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AN EFFICIENT METHOD FOR CONSTRUCTING A CHIMERIC BACTERIOPHAGE T4 TO ESTIMATE THE REGULATORY SIGNALS

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A system for the quantitative estimation of T4 regulatory signals in bacteriophage T4 was developed. A transmitter-reporter plasmid vector, pCV22, which is able not only to fuse transcriptional and translational signals to the coding region of lacZ gene (reporter) but also to transmit the fused gene into T4 phage genome (transmitter). The regulatory signals of T4 phage genes fused with the reportor gene was transmitted efficiently into T4 phage (T4dC-lac phage; a receiver phage) by a replacement type of recombination in the uvsY gene region. This type of chimeric phage was demonstrated to be able to quantitatively estimate the late regulatory signal of T4 phage in vivo.

A number of T4 genes have been cloned and sequenced. T4 early and middle type promoters are turned on at the prereplicative period and these sequences show partial homology with E. coli promoters (1,2). On the other hand, T4 late promoters were shown to have an AT rich consensus sequence, TATAAATA, corresponding to the -10 region of E. coli promoter (3, 4), but do not have any consensus sequence at the -35 region (5). Three T4 phage coded proteins, the products of gene 33, 45 and 55, were demonstrated to be required for in vivo expression of late genes (6, 7). These proteins were considered to interact with the host RNA polymerase for changing the enzyme specificity to recognize only the sequence of the T4 late promoters (3). A minimal sequence required for the function of T4 late promoters was identified using an in vitro transcription assay system; a consensus sequence found in the 5' upstream region of late genes was thought to serve as a T4 late promoter. However, the in vitro system was not able to quantitatively evaluate T4 regulatory signals or to reproduce the timing of T4 late gene expression.

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In this communication we describe the development of an *in vivo* system to estimate quantitatively the regulatory signals of bacteriophage T4 genes.

EXPERIMENTAL PROCEDURES

Bacteria, phage and plasmids. *E. coli* MC1061 (r^-, m^+, *lacZ*) was used as the host for β-galactosidase assay and transformation (8). Plasmid AG1 is a derivative of VX having a *supF* gene (14). *E. coli* C600rm (r^-, m^-, *supE*) was also used as the host for transformation. T4dC phage has mutations in genes *42* (*amC87*), *56* (*amE51*), *denB-s19* and *unf39* (=*alc*)(9). T4 *amNL292* is an amber mutant of gene *55* (7). T4dC-46 was constructed by the procedure described in the text. T4r46 and T4r46-55 are derivatives of T4dC-46, which were constructed by crossing with wild-type T4 and *amNL292* mutant, respectively. T4r46-55 was a blue plaque former having an *amNL292* mutation. Plasmid pTBA1 contains a 6.5 kb *EcoRI* fragment spanning T4 genes *uvsW* to *29* (10). Plasmid pMC1871 (11) was purchased from Pharmacia Co.

Media and culture conditions. Bacterial strains were cultivated in either 2 x YT medium (12) or M9S medium (13). Phage and bacteria were titrated on tryptone agar plates (10). For blue plaque formers, 5-bromo-4-chloro-3-indolyl- -D-galactoside (Xgal) was added in top-agar at a concentration of 100 ug/ml.

Recombinant DNA techniques. Plasmid DNA was prepared as described in a previous paper (10). Cleavage of DNA with restriction enzymes, agarose gel electrophoresis of DNA fragments, ligation, transformtion and other recombinant DNA techniques were carried out as described in a manual (14).

Assay of beta-galactosidase after T4 phage infection. MC1061 cells were grown at 37°C in M9S or 2 X YT medium, and 2 x 10^8 cells / ml were infected with T4 phage at a multiplicity of infection (m. o. i.) of 5 (13). After infection samples of the culture were periodically taken and beta-galactosidase was assayed by the method of Miller (15).

Enzymes and reagents. Restriction endonuclease, T4 DNA ligase, and T4 DNA polymerase were purchased from Takara Shuzo Co. (Kyoto). Xgal was purchased from Sigma Co. Linkers were also obtained from Takara Shuzo Co and phosphorylated with T4 polynucleotide kinase.

