USE OF DEFINED HOMOGENEOUS PROCARYOTIC DNA SEQUENCES TO ANALYZE GENETIC TRANSFORMATION IN PROKARYOTIC AND EUCARYOTIC CELLS

by

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Cloned *Bacillus subtilis* genes were used to transform *B. subtilis* auxotrophs. Intracellular forms of donor DNA were then analyzed by agarose gel electrophoresis and autoradiography. Only donor double stranded molecules were found in the cells and these were sensitive to restriction endonuclease in appropriate recipient cells. The same defined DNA segments were also used to cotransform mouse cells and a sensitive assay was developed to detect donor DNA sequences. Procaryotic carrier DNA was found to inhibit mammalian cell transformation. Addition of DNA to mammalian cells during S phase increased the frequency of stable transformation. The technology described here is sensitive and simple in analyzing intermediate steps in recombination in procaryotes and eucaryotes.

1. INTRODUCTION

In 1958 working at the Carlsberg Laboratories, I learned about genetic transformation and *Bacillus subtilis*. HEINZ HOLTER was trying to set up a transformation system in yeast and *B. subtilis* was used as a source of the protease, subtilisin by MARTIN OTTESEN and LINDERSTRØM-LANG. Since arriving at Stanford in 1959, I have concentrated on various aspects of molecular genetics in *B. subtilis*, especially the genetic control of DNA replication and recombination. More recently our studies of transformation using *B. subtilis* genes were extended to mammalian cells.

Bacilli have become very important in biological research in recent years. The genetic map of *B. subtilis* has been extensively analyzed. This organism has a simple developmental cycle with temporal gene expression controlled by variety of promoters and sigma factors. It has an effi-

cient, genetically controlled secretion mechanism which has been used to isolate products of foreign genes cloned in this organism (6). Some Bacilli produce toxic crystals that are selectively lethal to lepidopteran insects, a biological source of pest control.

B. subtilis can take up externally added DNA and undergo stable homologous genetic transformation. Study of molecular aspects of the process has been much facilitated by recombinant DNA technology and the results are useful in understanding the biology of the *Bacillus* group. We have used well defined homogeneous DNA sequences carrying easily assayable linked genetic markers as donor DNA in both pro- and eucaryotic transformation systems. Use of these molecules in concert with electrophoretic and autoradiographic analysis permit the detection of different intracellular forms of donor DNA and changes that might occur specifically at the

Figure 1. Vectors carrying the leucine genes of *Bacillus subtilis*. The position of the genes in the genome of phage λ along with the molecular weights, which are expressed in megadaltons are shown in a. Arrows indicate the sites of restriction enzyme cleavage by different enzymes as indicated. The vector is referred as λ -*leu* in the text. The plasmid pBR 322 carrying the same 4.5 megadalton segment with the leucine genes is shown in b. It is referred as *pleu* 16.

termini during or prior to actual recombination process. Any changes incurred by the gene or gene cluster could easily be judged by assaying the reisolated DNA from the cells in a second transformation of *B. subtilis* cells. The assays are simple and do not demand any specific donor DNA confirmation (e.g. super helicity).

2. MATERIALS AND METHODS

2.1. Vectors, bacterial strains and mammalian cell lines

These cell lines, bacterial hosts and vectors and the cloning of specific DNA segments are all described in detail in references 5, 7 and 14.

2.2. Donor DNA

Homogeneous donor DNA molecules of uniform length, termini and genetic content were made as follows. A 4.5 (Md) megadalton segment produced by *EcoRI* cleavage of *B. subtilis* DNA carrying three linked genes that control leucine biosynthesis (*leu* A, C, B) was cloned and amplified in *E. coli* using phage λ or plasmid pBR 322 vectors, respectively. They are referred as λ -*leu* and *pleu* 16 and are shown in Figure 1. For transformation of *B. subtilis* leucine auxotrophs, the *leu* segment was cleaved from the vectors and used as donor DNA. Although any of the auxotrophs carrying one of the leucine

