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THE MOLECULAR CLONING OF BACTERIOPHAGE T4:

THE IDENTIFICATION AND EXPRESSION OF A CLONE CODING FOR BACTERIOPHAGE THYMIDYLATE SYNTHETASE

by

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ABSTRACT

Bacteriophage T4 DNA containing cytosine has been obtained from cells infected with phage mutants in genes 56, denA, denB, and alc. This DNA can be cut with a number of restriction endonucleases. However, DNA containing nonglucosylated hydroxymethylcytosine is either refractory to restriction or the patterns of restriction are partial and irreproducible.

Fragments obtained by restriction of cytosine containing DNA with Pstl have been cloned using the plasmid vector pBR322. Plasmids containing ligated fragments were transformed into thymidylate synthetase negative (td^-) host cells. Two clones were isolated with the ability to grow in the absence of added thymine. Both isolates express thymidylate synthetase activity as determined by enzymatic assays.

A class of deletion mutants in T4 has been reported in the td-dfr region by Homyk and Weil. Our data show that these mutants cross react with antisera prepared against td and dfr, revert on nonpermissive bacterial strains, and produce normal levels of dfr upon removal of a second independent deletion. This evidence is in direct contradiction to the original work which reported that these deletions spanned the entire td-frd region.

INTRODUCTION

Basis of Research Problem

The basis of this research problem was to obtain a viable plasmid in E. coli containing, in addition to its own genome, that part of the T4 bacteriophage genome which codes for the enzymes thymidylate synthetase and dihydrofolate reductase (in this work dfr refers to the actual enzyme and frd refers to the gene coding for it). Both of these enzymes are involved in DNA precursor metabolism and their respective roles are shown (Fig. 1). As can be seen dfr is involved in the reduction of dihydrolic acid to tetrahydrofolic acid. Subsequent methylation produces Cl-methyl tetrahydrofolic acid which is the substrate for the methylation of dUMP to form TMP catalyzed by td. This plasmid would be capable of expressing these genes in vivo. The reason for using a recombinant DNA technique was to be able to amplify the DNA sequence coding for these enzymes and in turn the level of protein being made (Hershfield et al. 1974).

T4 is one of a class of E. coli viruses known as the T-even bacterlophages. It is a large virus with an icosahedral head. Attached to the head is a tail composed of a collar, a unique contractile outer sheath, a hollow inner tube, and a hexagonal baseplate (Fig. 2). The head contains one molecule of double stranded DNA whose molecular weight is 1.22 x 108 daltons, long enough to code for 160-170 average gene products (Wood and Revel 1976).

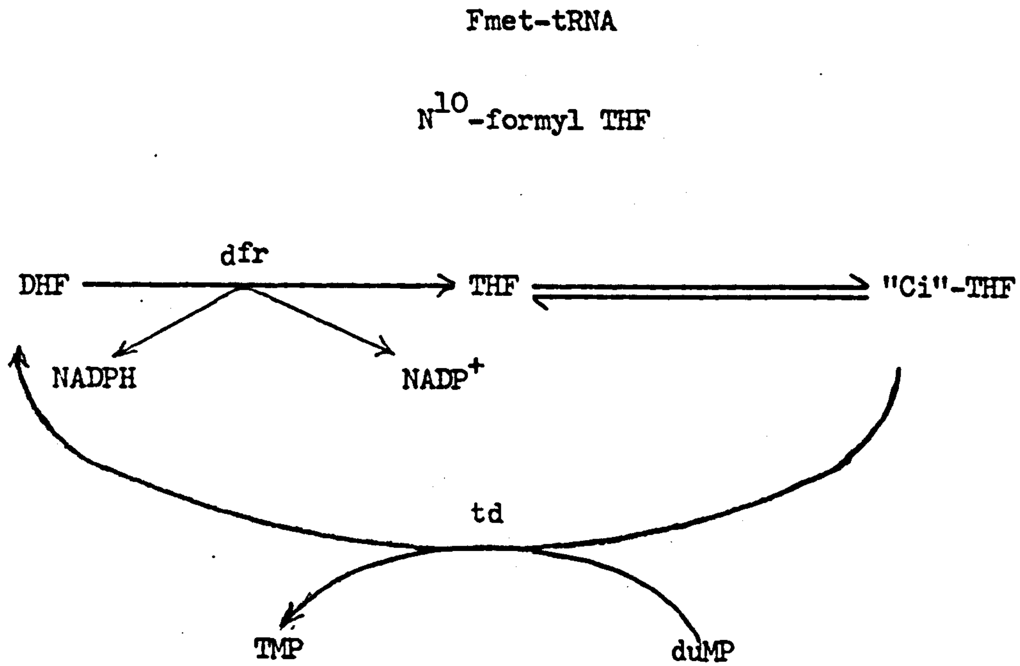


Figure 1. Folic acid metabolism.

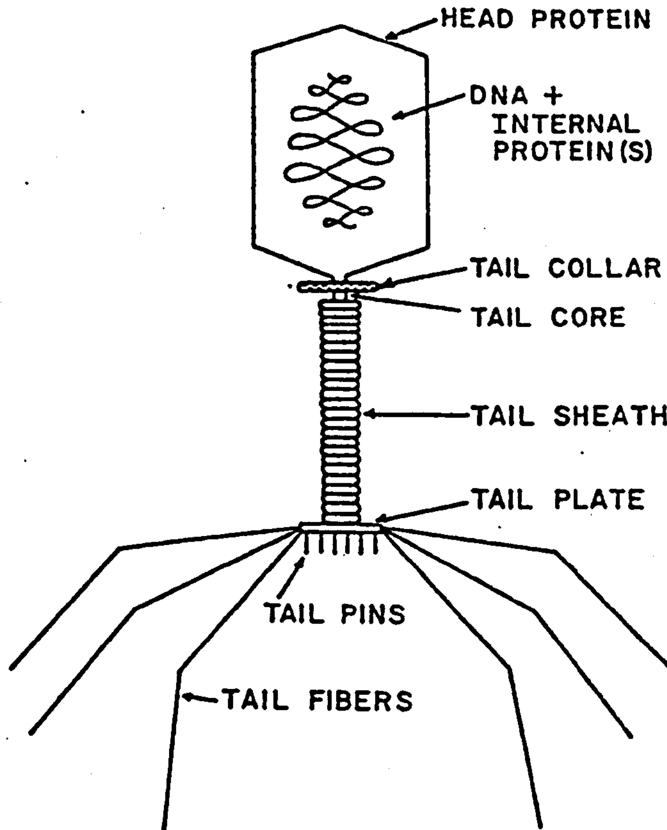


Figure 2. Schematic diagram of phage T4.

Essential Features of T-even Phage Growth

The essential features of T-even phage growth were revealed in the classical studies of Ellis and Delbruck (1939) and Doermann (1948). Subsequent studies in many laboratories (reviewed in Mathews 1971) have gone far to elaborate the phage life cycle.

The process of infection begins with the attachment of the virus to a suitable host cell (Fig. 3). The virus, after colliding with the cell at the proper orientation, makes contact with a cellular surface receptor via the tail fibers. The tail fibers change conformation to allow the tail to make contact. Once contact is initiated, the tail contracts, and the DNA is injected from the head through the hollow tube into the cell. The injected DNA is transcribed and translated into proteins which perform the various functional roles involved in degradation of the host cell's DNA, utilization of the cell's metabolic machinery, and assembly of mature phage particles. Intracellular phage production is completed in about 20 minutes; the cell lyses releasing approximately 200 phage, and a new infective cycle is begun by the viral progeny on other cells in the medium.

Our laboratory (Department of Biochemistry, The University of Arizona, Tucson) has been involved in the study of DNA precursor biosynthesis in T4 for several years. This is an attractive system for studying precursor biosynthesis since both the virus and its host are well characterized genetically. Also bacteria infected by T4 synthesize viral DNA at very high rates facilitating the need for an easily adaptable system whereby precursor nucleotides can be shuttled into the replicative apparatus of the virus.

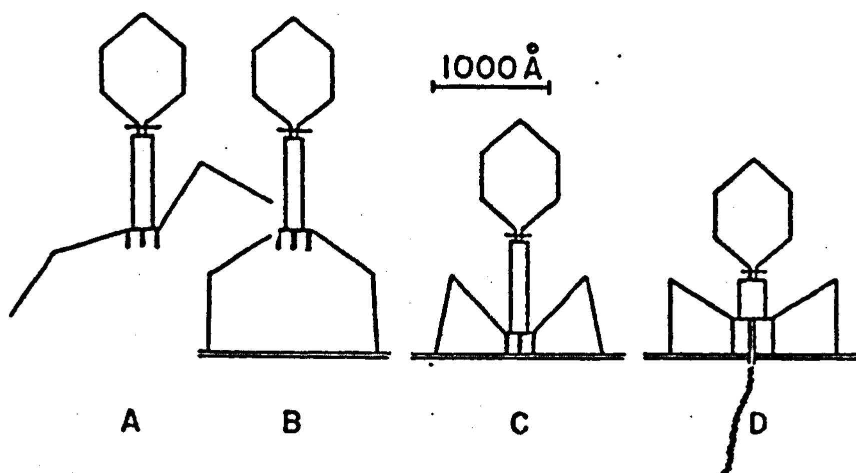


Figure 3. Schematic representation of steps involved in attachment of T4 to its host. -- A, unattached virus; B, interaction of tail fibers with cellular receptors; C, pinning; D, injection of DNA.

