (*) at position 86, show the mismatched C-C base pair in order to create another BglII site by hybrid gene synthesis approach (manuscript submitted).

B: Scheme for in vivtro joining of six synthetic fragments (R-1 to R-6). Six fragments were mixed with linearized plasmid pUC8 at EcoRI and BamHI restriction sites and transformed HB101 competent cells.

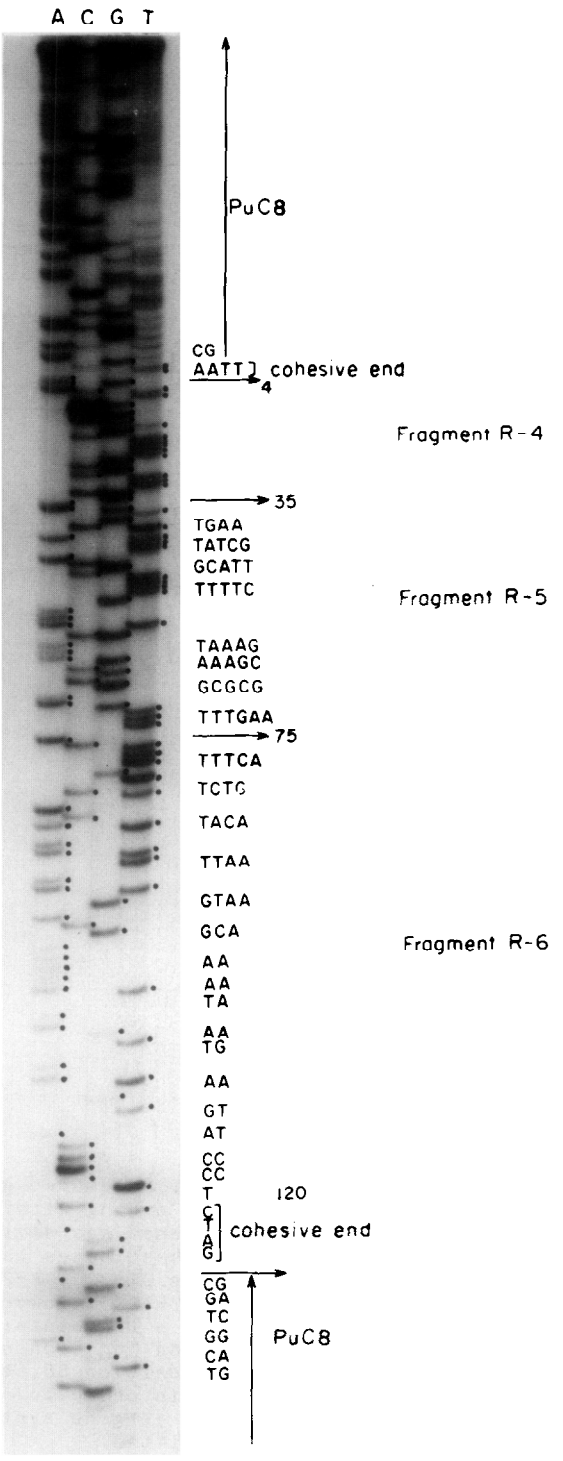


Figure 2. Dideoxydeoxy DNA sequence analysis of pUC8 containing assembled DNA sequence corresponding to the sequence depicted in A of Figure 1 starting from the GATC cohesive end.

experiment with unphosphorylated fragments gave the expected clone but with lower efficiency (the result not shown).

The results confirmed our expectation that enzymatic ligation systems inside the cell can be used in the joining of chemically synthesized oligodeoxyribonucleotides. It is likely that the synthetic fragments and plasmid formed a relaxed but considerably stable circle held by the hydrogen bonds outside the cell. On entry, such a structure was covalently linked by a ligation system inside the cell to form a normal plasmid for replication. We termed this approach as '*in vivtro*' which combined both '*in vitro*' and '*in vivo*' features as predicted by Kornberg (8).

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