Figure 2. Fate of leucine gene DNA in *Bacillus subtilis*. A competent culture of *B. subtilis* leucine auxotroph (10^9 cells) were incubated with the pure leucine genes that were amplified in *E. coli* and were freed of vector DNA at 0.5 micrograms per ml. Aliquots were removed from the transformation mixture at various times and uptake was terminated by treatment with DNase I. A portion of the cells was used for plating to detect the leucine positive transformants. The rest of the culture was used to isolate DNA. Each DNA preparation was divided into two aliquots. One was run on the gel as such (native) and the other was denatured (denatured) prior to electrophoresis on a 0.8% agarose. Included as reference positions are denatured (ss *leu*) and native (ds *leu*) leucine genes of the 4.5 megadalton segment, the donor DNA. The DNA was transferred to nitrocellulose filter paper and was hybridized to ^{32}P labelled nick-translated probe made from *pleu* 16 and subjected to autoradiography. In the "native" lanes there is a gradual increase in the donor DNA entering the cells. No single stranded DNA is detectable at these points. There is a small contaminating chimeric plasmid DNA and

the sequences of pBR 322 (2.6 megadalton) are seen on both sides of the donor DNA. The ds leu lane has also the pBR 322 sequences as marker. The lanes showing the denatured DNA do show their presence at the expected position as the standard, ss leu. Included are two electron microscope pictures of donor DNA molecules isolated from the gel. Plasmid pBR 322 was included as an internal molecular weight standard. The bar scale represents 0.2 μ m.

Figure 3. Fate of leucine donor DNA in a *Bacillus subtilis* strain that is constitutive for the Bsu restriction-modification system (Bsu-R). The experimental protocol was similar to that described for Figure 2. As before there is intense hybridization of the probe at the position of genomic DNA (origin). But unlike Figure 2 native lane where the donor (4.5 megadalton) molecules were clearly seen, here these molecules are restricted and the cleavage products are clearly seen. At 45 minutes most of the fragments have been degraded. At the extreme right hand lane is the molecular weight standard. It is an Eco RI digest of phage Ø3T. The size of the fragments range from 9.4 to 0.46 megadaltons.

mutations could be used for transformation assay, we have used mostly the strain carrying the *leu B* mutation in these experiments (4,14). For cotransformation experiments using mouse L^{MTK}-(thymidine kinase-) cells as recipient, the λ -*leu* DNA was used as such (14).

2.3. End labelling

The 5' end labelling was done after removing the terminal phosphate with alkaline phosphatase and then kinasing the end with T4 polynucleotide kinase using $\gamma^{32}\text{P}$ ATP as substrate. The 3' end labelling was done by "repair synthesis". The purified *leu* DNA segment was digested under very limited conditions with exonuclease III, removing approximately 150 bases from each end. These molecules were then used as a substrate for synthesis with AMV reverse transcriptase and $\alpha^{32}\text{P}$ dATP along with other three deoxyribonucleotide triphosphates. Hind III cleavage of the molecule results in two small terminal segments of 160 and 190 base pairs in length. The label was confined to only these fragments indicating that the labelled regions comprise less than 2.3% of molecular length of the *leu* DNA (4).

2.4. Nick translation

Highly radioactive DNA used as a probe for leucine DNA sequences was made by using *pleu 16* or λ -*leu* DNA as substrate and $\alpha^{32}\text{P}$ dATP as a label along with three other deoxyribonucleotide triphosphates and DNA polymerase I. Specific activities obtained by this method were in the range of 1×10^8 cpm per μgm of DNA (4).

2.5. Transformation and analysis of donor DNA molecules

The *B. subtilis* transformation, analysis of intracellular donor DNA by agarose gel electrophoresis and autoradiography after transfer to nitrocellulose filter paper and hybridization to with the probe (11) are described in the legend to Figure 2. Calcium phosphate coprecipitation method (8) was used to transform mouse L^{MTK}-cells with genomic DNA from HeLa cells and

λ -*leu* DNA. TK⁺ transformants were selected on HAT medium.