The specific protein whose DNA sequence was cloned, thymidylate synthetase, was first described by Flaks and Cohen (1957). It is a dimeric protein composed of two identical subunits, totaling 58,000 daltons (Weber and Osborn 1969; Capco, Krupp and Mathews 1973). This protein is synthesized early in the life cycle of the phage and augments a pre-existing host cell activity. Recent studies in our laboratory (Capco and Mathews 1973; Mosher, DiRenzo and Mathews 1977), and that of Kozloff, Crosby and Lute (1975) and Kozloff, Lute and Crosby (1977) have established that the viral enzyme is also a structural component of the phage particle, thus serving an interesting dual structural and functional role.

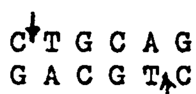
Thymidylate synthetase in higher organisms is the target site for the inhibition of DNA synthesis by drugs such as fluorouracil and fluorodeoxyuridine which have been used in the treatment of cancer (Cohen et al. 1958). Although other anti-cancer drugs which inhibit enzymes involved in DNA synthesis are known (for example, trimethoprim, an inhibitor of dfr), thymidylate synthetase is of particular interest because the product of the reaction is involved solely in the synthesis of DNA and unlike dfr has less potential for causing undesirable side effects (Fig. 1).

Involvement of Recombinant DNA Techniques

Recombinant DNA techniques have evolved and progressed quite rapidly in the past few years to the point where this technology is having widespread applications (Scott and Werner 1977). One reason for its rapid progression and actually its onset has been the discovery

of restriction enzymes (Meselson and Yuan 1968). These are enzymes which recognize the cleave DNA at specific base sequences. Nearly 200 sequence specific restriction enzymes are known with most cleaving at a certain four to six nucleotide sequence in DNA (Roberts 1976).

The predominant enzyme used in these studies, Pstl, recognizes a specific hexanucleotide sequence (Smith, Blattner and Davies 1976).



Staggered cleavage at the vertical arrows generates two double stranded molecules, each with a single stranded complementary end. These single stranded pieces have "sticky" ends, for under certain conditions they will form hydrogen bonds (anneal) and then can be covalently rejoined by the action of a ligation enzyme. In this manner any two DNA molecules possessing sequences for a restriction enzyme can be cut and then rejoined together producing hybrid or recombinant molecules.

A second important component in recombinant work are plasmids. Plasmids are covalently closed circular (CCC) pieces of extrachromosomal DNA which are endowed with the property of self replication (Helinski 1976). They are also small; the plasmid used in these studies (pBR322) has a molecular weight of 2.6×10^6 daltons (4300 base pairs) (Bolivar et al. 1977). An additional feature is that in the presence of chloramphenicol, which inhibits protein synthesis and hence DNA synthesis and cell division, they continue to replicate increasing the number of copies present in the cell by as much as 100-fold (Clewell 1972).

With the use of restriction enzymes and plasmids it is possible to cleave the plasmid and DNA of interest (i.e., T4) with the same enzyme and then covalently join the fragmented DNA molecules together in the presence of ligase (Sgaramella, Van de Sande and Khorana 1970). The plasmid vector used in this work is cut once with Pstl generating linear molecules with sticky ends. Hydrogen bonds are formed between the termini of the plasmid and the DNA to be cloned. The ligase then fuses the DNA sequences together to yield a mixture of products: recircularized plasmids, rejoined fragments, and recombinant plasmids containing inserts.

This is all done _in vitro_. The final step, transformation, allows a bacterial cell to do the replication and amplification of a particular recombinant _in vivo_. This procedure involves treatment of the bacterial cells with $CaCl_2$ and a heat shock step (Mandel and Higa 1970). This procedure renders the bacterial cell permeable to free DNA. Only DNA fragments attached to plasmid vectors can replicate autonomously and hence survive _in vivo_. Isolation of significant quantities of any recombinant can be achieved by chloramphenicol treatment as mentioned previously (Clewell 1972). The basic steps of this procedure are shown in Figure 4.

There are two ways to perform a cloning experiment. By far the easiest, in terms of detection, is to first purify the specific piece of DNA to be cloned (i.e., the restriction fragment containing the td and frd region of T4). This route insures that any recombinants obtained will be the desired ones. Initially this pathway was undertaken but, as shall be seen, was not successful.

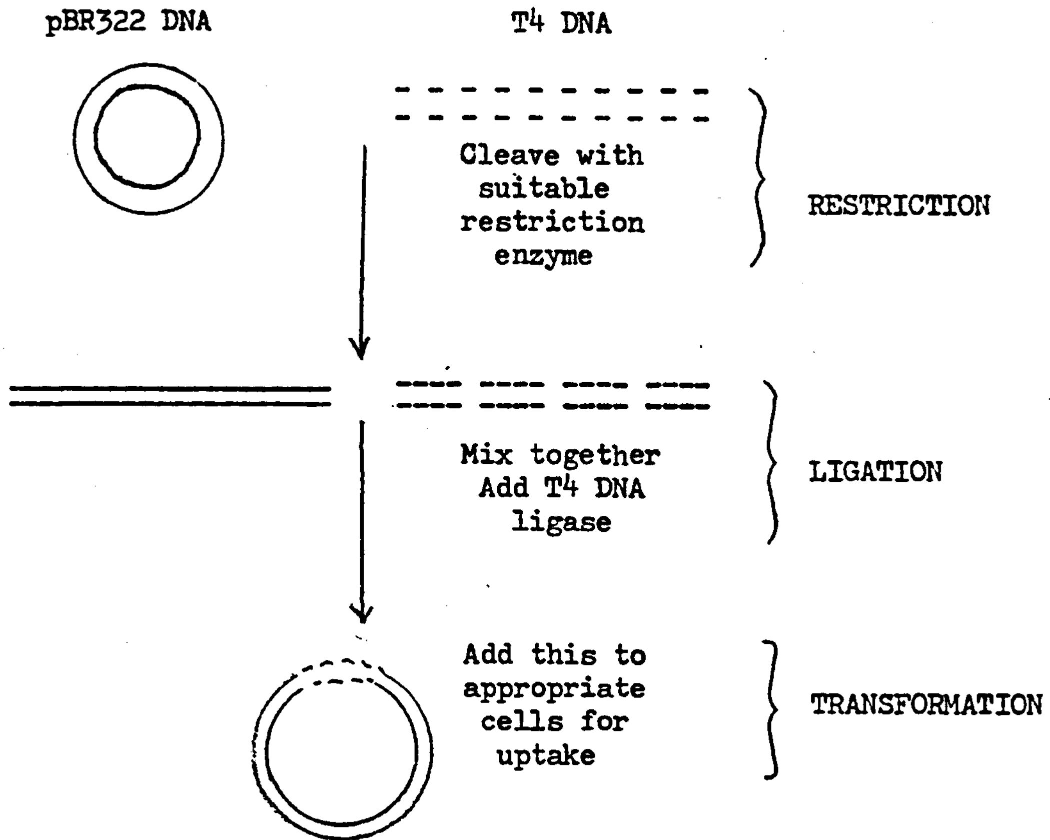


Figure 4. Three basic steps involved in recombinant DNA work.

The second method involves a shotgun experiment where the entire T4 genome is restricted and subsequently used for ligation and transformation. With this procedure restriction, ligation, and transformation are essentially preliminary steps. Suitable selection procedures must then be developed to identify and isolate the desired clone among possibly hundreds of recombinants. As will be seen, two selection procedures were used to identify any recombinants (as opposed to the original plasmid) and then to isolate the recombinant(s) containing the desired region of T4 DNA.

MATERIALS AND METHODS

Materials

All reagents were from commercially available sources. The restriction enzyme Eco R1 and T4 DNA ligase were purchased from Miles Laboratories. The restriction enzymes Hindlll, BamH1, Sall, and Pstl were purchased from New England Biolabs. UDPG (^{14}C-glucose) was purchased from New England Nuclear.