RESULTS AND DISCUSSION

(a) Strategy for the quantitative estimation of T4 regulatory signals.

The design of our system for quantitative estimation of T4 regulatory signals in vivo is schmatically illustrated in Fig. 1. We have previously reported a simple method for introducing foreign DNA fragments into T4dC phage genome (10). Any DNA fragments inserted into the *uvsY* gene of T4 cloned in a plasmid vector (transmitter) can be efficiently transferred into T4dC phage genome by a replacement type of recombination. To use this same strategy we constructed a transmitter vector which allows the fusion of the regulatory signals with a reporter gene which has been inserted into the *uvsY* gene. Then the T4 regulatory signlas with the reporter gene was transferred into T4 phage genome as

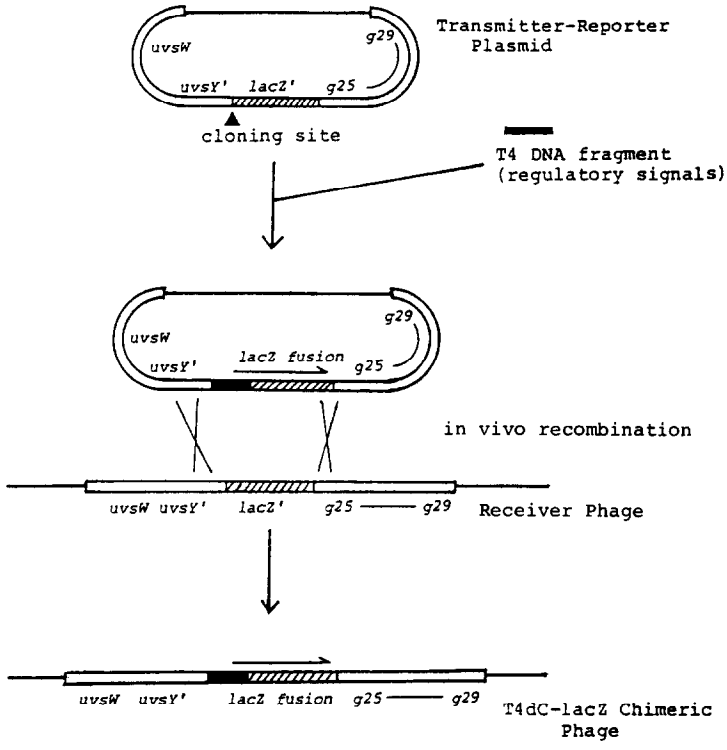

Fig. 1. Schematic representation of the system for the quantitative estimation of T4 regulatory signals.

An outline of the system is shown. A reporter gene (lacZ coding region) was inserted into the uvsY gene of T4 cloned in a plasmid (transmitter). The transmitter-reporter plasmid was used to clone regulatory signals of T4 bacteriophage. A receiver phage which has the same lacZ coding region as the transmitter-reporter plasmid is used to ensure an efficient recombination between the transmitter-reporter plasmid and T4dC phage. The transmitter-reporter plasmid inserted regulatory signals of T4 was recombined with the receiver T4dC phage and the progeny phages which make blue plaques are screened. Open boxes show the T4 genes and hatched boxes show the lacZ coding region. Closed boxes indicate the T4 DNA fragment containing T4 regulatory signals.

described above. The T4-chimeric phages thus constructed were used to estimate quantitatively expression from the regulatory signal by assaying the activity of reporter gene products.

We used the coding region of E. coli lacZ gene as a reporter gene. The activity of lacZ gene product (β-galactosidase) can easily be detected using Xgal and assayed quantitatively.