3. RESULTS AND DISCUSSION

3.1. Studies of procaryotic cells (*B. subtilis*)

In all procaryotic transformation systems analyzed, DNA molecules are actively transported in to the cells at a particular stage of the cell cycle where they undergo homologous recombination with the resident chromosome resulting in a heteroduplex molecule. Early experiments to study the fate of donor DNA inside the cell made use of DNA molecules heterogeneous in size, termini and genetic content. It was difficult to separate catabolic degradation products from metabolic intermediates in recombination. Literature is abundant on the mode of entry of genomic DNA, viral and plasmid DNA and on the products formed inside the cell (3). Competent cells were sampled at various times after addition of the *leu* DNA segment. They were analyzed for transformation frequency by plating on selective media and the total cellular DNA was purified and analyzed as described in the legend to Figure 2. The DNA labelled at the 5' end lost its terminal label by phosphatases in the recipient cells. The 3' end labelled donor DNA entered the cell with the label since we could detect its presence on the gel in the double stranded *leu* DNA position. However there was no label associated with the genomic high molecular weight DNA at the origin even though the transformation frequency was not affected. This observation suggests that during recombination the two terminal regions of at least 200 nucleotides long are removed. This event might be crucial in recombination. Thus neither forms of labelling is satisfactory to study the intracellular forms of donor DNA. The internalized donor DNA in the cell was identified by hybridizing with nick translated *pleu 16* DNA probe. As early as two minutes after the addition of donor DNA to the cells, native donor DNA molecules were found inside the cell (Figure 2). As one would expect there was extensive hybridization to the cellular DNA.

No single stranded donor DNA molecules or complex renatured molecules were seen at any time. The significance of the observation is

discussed later. This type of analysis is very sensitive, fast and eliminates complicated interpretations associated with centrifuge technology. Thus unintegrated donor DNA migrates at the position of intact double stranded *leu* segment (separate from high molecular weight cellular DNA). Supporting evidence for the *leu* segment being double stranded are its resistance to S1 nuclease after its isolation from the cells (5), the configuration of the molecules under electron microscope (Figure 2) and its *in vivo* restriction in strains constitutive for Bsu-R restriction modification system (12).

In *B. subtilis* transformation system it was observed (12) that a modified or unmodified donor DNA gave the same frequency of transformation with a strain that has the Bsu-R restriction-modification system. In other words the homologous transforming DNA is immune to restriction *in vivo* although the same unmodified DNA is cleaved by the Bsu-R restriction enzyme *in vitro* (1). The mechanism of *in vivo* protection is not known. However, unmodified viral or plasmid DNA are restricted in these cells. The *leu* DNA was given to *B. subtilis* cells with Bsu-R restriction-modification system. The DNA was restricted in the cells, unlike the genomic DNA but like the plasmid or phage DNA. Thus propagation of *B. subtilis* genes in *E. coli* prevents the usual protection given to incoming *B. subtilis* genes. Figure 3 shows the *in vivo* restriction pattern of the *leu* DNA. This pattern resembles the *in vitro* cleavage pattern of the *leu* DNA by Hae III, an isoschizomer of Bsu-R (1). This observation also supports that the donor molecule is in a double stranded form to be a substrate for the restriction enzyme.

The finding that double stranded DNA of donor origin inside the cell was unexpected. There was no detectable single stranded molecules were found at any time. All observations using plasmid, viral or genomic DNA as donor indicate that these DNA are converted to single strands during entry (3). The genomic DNA as single strands are protected by "proteins" and quantitatively converted to a donor recipient complex. In the case of viral and plasmid DNA these single strands, at least some of them renature to form a complete molecule (3).