Media

Minimal media contained per liter 0.6% Na_2HPO_4, 0.3% NaH_2PO_4, and 0.1% NH_4Cl. After autoclaving and cooling sterile solutions of the following were added (final concentrations indicated): 0.02% $MgSO_4$, 0.001% $CaCl_2$, 0.3% Glucose, and 0.001% thiamine. L broth contained per liter 1.0% Bacto-Tryptone, 0.5% Yeast Extract, and 0.5% NaCl. After autoclaving and cooling sterile solutions of the following were added (final concentrations indicated): 1mM $CaCl_2$, 0.02% Dextrose, and 0.002% thymidine. Nutrient broth contained per liter 0.8% nutrient broth and 0.5% NaCl. TYE broth contained per liter 1.5% Bacto-Tryptone, 1.0% Bacto yeast extract, and 0.5% NaCl. The pH was adjusted with NaOH to 7.0. ABCDE minimal media contained per liter 0.7% K_2HPO_4, 0.2% KH_2PO_4, 0.05% Sodium citrate, 0.1% $(NH_4)_2SO_4$, and 0.01% $MgSO_4$. GPTG media contained per liter 0.58% NaCl, 0.37% KCl, 0.015 $CaCl_2$, 0.01% $MgCl_2$, 0.11% NH_4Cl, 1.21% Tris base, 0.0322% Na_2SO_4, 0.01% β-glycerophosphoric acid disodium salf, 0.5% Glucose,

and 0.1% Casamino acids. The pH was adjusted with NaOH to 7.4. Where indicated ampicillin (20 μg/ml) and tetracycline (5 μg/ml) were added. L top agar contained per liter 1.0% Bacto-Tryptone, 0.5% Yeast extract, 0.65% Difco agar, 1.0% NaCl, and 0.01% NaOH. Nutrient broth top agar contained per liter 0.8% Nutrient broth, 0.5% NaCl, and 0.65% Difco agar. λ plates contained per liter 1.0% Bacto-Tryptone, 0.5% NaCl, 1.0% Difco agar, and 0.0001% thiamine. ABCDE plates contained per liter 500 mls ABCDE media (see above), 500 mls distilled water, and 1.5% Difco agar. TYE plates contained per liter 1.5% Bacto-Tryptone, 1.0% Bacto yeast extract, 0.5% NaCl, and 1.5% Difco agar. Where indicated ampicillin (20 μg/ml) and tetracycline (5 μg/ml) were added.

Bacterial Strains

Bacterial strains used are indicated in Table 1.

Phage Strains

Phage strains used are indicated in Table 2.

Growth and Infection of Cells

Bacteria were routinely inoculated from solid slants and incubated overnight with shaking at 37°C. Then 0.05 mls of this overnight culture was added to 20 mls of the indicated media (or multiple volumes thereof). These cultures were grown to a reading of 80 on a Klett meter (corresponding to $3X10^8$ cells/ml) prior to infection with phage. Incubation was usually at 37°C from 3-6 hours at which time chloroform was added to lyse any remaining cells. Debris was removed by centrifugation and phage stored with a drop of chloroform.

Table 1. Bacterial strains.

Strain	Relevant Characters	Source
B	wild type, Su^-	a
B201	same as B, Thy^-	a
B834	$r_B^- m_B^-$, gal^-, Su^-	c; Snyder, Gold and Kutter 1976
B834galU56	same as B834, UDPG $PPase^-$	c; Snyder, Gold and Kutter 1976
$B834T^-$	same as B834, Thy^-	d
CR63	wild type, Su^+	a
CT271	nonpermissive for deletions	Wilson 1973
CT526	nonpermissive for deletions	Wilson 1973
HB101	$r_B^- m_B^-$, rec^-, rgl^+	b
K803	$r_K^- m_K^-$, rgl^-, Su_{II}^+	c; Snyder et al. 1976
OK305	$uracil^-$, cd^-	Hall, Tessman and Karlstrom 1967
RRI(pBR322)	$r_K^- m_K^-$, lac^-, tc^r, ap^r	b; Bolivar et al. 1977
U95rgl	rgl^-, UDPG $PPase^-$, Su^-	e; Revel and Georgopoulos 1969
W4597	r_K^+, m_K^+, UDPG $PPase^-$, Su^-	e; Revel and Georgopoulos 1969

a. laboratory strain collection
b. Kindly provided by H. Boyer, Univ. of Calif., San Francisco.
c. Kindly provided by L. Snyder, Michigan State University.
d. this work
e. Kindly provided by H. Revel, Cal. Tech.

Table 2. Phage strains.

Strain	Relevant Characters	Source
T4D	wild type	a
T4αgt57βgt14	αgt^-, βgt^-	b; Revel & Georgopoulos 1969
dec8	am51(56^-), nrd28($denA^-$) rIIH23	c; Snyder et al. 1976
del 1	reported as rII^-, td^-, dfr^+	Homyk and Weil 1974
del 7	reported as rII^-, td^-, dfr^-	Homyk and Weil 1974
del 7rII^+	rII^+, td^-, dfr^+	d
del 9	reported as rII^-, td^-, dfr^-	Homyk and Weil 1974
del 9rII^+	rII^+, td^-, dfr^+	d
r1587	rII deletion mutant	a
ruv363	rII point mutant	a
wh2	dfr^-	a; Hall et al. 1967

a. laboratory strain collection.
b. Kindly provided by D. Kaplan, Univ. of Calif., Los Angeles.
c. Kindly provided by E. Kutter, Evergreen State Univ.
d. this work.

Preparation of Thy$^-$ Bacteria

The following preparation was according to Stacey and Simson (1965). The appropriate bacterial strain (B834galU56) was grown overnight at 37°C in L broth plus 0.01% thymine. Three tubes labeled MC (media control), SC (strain control), and TMP (trimethoprim) were prepared. Two mls of ABCDE media plus 0.01% thymine were added to each tube while 0.02 mls of the overnight was only added to SC and TMP. To MC and TMP were added 0.04 mls of sterile TMP solution (500 µg/ml). The tubes were incubated at 37°C for two days or until the TMP tube became turbid. The MC tube should remain clear; the SC tube should be cloudy after overnight incubation. A loopful of cells was taken from the TMP tube and patched onto L plus thymine plates. These plates were incubated at 37°C for four to five hours, replica plated onto ABCDE minimal plates without thymine and incubated overnight at 37°C. Three patches were picked which did not grow on minimal plates, streaked out to obtain single colonies and grown in liquid culture. A known concentration of cells is spread onto minimal plates without thymine and incubated two days at 37°C to determine the reversion frequency.

Halo Plating Technique

The following technique was according to Hall, Tessman and Karlstrom (1967). _E. coli_ OK305 was grown in GPTG media plus 20 µg/ml uracil to a cell density of $2X10^8$ cells/ml. The culture was then centrifuged to pellet cells which were then resuspended in one twentieth the original volume of GPTG containing no uracil. Two to three drops of this is added to top agar (GPTG media plus 0.7% Bactoagar and

0.002% L-tryptophan) and then poured onto GPTG plates (GPTG media plus 1.2% Bactoagar and 0.002% L-tryptophan). Phage were added in an appropriate amount to give approximately one hundred plaques per plate. Incubation was at 37°C from 8-12 hours at which time halos were very pronounced due to lytic phage growth producing a uridine compound that uninfected bacteria could use to grow.

Genetic Crosses

The indicated bacteria were grown to a cell density of $3X10^8$ cells/ml at 37°C. At this time phage were added at the indicated ratios and MOI's (refer to results). Incubation was continued at 37°C for ninety minutes at which time chloroform was added to lyse any remaining cells. Plating of progeny was done according to the specified selection procedure.

Assays

Protein Determination (Biuret Method)

The following method was according to Layne (1957). The Biuret reagent is prepared by dissolving 1.50 grams of cupric sulfate ($CuSo_4 \cdot 5H_2O$) and 6.0 grams sodium potassium tartrate ($NaKC_4H_4O_6 \cdot 4H_2O$) in 500 mls of water. To this is added with constant swirling 300 mls of 10% NaOH solution. The final dilution is to one liter with distilled water. Routinely .25 mls of cell extract was added to 1.0 ml of reagent and allowed to stand at room temperature for 20 minutes. The absorbance was then measured at 540nm against a blank which contained .25 mls of buffer instead of extract. The protein concentration

was determined by reference to a calibration curve established by adding .25 mls of known amounts of bovine serum albumin solution to the reagent and measuring the absorbance at 540 nm.

UDPG Pyrophosphorylase

Cells were grown in 100 mls of nutrient broth to $3X10^8$ cells/ml, then centrifuged (4000Xg for 20 minutes), resuspended in three mls of Tris-HCl buffer (0.1M, pH 7.8), and then disrupted by sonic oscillation for 40 seconds with a Branson sonifier. Extracts were centrifuged at 12,000Xg for 30 minutes. The reaction mixture contained in one ml, 0.8 ml Tris-HCl (0.1M, pH 7.0); 0.1 ml cell extract; 0.01 ml $MgCl_2$ (0.05M); 0.01 ml cysteine (0.25M, pH 7.0); 0.02 ml NADP (12.5 mM); 0.01 ml phosphoglucomutase (12 units/ml); 0.01 ml glucose-6-phosphate dehydrogenase (12 units/ml); 0.01 ml UDPG (40 mM); and 0.01 ml glucose-1,6-diphosphate (0.24 mM). The reaction was initiated by the addition of 0.05 mls sodium pyrophosphate (0.1 M). A total volume of one ml was contained in a quartz cuvette (1 cm light path). A blank cuvette for reference contained all ingredients except sodium pyrophosphate. The overall reaction results in a reduction of NADP and is observed by monitoring the increase in absorbance at 340 nm. The enzyme activity (μmoles NADPH formed/mg protein/hour) at 34°C is calculated assuming a molar extinction coefficient for NADPH at 340 nm of 6.22 cm^2/μmole.