(b) **Construction of a transmitter-reporter plasmid, pCV22.**

A trasnmitter-reporter plasmid is the most important component of assay system. The plasmid should have two functions; (a) a cloning site for fusing a DNA fragment having regulatory signals to the reporter, and (b) DNA homology used to transmit the fused gene into T4 phage genome. To fulfill the two functions, a BamHI site was placed in the 5' upstream

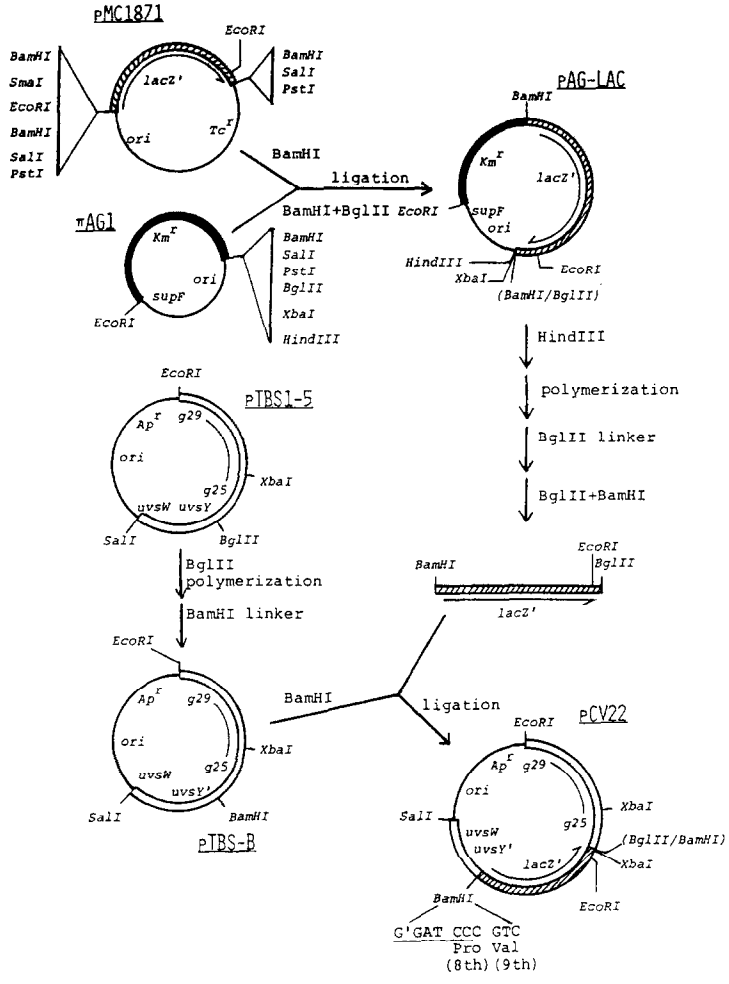

Fig. 2. Procedures for the construction of transmitter-reporter plasmid pCV22.
A 3.1 kb BamHI fragment of pMC1871 containing lacZ coding region was inserted into the BglII site of uvsY gene in a derivative of the transmitter plasmid pTBS1-5. Open, hatched and filled boxes show the T4 uvsY region, the lacZ coding region and a PvuII fragment containing kanamycin-resistance gene derived from Tn903, respectively. A BamHI site in pCV22 is located at the 6th to 8th codon of the lacZ coding region. An arrow aside the lacZ gene (lacZ') indicates the orientation of the gene.

region of the lacZ gene which had been inserted into the BglII site of T4 uvsY gene (16).

Procedures for the construction of a transmitter-reporter, pCV22, are shown in Fig. 2. A 3.1-kb BamHI fragment derived from pMC1871 spanning most of the lacZ coding region except the first nine codons was cloned in the BamHI-BglII site of AG1. A plasmid clone in which the BglII site joined to the 3'-BamHI site of lacZ gene was screened and named pAG-LAC. To convert the BamHI site in the 3'-proximal of lacZ gene to BglII site, the restriction sites of pAG-LAC with HindIII were filled-in with the

stan dard method and then joined with BglII linker. By cleaving the resultant DNA with BamHI and BglII, we obtained a BamHI-BglII DNA fragment that contained the lacZ coding region having a BamHI site at the 5'-proximal and a BglII site at the 3'-proximal of lacZ gene.