We have used different methodologies that are

supposed to protect intracellular donor DNA single strands and analyzed by the regimen described above. The results (5) were the same i.e. there was no single strands and only double stranded donor DNA was found. This is very similar to what was found in *Haemophilus* (10). It is also known that double stranded plasmid DNA containing a short region of homology to the host chromosome can undergo insertion in to the chromosome by Campbell model of integration (2,9). One can always postulate that the donor molecules enter as single strands but immediately renature inside the cell. Then why the other types of donor DNAs are not allowed to perfectly renature as double stranded DNA molecules? The most economic interpretation is that the *B. subtilis* genes cloned in *E. coli* and

Figure 4. Transformation of a *B. subtilis* leucine auxotroph with λ -*leu* DNA in a background of LMtk-DNA. λ -*leu* DNA was mixed with LMtk- genomic DNA in a known genomic equivalents and transformed into *B. subtilis* leucine auxotroph. One genomic equivalent of λ -*leu* DNA in 1 microgram LMtk-DNA is 7.6×10^{-6} microgram. The number of cells transformed to leucine prototrophy is plotted against the genomic equivalents of λ -*leu* sequences present in the transformation mix.

Table I.

Clone number	Source of donor tk+ DNA	Number of λ - <i>leu</i> equivalents	
		Gel estimate	Bioassay estimate
17	HeLa S3	0	0
23		1	2
27		20	25
102		0	0
108		2	3
109		5	13
313		40	60
314		1	0
317		5	3
319		10	10

Number of λ -*leu* copies per transformant mouse cell genome. The genomic equivalents of λ -*leu* gene copies present in each transformant was estimated from gels either by scanning the radiograph or by counting the desired regions of the nitrocellulose paper. The bioassay estimate was obtained by transformation of *B. subtilis* leucine auxotroph by the DNA isolated from the tk+ transformants. ptk+2 represents the chimeric plasmid of pBR 322 containing 3.5 kb segment of herpes simplex virus type 1 which has the tk+ gene in it and was kindly provided by P. BERG of Stanford University.

used in these experiments enter the cells as double stranded form.

3.2. Studies of mammalian cells (mouse cells)

Since the technology is simple and sensitive we decided to use the same *leu* genes, λ -*leu* as a donor in cotransforming mouse cells. If transformation occurred then one can follow the fate of the donor DNA in eucaryotic cells. The DNA could be reextracted and used to transform *B. subtilis* cells. Any gene rearrangements or amplifications could be studied easily by the biological assays. It is initially important to quantitate the sensitivity of the assay.

Mouse LMtk-cells was used as recipients. Human TK+ DNA was included as a selectable marker along with the *leu* genes in the λ vector. The LMtk-DNA was used as carrier. Cellular DNA isolated from TK+ colonies was used to transform leucine auxotrophs of *B. subtilis*. Each auxotroph was transformed to prototrophy at a similar frequency indicating that the number of *leu* genes per transformant genome of mouse deduced by transformational assay strongly correlated with the number of copies estimated by hybridization and autoradiography. These experiments support the use of *B.*

subtilis genes as a donor DNA in eucaryotic system. The transformation of reisolated DNA requires no special conformation and the assay can detect the presence of single copy of *leu* gene in a mouse genome. If these genes are amplified in cells, the extent of this amplification is assayed very easily. The results of the reconstruction experiment to standardize the detection of number of copies of *leu* genes are given in Figure 4. The number of *leu* gene copies found in different TK+ transformants are presented in Table I. Thus the results of both physical and biological assays are in agreement. We also found that during incorporation of the procaryotic genes in mammalian genome, there was no alteration in the linkage relationship between genes. Further extension of these experiments indicate (15) that the choice of carrier DNA is important. Procaryotic DNA and phage DNA was found to be consistently poor as a carrier DNA and inhibited transformation frequency be several folds.

GIULOTTO and ISREAL working in this laboratory examined whether the ability of the cells to take up and express stable TK+ transformants is related to a particular stage in the cell cycle. Using synchronized cells and adding TK+ DNA at different stages of the cell cycle they found (7) that the frequency of transformation is signi-

ificantly higher (by several folds) if the cells were exposed to DNA during S phase.

In conclusion, it is shown that use of purified homogeneous procaryotic DNA with known set of genes as donor would enable the analysis of recombinational process in both pro- and eucaryotic systems very easy. Several previously unknown features have been identified by using the technology described here. These are all outlined in this communication.

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