DNA Glucosylation

Cells were grown in 100 mls of minimal media to a cell density of $3X10^8$ cells/ml and then infected with T4D at an MOI of 5. At 12 minutes post infection the culture was centrifuged (5000 rpm, 20

minutes) and the pellet resuspended in 3 mls glycylglycine buffer (0.05M glycylglycine, 0.002M EDTA, 0.002M reduced glutathione, pH 7.0). The cells were then sonicated for 30 second intervals while on ice until clear and then centrifuged (9000 rpm, 30 minutes). The resulting supernatant served as the glucosyl transferase crude enzyme preparation. T4 DNA was extracted from the appropriate infections by the phenol procedure (described later). The reaction mixture contained 0.7 mls Tris-HCl (0.1M, pH 7.0); 0.01 ml $MgCl_2$ (0.5M); 0.02 mls KCl (0.1M); 0.005 mls UDPG (4 mM); 1 µCi UDPG (^{14}C-glucose); and 10 µg DNA. The reaction was initiated by the addition of 0.1 ml crude enzyme preparation. The mixture was incubated at 37°C and, at various times, 0.1 ml samples were transferred to Whatman #3 filter papers containing 0.1 ml of 5% TCA. The filters were dried, washed three times with 5.0 mls each cold 5% TCA and acetone. The filters were dried and counted in 5.0 mls Omnifluor scintillation cocktail.

Dihydrofolate Reductase

Cells were grown in 100 mls of the indicated media to a cell density of $3X10^8$ cells/ml and then infected with the indicated phage at an MOI of 5. The infection was carried out at 37°C for 15 minutes at which time the culture was centrifuged (6000 rpm, 10 minutes) and resuspended in 2.5 mls of 0.1M tris, pH 7.4. The cells were then sonicated for 30 second intervals on ice until clear and then pelleted (12,000 rpm, 15 minutes). This served as the enzyme source. The reaction mixture contained 0.5 mls KPO_4 (0.1M, pH 7.4); 0.38 mls distilled H_2O; 0.05 mls NADPH (1.9 mg/ml dH_2O); and 0.05 mls dihydrofolic

acid -- FH_2 (3 mg in 5 mls 0.1M Tris, pH 7.4; 0.1M β-mercaptoethanol). The reaction was initiated by the addition of 0.02 mls enzyme so the total volume was 1.0 ml in a quartz cuvette (1 cm light path). A blank cuvette for reference contained everything except enzyme. The overall reaction results in the oxidation of NADPH to NADP and is observed by monitoring the decrease in absorbance at 340 nm. The enzyme activity (nmoles NADP formed/minute/mg protein) is calculated assuming a molar extinction coefficient for NADP of $6.6X10^3$ cm^2/nmole.

Thymidylate Synthetase

Cells were grown (usually B201 since it is td^-) in 100 mls of the indicated media to a cell density of $3X10^8$ cells/ml then infected with the indicated phage at an MOI of 5. The infection was carried out at 37°C for 15 minutes at which time the culture was centrifuged (6000 rpm, 10 minutes) and resuspended in 2.5 mls of 0.1M tris, pH 7.4. The cells were sonicated for 30 second intervals on ice until clear and then pelleted (12,000 rpm, 15 minutes). This served as the enzyme source. The reaction mix contained in 50 mls, 40 mls Tris (0.1M, pH 7.4); 0.7 mls β-mercaptoethanol; 0.12 mls HCHO (37% solution) 0.51 grams $MgCl_2 \cdot 6H_2O$; and 0.038 grams $EDTA \cdot 2H_2O$. To 0.5 mls of this mix was added 0.1 ml FH_4-tetrahydrofolic acid (5.4 mg in 5.0 mls of 0.1M Tris, pH 7.5 and 0.1M β-mercaptoethanol); 0.1 ml enzyme, and 0.2 mls dH_2O. The reaction was initiated by the addition of 0.1 ml dUMP (1 mM). A blank cuvette for reference contained everything except dUMP. The overall reaction results in the oxidation of FH_4 to FH_2 and is observed by monitoring the increase in absorbance at 338 nm. The

enzyme activity (nmoles FH_2 formed/minute/mg protein) is calculated assuming a molar extinction coefficient for FH_2 of $12X10^3$ cm^2/nmole.

Preparation of DNA

Phage DNA

Routinely 200 mls of bacteris were grown to a cell density of $3X10^8$ cells/ml and infected with the indicated phage at an MOI of 0.01. Infection proceeded at 37°C for at least 8 hours and frequently overnight. Chloroform was added and then the cultures were pelleted (6000 rpm, 10 minutes) to remove bacterial debris. DNase and RNase were added (1 ug/ml each) and the cultures sat at room temperature for 1 hour. This was followed by two rounds of high speed spin (35000 rpm, 45 minutes), resuspension in 0.1M Tris, pH 7.4 and low speed spin (6000 rpm, 10 minutes). The resultant supernatant contained phage virions. Purification of phage DNA utilized a phenol extraction technique (Kozinski and Lin 1965). Phenol was initially distilled over metallic zinc into water. Phenol prepared in this way was stored at -20°C under nitrogen in the dark. For use the phenol was heated to liquify and one fiftieth the volume of 1.0M KPO_4 buffer, pH 6.8 was added along with about one fourth volume of dH_2O to saturate the phenol. Phage were adjusted to a 260 nm absorbance of between 5-15 cm^{-1}. An equal volume of water saturated phenol was added to the phage mixture and rolled for 30 minutes in conical pyrex tubes. The tube was chilled to 0°C and centrifuged (3000 rpm, 5 minutes). The phenol layer (bottom) was removed and the extraction repeated twice with briefer periods of rolling. The aqueous phase should be clear at this time.

This phase was transferred to a Visking No. 20 dialysis bag that had previously been boiled in 5% sodium bicarbonate solution for 20 minutes and washed extensively with dH_2O. The DNA was dialyzed against 0.1 SSC buffer (0.15M NaCl, 0.015M sodium citrate) to remove any residual phenol. DNA was stored at 4°C with a drop of chloroform.

Plasmid DNA

Routinely 200 mls of the appropriate plasmid bearing bacterial strain was grown to a cell density of 6×10^8 cells/ml with the necessary antibiotics included in the media. At this time chloramphenicol was added to a final concentration of 150 µg/ml and the culture incubated overnight with shaking. The next morning the culture was chilled, centrifuged (8000 rpm, 15 minutes), and resuspended in one twentieth the volume of 50 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 8.0. This is centrifuged (8000 rpm, 15 minutes) and the pellet frozen in a dry ice-ethanol bath. The following steps are all performed at 0°C. The pellet is resuspended in 2 mls of 25% Sucrose-50 mM Tris-HCl, pH 8.0. Cold lysozyme solution (.4 mls of a 10 mg/ml solution in 0.25M Tris-HCl, pH 8.0) was added. After 5 minutes 1.6 mls of 0.25M EDTA, pH 8.0 was added. After 10 minutes 3.2 mls of Triton X 100 mix (1 ml of 10% Triton X 100 in 10 mM Tris, pH 8.0; 25 mls of 0.25M EDTA, pH 8.0; 5 mls of 1M Tris-HCl, pH 8.0, and 70 mls dH_2O) were added and the mixture set at 0°C for 15 minutes. This was centrifuged in a Spinco Type 30 rotor at 30,000 rpm for 45 minutes. The pellet is thick and viscous and can be removed by picking it out with tweezers. To the supernatant was added 10 µg/ml of stock RNase solution (10 mg/ml in 50 mM sodium

acetate, pH 4.8; boiled for 5 minutes) and incubation was for 10 minutes at room temperature. The DNA was then extracted with water saturated phenol by adding an equal volume of this phenol. The solution was mixed thoroughly, centrifuged (5000 rpm, 5 minutes), and the aqueous phase removed. The DNA was precipitated by adding 2 volumes of cold ethanol and 0.2 volumes of 1.5M sodium acetate and incubating at -20°C for at least three hours (frequently overnight). The solution was centrifuged (6000 rpm, 5 minutes) and resuspended in agarose column buffer (0.5M NaCl; 10 mM Tris-HCl, pH 8.0; and 1 mM EDTA) prior to loading onto an agarose A50m column which has first been extensively washed with column buffer. The sample is run through at 10 ml/hour and 1.5 ml fractions are collected. Absorbances are read at 260 nm and peak fractions are pooled, ethanol precipitated, and resuspended in 10 mM Tris-HCl, pH 7.4; 1 mM EDTA. A final absorbance was read at 260 nm to determine the DNA concentration.