Then the BamHI-BglII DNA fragment was ligated with the corresponding sites of pTBS1-B which had been changed one BglII site into BamHI site of pTBS1-5 using filling-in and BamHI liker insertion. The BamHI-BglII fragment containing the lacZ coding region was joined with the BamHI site of pTBS-B DNA and a transmitter-reporter plasmid, pCV22, was obtained; this plasmid had a unique BamHI site at the 5'-end of lacZ available for the cloning of DNA fragments. The BamHI site can be used for the insertion of DNA fragments cleaved with either Sau3A, BamHI, BclI or BglII. Proper insertion of DNA fragments containing regulatory signals into the unique BamHI site of pCV22 should allow the expression of lacZ gene.

(c) Use of receiver phage for an efficient transfer of the fused lacZ gene into T4 genome

The transfer of the fused lacZ gene from the transmitter-reporter plasmid pCV22 into T4dC phage genome can be attained by a replacement type of recombination between pCV22 derivatives and T4dC phage. The same principle had been used to introduce foreign genes into T4dC phage genome (10). However, the size of non-homologous lacZ sequence was so large that it reduces the recombination frequency between the plasmids and T4dC phage. In fact, frequency of the replacement type recombination between pCV22 and T4dC phage was less than 10^{-3} per progeny phage. One way to increase the recombination frequency between the pCV22 derivatives and T4dC phage genome was to use a T4dC-lacZ hybrid phage (called receiver phage) which already contained the lacZ coding region in the uvsY gene of the phage. Accordingly we constructed a T4dC-lacZ hybrid phage using pCV22 and T4dC phage by *in vivo* recombination. The uvsY-defective phenotype can be identified by the inability to propagate in suppressor-negative hosts because T4dC phage with a defective mutation in uvsY gene cannot make plaque on cytosine-substituting growth condition (Takahashi, unpublished results). One of these recombinant phages which was replaced its active uvsY gene with the coding region of lacZ was named as T4dC-lac22. When the T4dC-lac22 was used as a receiver of the lacZ-fused gene from pCV22 derivatives which contained regulatory signals of T4, recombination frequency between the receiver phage, T4dC-lac22 and pCV22 derivatives was very efficient and about 10 % of the progeny phages which was at least two orders of magnitude higher than of that between the original T4dC phage and pCV22 derivatives. Since the

lacZ gene in T4dC-lac22 was promoterless and located within the uvsY gene in an inverted orientation, the receiver phage infection itself did not induce beta-galactosidase activity from the uvsY gene promoter.

(d) **Evaluation of the transmitter-reporter system**

To test the usefulness of the reporter-transmitter system developed in this paper, we used a 1.6 kb Sau3A DNA fragment spanning T4 genes 68, 21 and the 5' part of gene 22 (17). This region contains two typical promoters, p21 and p22 (3)(TATAAATA) and the nucelotide sequence data predicted generation of a gene 22-lacZ fused gene which produces an active β-galactosidase. The Sau3A fragment was joined with the BamHI-cleaved pCV22 and transformed MC1061 cells. These transformants gave scarecely blue colonies on Xgal plates. The expression from the 22-lacZ fused gene in plasmids, however, was clearly detected by blue plaques by T4dC phage infection. This was considered to be due to complementation between the infected phage and fused gene in plasmid. Plasmids from the transformants giving blue plaques to T4dC phage infection were examined by cleavage analysis and shown to give an identical cleavage pattern. One of such plasmids, pCV22-46, was examined by Southern hybridization with lacZ and gene 22 fragments as a probe, which indicated the proper insertion of the Sau3A fragment into the BamHI site of pCV22. Nucleotide sequence the 5' upstream and N-terminal region of gene 22 including the lacZ junction was determined and the in frame fusion of gene 22 and lacZ was confirmed (Noguchi and Takahashi, unpublished results).