Restriction Conditions

Restriction buffers for Hind III, Bam HI, Eco RI, and Pst I contained 80 mM Tris-HCl, pH 7.4; 8 mM $MgCl_2$; and 2 mM β-mercaptoethanol and 100 µg/ml of gelatin. The restriction buffer for Sal I contained 8 mM Tris-HCl, pH 7.4; 6 mM $MgCl_2$; 0.2 mM EDTA; 150 mM NaCl; and 50 µg/ml of gelatin. All buffers were prepared in 10X concentrations. To this was usually added 200 ng DNA, 1-2 units enzyme, and dH_2O to give a final volume of 10-20 µl. Restrictions were performed at 37°C (except Pst I which was at 30°C) from one to three hours. The reactions were stopped by the addition of a solution containing 5% SDS, 25% Glycerol, and 0.025% Bromphenol blue.

Gel Electrophoresis and Photography

Electrophoresis was performed as indicated in figures. Agarose buffer consisted of 40 mM Tris-Acetate, pH 8.05; 20 mM Sodium acetate; 2 mM EDTA; and 18 mM NaCl. After electrophoresis the gels were stained by soaking in 1 µg/ml of ethidium bromide for 15 minutes, rinsing with dH_2O, and blotting with Saran wrap for one hour to reduce background staining. The gels were filuminated directly on the stage of a long wavelength ultraviolet lamp (Blak-ray transilluminator, Ultraviolet products). Photography was with Polaroid 57 positive film through the following filters: Wratten 9, Wratten 2B, and Ultraviolet filter J-344 purchased from Ultraviolet products.

Ligation Conditions

The ligation reaction mixture included 74.6 ul of 10X ligation buffer (660 mM Tris-HCl, pH 7.6; 50 mM DTT; 1 mM ATP); 673 µl of Pst I cleaved pBR322 (63 µg/ml); 73 ul of Pst I cleaved T4 dec 8 DNA (290 µg/ml); and 1 µl of T4 DNA ligase (0.8 units). Incubation was at 14°C for 48 hours. At this time the ligation mixture was dialyzed against 2 liters of 10 mM Tris-HCl, pH 7.4 and .25 mM EDTA.

Transformation of Cells

An overnight culture was grown from a single colony of the appropriate strain in 2 mls of L broth at 37°C. Then 0.5 mls of this overnight was diluted into 10 mls of L broth and grown for one hour with shaking (no aeration) to give an approximate cell density of $5X10^8$ cells/ml. This was centrifuged (5000 rpm, 5 minutes) and the pelleted cells were resuspended in 5 mls of iced 0.03 M $CaCl_2$. This

was held at 0°C for at least 20 minutes, centrifuged (5000 rpm, 5 minutes) and the pellet resuspended in 1.0 ml of iced 0.03M $CaCl_2$. The appropriate DNA was added to a thin walled glass tube with two volumes of the above cells. This was held at 0°C for one hour and then heat shocked at 42°C for 2 minutes. Forty volumes of L broth was added and the mixture incubated at 37°C for 2 hours in order for the antibiotic resistance of the transformants to be expressed. One tenth ml was spread out on appropriate plates and incubated overnight to screen for transformants.

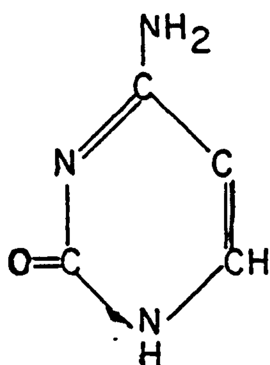
RESULTS

Restriction of HMC DNA

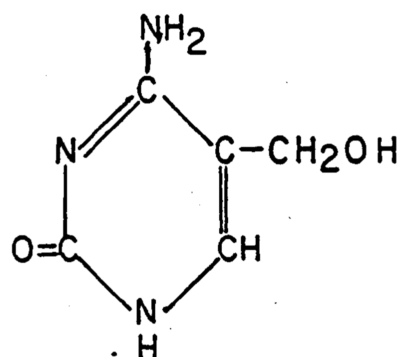
Pertinent Properties of T4 DNA

A unique feature of T4 DNA is that its pyrimidine content consists of thymine and 5'-hydroxymethylcytosine substituted in place of cytosine (Wyatt and Cohen 1952). This DNA is commonly referred to as HMC DNA. In addition, the hydroxymethyl residue is glucosylated in an α or β linkage (Fig. 5). The apparent reason for this is to prevent the degradation of infecting T4 DNA from host nucleases which recognize and restrict nonglucosylated HMC DNA. T4 DNA containing cytosine (C DNA) is degraded by host and phage enzymes (Kutter and Wiberg 1968); host mutants are available which are unable to degrade C DNA. Replication of this DNA proceeds but no late transcription occurs which is necessary for packaging of the DNA and maturation to an infective viral particle (Kutter et al. 1975). This step is prevented by the phage itself.

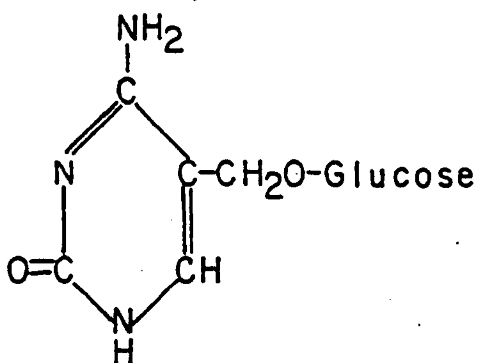
As a consequence of this inability to produce virus with C DNA the first series of experiments involved HMC DNA where an attempt was made to restrict this DNA and locate the specific fragment(s) coding for the viral enzymes dihydrofolate reductase (dfr) and thymidylate synthetase(td). Since the td and dfr genes are adjacent on the T4 genetic map (Fig. 6) one might hope to clone fragments containing both of these genes in a single insert.



Cytosine



5–Hydroxymethyl Cytosine



Glucosylated 5–Hydroxymethyl Cytosine

Figure 5. Cytosine derivatives found in T4 DNA.

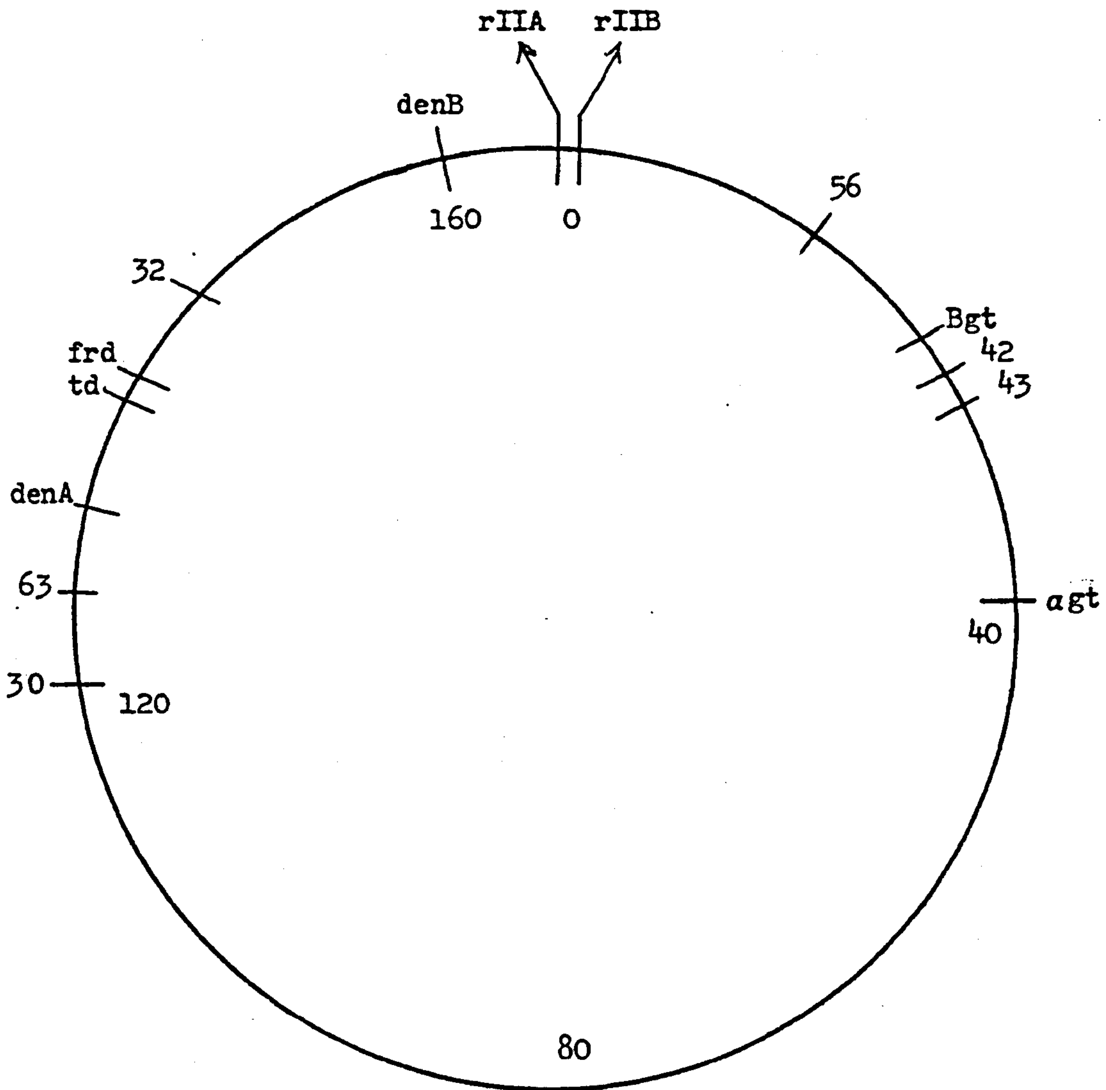


Figure 6. Approximate positions of relevant markers on T4 genome. — Numbers refer to distances in kilobase pairs.

A recent paper had reported the existence of a set of deletion mutants in the td-dfr region of T4 (Homyk and Weil 1974). Data were presented for two of the mutants (del7, del9) showing a deletion which extended through the DNA coding for td and dfr. These particular deletions had no td or dfr activity as analyzed by spectrophotometric enzyme assays (Mosher et al. 1977). The initial protocol of this work was to restrict wild type DNA and compare it with the restriction of these deletion mutants looking for differences in banding patterns when analyzed by agarose gel electrophoresis. Any bands seen with wild type DNA would be a candidate for cloning if the corresponding band was missing in these deletions.

Isolation of Nonglucosylated HMC DNA

Initially wild type and deletion HMC DNA was to be isolated. However, this DNA had to be nonglucosylated. As mentioned previously the presence of glucose in phage DNA serves as a protection against host restriction upon passing through the cell membrane. Unfortunately the presence of glucose on HMC residues also protects against most restriction enzymes (Kaplan and Nierlich 1975). An elaborate pathway exists whereby glucose is added to HMC residues after replication (Fig. 7) (Revel and Luria 1970). As shown this pathway uses both host (UDPG pyrophosphorylase) and phage (glucosyl transferase) enzymes such that glucosylation can be prevented by mutation in either enzyme.

Glucosyl transferase mutants of T4 do exist (Georgopoulos 1967). One of these (T4αgt57βgt14) was kindly provided by H. Revel and used in preparing nonglucosylated phage. T4D was also grown in

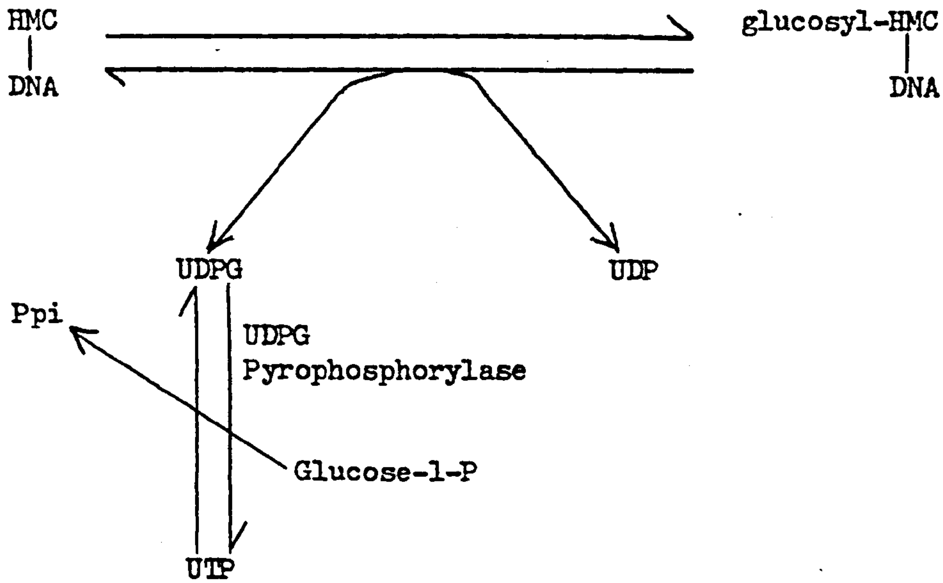


Figure 7. Pathway involved in the glucosylation of HMC-DNA.

*E. coli*U95rgl a strain lacking UDPGpyrophosphorylase activity (Revel and Georgopoulos 1969). Due to technical reasons del7 and del9 were only grown in U95rgl to produce nonglucosylated DNA. Nonglucosylated DNA prepared with the host mutant will be referred to with a "*" (i.e., T4D*).

As a check for glucosylation, assays were performed on all DNA to be used according to a published procedure (Revel and Georgopoulos 1969). This assay involved incubating the DNA with ^{14}C-UDPGlucose and a crude phage extract containing the glucosyl transferase enzymes. Aliquots were removed and counted at certain times. The degree of nonglucosylation is indicated by the amount of label incorporated into the DNA. As can be seen (Figs. 8 and 9) T4D DNA should and does incorporate minimal label when compared to T4*, del7*, del9*, or T4αgtβgt.

HMC DNA Restriction

Restrictions were then carried out on the above mentioned DNA's. At the time of this work Eco RI was the only enzyme commercially available. This enzyme had been shown to fragment T4 HMCglu$^-$ DNA into approximately forty fragments (Kaplan and Nierlich 1975). Because of this number a large gel electrophoresis apparatus designed by Don Kaplan at UCLA specifically for this type of work was purchased. When the technical problems of using so large an apparatus were worked out it became apparent that restriction abnormalities also existed. Several times no restriction was observed and when restriction did occur it was not reproducible (data not shown).

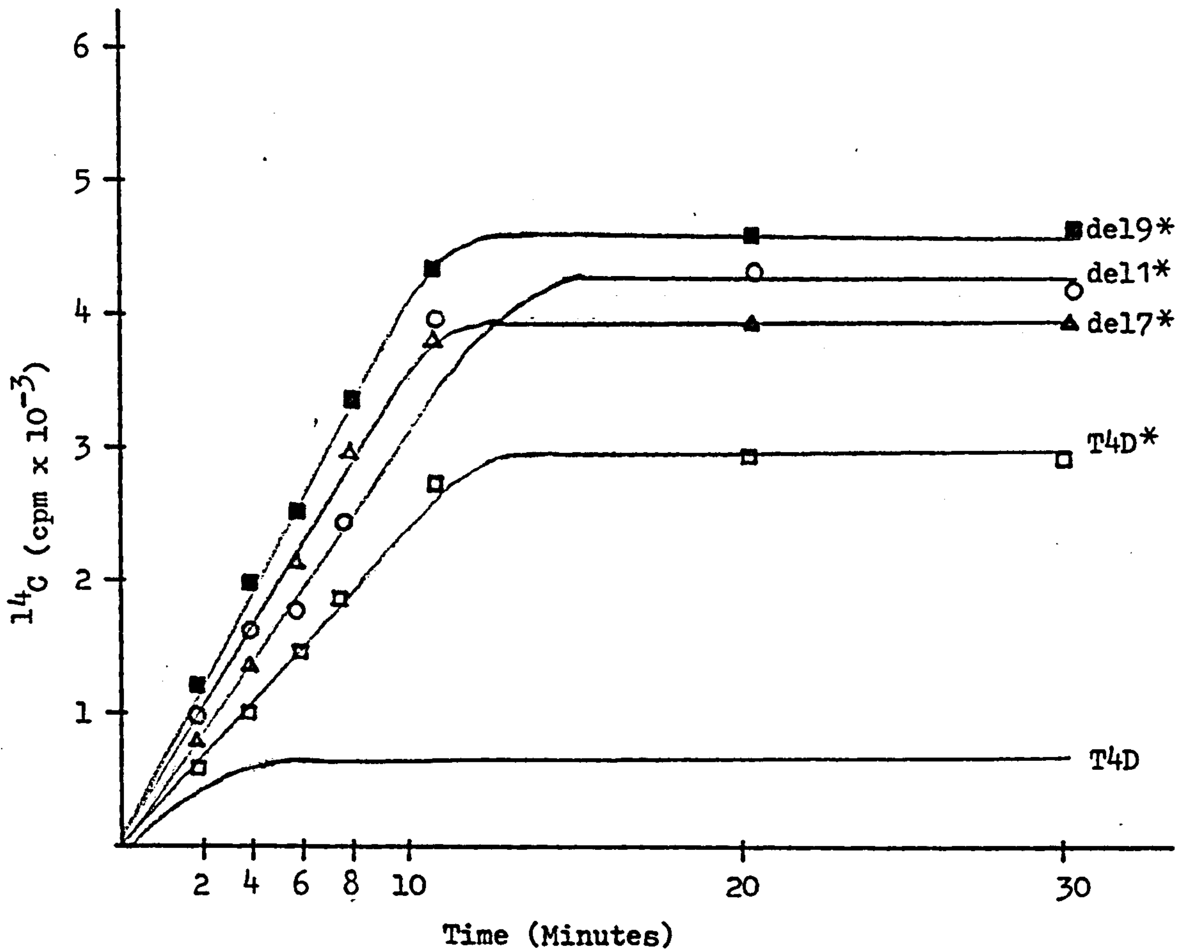


Figure 8. Incorporation of ^{14}C-glucose into phage DNA with a UDPG -host strain. -- Infection of following strains was in E. Coli U95rgl (UDPG PPase^{-}). T4d, ◻ T4D*, △ del7*, O del1*, ■ del9*. Assay performed as described in Materials and Methods.