To transfer the fused gene into T4 phage genome, MC1061 cells carrying plasmid pCV22-46 were infected with T4dC-lac22 phage and the progeny phages were tested as to the blue plaque formability. About 10 % of the progeny phages was shown to give blue plaques, which indicates that the recombination frequency between the pCV22 derivative and the receiver phage, T4dC-lac22, was quite efficient. The genome structure around the 22-lacZ fused gene was confirmed to be identical to that of pCV22-46 by Geomic Southern hybridization and one of these blue plaque formers was named T4dC-46. This chimeric phage was used to contrcut its derivatives, T4r46 and T4r46-55 (See experimental procedures). The latter phage is a blue-plaque former having an mber mutation in gene 55. Gene 55-dependent expression of 22-lacZ gene in T4 genome is shown in Fig.3. The lacZ expression from the late promoter, p22, was induced at about 10 min after infection of T4r46-55 in the presence of an amber suppressor but not in the absence of it (Fig.3B). This result clearly indicates that the lacZ reporter gene in T4 genome was faithfully expressed with the T4 late regulatory signal. Accordingly we have conlcuded that the transmitter-reporter system described in this paper is quite efficient and

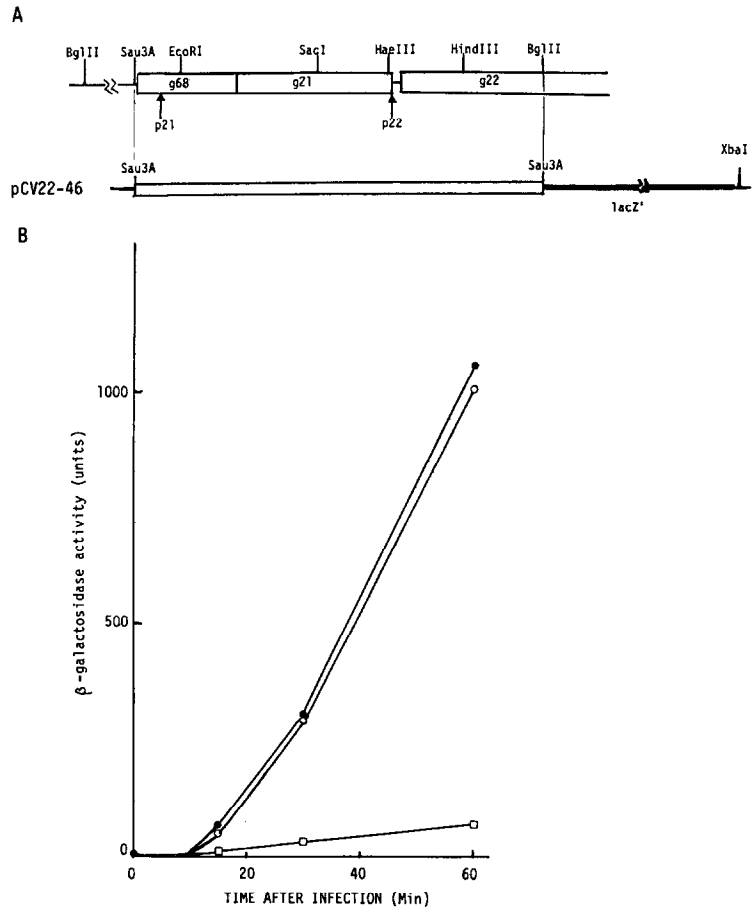

Fig. 3. Gene 55-dependent expression of the 22-lacZ fused gene in T4 phage.

A. Restriction and genomic maps of genes 68-22 region of T4 genome. The 1.6 kb Sau3A fragment spans genes 68, 21 and the 5' part of 22. Arrows indicate two promoters, p21 and p22 (TATAAATA sequence). The lower part shows the Sau3A fragment and the lacZ fused region in pCV22-46 (and T4dC-46 , T4r46 and T4r46-55 phages). B. Kinetics of β-galactosidase production in MC1061 cells (□) and MC1061 carrying TTAG1 (supF) (○) after the infection of T4r46-55. T4r46 phage infection to MC1061 was also shown (●). Cells were grown in M9S medium and infected with T4 phage at 37°C.

useful to construct chimeric phages with the lacZ reporter gene to analyze the kinetics and strength of T4 regulatory signals in vivo.

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