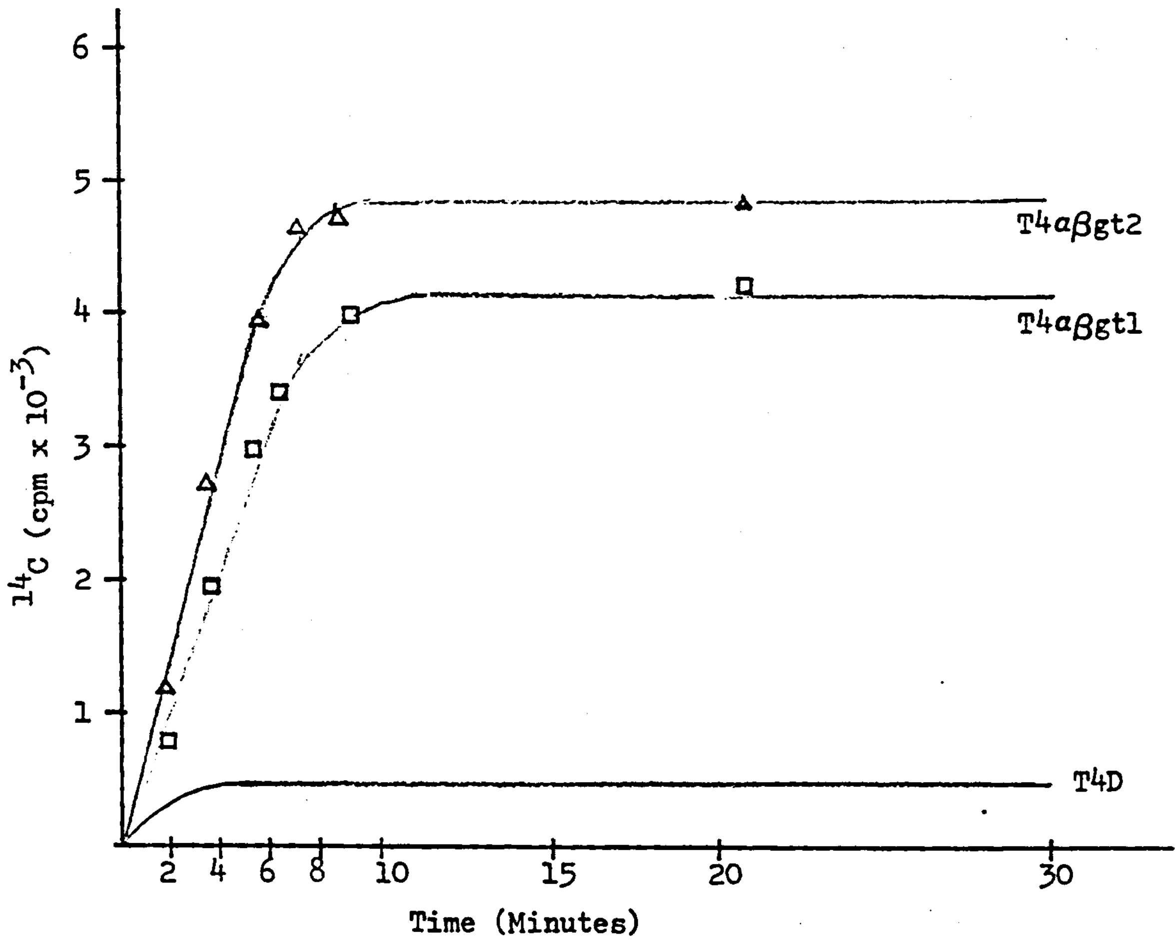


Figure 9. Incorporation of ^{14}C-glucose into phage DNA with T4α57βgt14. -- Infection was with two independent isolates of the glucosyl transferase mutant T4α57βgt14. Assay performed as described in Materials and Methods.

Don Kaplan at UCLA was having similar problems (Kaplan, personal communication 1978) and had stopped working with this type of T4 DNA. He and others thought that the hydroxymethyl group was interfering (inhibiting) attack by the restriction enzyme. At this point restriction of HMC DNA was discontinued and work turned to examining the deletion mutants in greater detail.

Studies with Del7 and Del9

Isolation of Deletion Mutants

The original set of deletion mutants were isolated by Homyk and Weil by virtue of a duplication in the rII region of the T4 genome (Homyk and Weil 1974). Virion morphogenesis results in the packaging of a specific quantity of DNA (Streisinger, Emrich and Stahl 1967) such that an apparently general method for isolating deletion mutants became available with the demonstration that phage carrying a duplication of the rII region (Weil, Terzaghi and Craseman 1965) often contain a deletion as well (Weil and Terzaghi 1970). Using the rII duplication as a reference marker they determined the distance from this marker where deletions occurred by hybridization to wild type T4 DNA and visualization by electron microscopy. One set of deletions were reported to span the td-dfr region according to these measurements. This set included del7 and del9.

Properties of These Mutants in Contradiction to the Reported Mapping Data

As mentioned previously both deletions possessed no td or dfr activity as analyzed by spectrophotometric enzymatic assays. However,

several observations were noted in our lab in apparent contradiction to the mapping as reported.

Del7 and Del9 Produce Material that Cross Reacts with Antisera Prepared Against td and dfr. Pure antisera to both td and dfr were incubated with these deletions. Previously it had been shown that the phage enzymes are immunologically distinct from the corresponding host enzymes (Krauss, Stollar and Friedkin 1973; Mathews, Crosby and Kozloff 1973). By using an immunodiffusion technique it was shown that both of these mutants synthesized material in infected cells that cross reacted with both antisera (Capco and Mathews 1973; Mosher et al. 1977). This is totally unexpected if the DNA coding for these proteins is deleted. However, part of each gene may be present resulting in incomplete proteins containing the antibody recognition to td and dfr antisera. Deletions containing portions of both genes would be unlikely since td and dfr are adjacent on the T4 genetic map whereas the size of these deletions is large enough to span several genes.

Phage Infectivity is Neutralized by Antisera Prepared Against td or dfr More Readily Than Wild Type. As mentioned in the Introduction, evidence has accumulated implicating td and dfr as structural components in the T4 baseplate. If td and dfr gene products in the virion represent the neutralizing targets of the respective antisera, then the deletions should be resistant to neutralization by the antisera. This consistently was not the case (Capco and Mathews 1973; Mosher et al. 1977).

Reversion is Consistently Obtained With These Mutants. For as yet unknown reasons these deletions are restricted on a set of wild type *E. coli* hospital strains isolated by Cal Tech scientists (Wilson 1973). One of these strains (CT526) was always used as a marker to insure the deletions still existed. However, reversion (the ability to grow on CT526) occurred repeatedly even infections were started from single plaques. If these mutants were truly deletions in the specified area no reversion should or could occur; the DNA physically is not present.

One additional anomally surfaced when certain genetic crosses were performed. Initially the rII duplication region was obtained from a cross between two overlapping rII deletion mutants (Homyk and Weil 1974). Because the deletions overlap a cross cannot give rise to wild type recombinants. Hence del7 and del9 contain an rII duplication but still do not possess the entire rII region. Restriction of HMC DNA was still in progress at this time. The restriction patterns between these deletions and wild type DNA was compared. For this purpose it was necessary to have del7 and del9 as isogenic to wild type as possible. To accomplish this an effort was made to eliminate the missing rII region. A selection procedure was devised making use of the fact that rII mutants do not grow on *E. coli* lambda lysogens. Crosses were performed between del7 and del9 and wild type where the selection was for growth in a lambda lysogen (*E. coli* 594λ) indicating an rII+ phenotype. Restriction of any potential recombinants by CT526 was also checked as an indication that the td-dfr deletion was still intact.

Five separate "single deletion" phage strains were isolated — two from del7 and three from del9. As a routine check enzymatic assays were performed. The totally unexpected result was that in all cases, although the phage still lacked td activity, they were now producing active dfr at wild type levels!

A Possible Model to Explain These Discrepancies Postulating the Existence of a Regulatory Gene Separate from the Actual Deletion

With the above information our lab seriously questioned the validity of the mapping data as reported. In addition, it was noted that these mutants were originally isolated under heavy irradiation (Homyk and Weil 1974). A proposal was put forth stating the existence of a regulatory gene associated with the production of functional dfr. This would serve to clear up some of the aforementioned anomalies.

1. The original deletion mutants actually contained the DNA coding for dfr, but also contained a mutation in this regulatory region. This would explain the dfr negative phenotype.

2. The cross reactivity and inactivation by antisera would be interpreted as protein being made which is enzymatically inactive (perhaps the regulation would involve cleavage of a precursor to active form) but does contain the antibody recognition site.

3. Reversion that was seen would be explained by reversion of this regulatory gene to wild type if restriction initially occurred due to a dfr negative phenotype.

4. Genetic crosses with wild type T4 would have allowed isolation of "single deletion" phage containing the regulatory region from wild type T4; hence the dfr positive phenotype.

At this point it must be mentioned that no explanation exists for the failure to observe a td positive phenotype in these single deletions since antisera prepared against phage td was able to cross react with and inactivate phage from these deletions similarly to dfr antisera.

Experiments to Prove or Disprove This Model

Further experiments were conducted in an attempt to prove or disprove, reinforce or contradict this proposal. To show that the rII region had no involvement in the production of enzymatically active dfr further crosses were performed where the rII region was again mutated, either selecting for phage that had the same deletion (producing the original del7 or del9) or introducing an rII point mutation. In all cases dfr activity was retained whereas all strains constructed were td^- (Tables 3 and 4).

Next, a series of experiments was undertaken where cell extracts were prepared from cells simultaneously infected with differing ratios of two phage — del7 or del9 and T4D. The purpose was to examine the dfr levels with differing ratios of input phage. The result showed that while dfr activity with del9 and T4D infected cells remained essentially constant, the level in del7 and T4D infected cells decreased as the del7:T4D ratio increased (Table 5). So, in addition to preventing del7 from making active dfr (assuming this phage has the DNA coding for dfr) it appears that the presence of this regulatory abnormality inhibits wild type production of dfr.

Table 3. Properties of recombinants in crosses of del7 or del9 to T4D.[a]

Group	Phage	Enzyme Spec. Act. (nmol/min/mg protein) dfr	td
A	T4D	22.9	4.1
	del7	1.5	<0.1
	del9	1.8	<0.1
B	del7 rII^+	25.0	<0.1
	del7 rII^+	34.5	<0.1
	del9 rII^+	18.1	ND[b]
	del9 rII^+	20.3	ND
	del9 rII^+	25.7	ND

[a]Results in group A represent properties of our original stocks. Del7 and del9 contain the r1589 deletion (rII^-). Group B represents five independently derived recombinants from crosses to T4D.

[b]ND, not determined.

Table 4. Properties of recombinants in crosses of del7 or del9 to rII mutants.[a]

Group	Phage	Enzyme Spec. Act. (nmol/min/mg protein) dfr	td
A	T4D	22.9	4.1
	del7	1.5	<0.1
	del9	1.8	<0.1
B	del7 r1589	24.8	0.4
	del7 rUV363	23.3	0.4
	del9 r1589	23.7	<0.1
	del9 rUV363	24.2	<0.1

[a]Group A is as described in Table 1. Group B represents rII mutations, either the original r1589 deletion or a point mutation (rUV363), which were crossed back into del7 or del9 to show that the state of the rII gene does not affect the expression of dfr.

Table 5. Dfr activities with mixed infections.[a] -- Procedure for assay described in Materials and Methods.

Strain	Input Ratios (M.O.I.'s)[b]	dfr Activity[c]
del7 x T4D	2:6	0.50
del7 x T4D	4:4	0.23
del7 x T4D	6:2	0.12
del9 x T4D	2:6	0.71
del9 x T4D	4:4	0.50
del9 x T4D	6:2	0.65

[a]Both phage were added simultaneously prior to preparation of cell extracts.

[b]M.O.I., multiplicity of infection.

[c]Average of three separate assays normalized with respect to T4D.

Attempts to Isolate This Regulatory Gene

As a conclusion to these studies, attempts were made to isolate this proposed regulatory mutant. Initially a cross was prepared between equal MOI's of del7 or del9 and T4D. The selection procedure was devised such that phage would be isolated with no deletion in the td-dfr region but have a mutation in this regulatory region. This involved selecting against both parents. The hospital strain CT526 was used to screen out del7 or del9 (where this strain would be permissive for regulatory mutant candidates). To select against the wild type parent a halo plating technique was used (Hall et al. 1967).

Briefly, this technique uses a bacterial strain which requires uracil to grow (E. coli OK305). The bacteria were grown in liquid media containing uracil, pelleted, resuspended in media lacking uracil, then added to plates without uracil and infected with the appropriate phage. Any phage which lack td or dfr activity will accumulate and excrete dUMP upon cell lysis (Fig. 1). Uninfected bacteria surrounding this area will be able to utilize this nucleotide and growth will occur forming a dense "halo" around plaques (Fig. 10).

The interest was in obtaining mutants defective in the regulatory region alone which would lead to low or no dfr activity so this technique was used to screen out wild type parents and to pick up any phage producing insufficient levels of dfr (or td). Any halo forming plaque was picked for further investigation; biochemical assays were carried out on cells infected with these halo formers.

The first time this protocol was followed halo formers were obtained with significantly reduced dfr activity (Table 6).

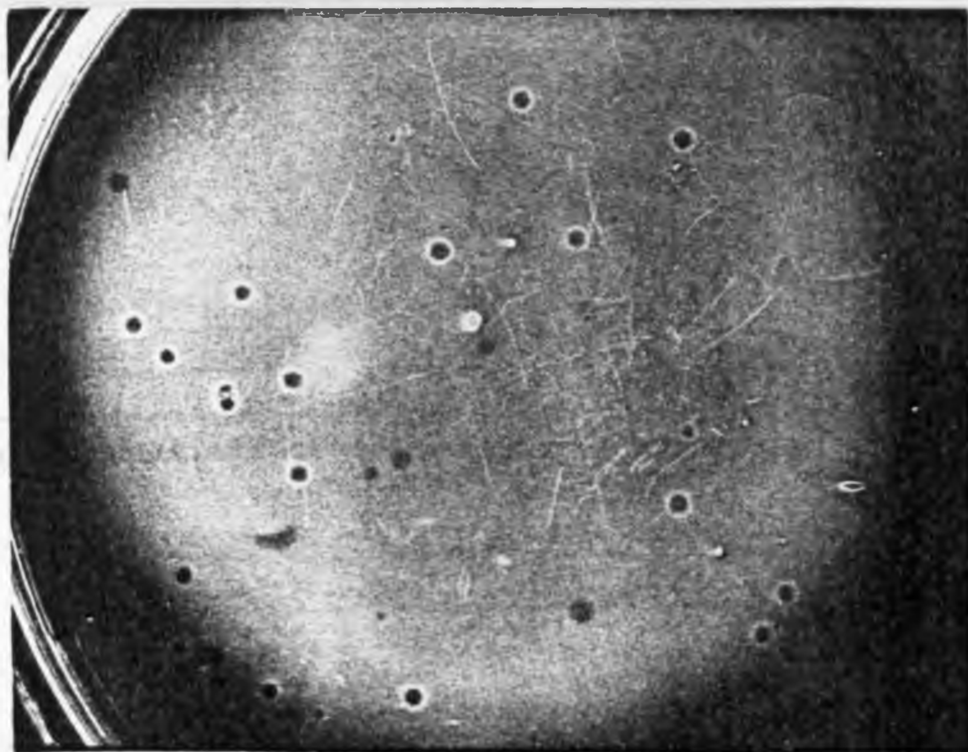


Figure 10. Halo formation with wh2 a dfr^- phage strain. -- Halo plating procedure described in Materials and Methods.

Table 6. Dfr assays of halo formers obtained with del7 or del9 crossed with T4D. -- Procedure for assays described in Materials and Methods.

Isolate	dfr Activity[a]
7.3	0.25
7.5	0.28
7.6	0.33
7.7	0.57
9.4	0.12
9.7	0.45

[a]Values normalized with respect to T4D.

Unfortunately the results were not reproducible and many subsequently isolations (close to 50) produced phage with significant if not wild type levels of dfr activity (Table 7). One major drawback of this technique was the difficulty associated with setting up and interpreting plating results. Many factors influenced the extent of halo formation such as amount of phage added, age of bacteria, age of plates, dryness of plates, incubation time, etc. Subsequently subtle changes frequently meant the difference between a positive or negative halo test with the same phage strain.

Restriction and Cloning of C DNA

Genetic Isolation of C DNA

The isolation of T4 cytosine containing DNA (C DNA) became facilitated by the appearance of a paper describing the production and viability of T4 virions containing C DNA (Snyder et al. 1976). The phage (dec8) is a quadruple mutant (denA, denB, gene 56, alc) where all pathways involved in the production of 5'HMdCMP are blocked (Fig. 11).

In wild type T4 DNA, 5'HMdCMP is formed by modification of dCMP, deoxycytidine monophosphate, derived from two sources:

1. The breakdown of *E. coli* DNA by an endonuclease coded for by the T4 denA gene.

2. The breakdown of intracellular pools of dCTP and dCDP by T4 gene 56 (dCTPase).

Mutations in denA and gene 56 serve to eliminate any dCMP that would be availablr for hydroxymethylation. A mutation in denB served to

Table 7. Subsequent dfr assays with halo formers.

Isolate	dfr Activity[a]
7.3	0.83
7.5	0.70
7.6	0.89
9.4	0.75
9.7	1.10

[a]Average of three separate assays normalized with respect to T4D.

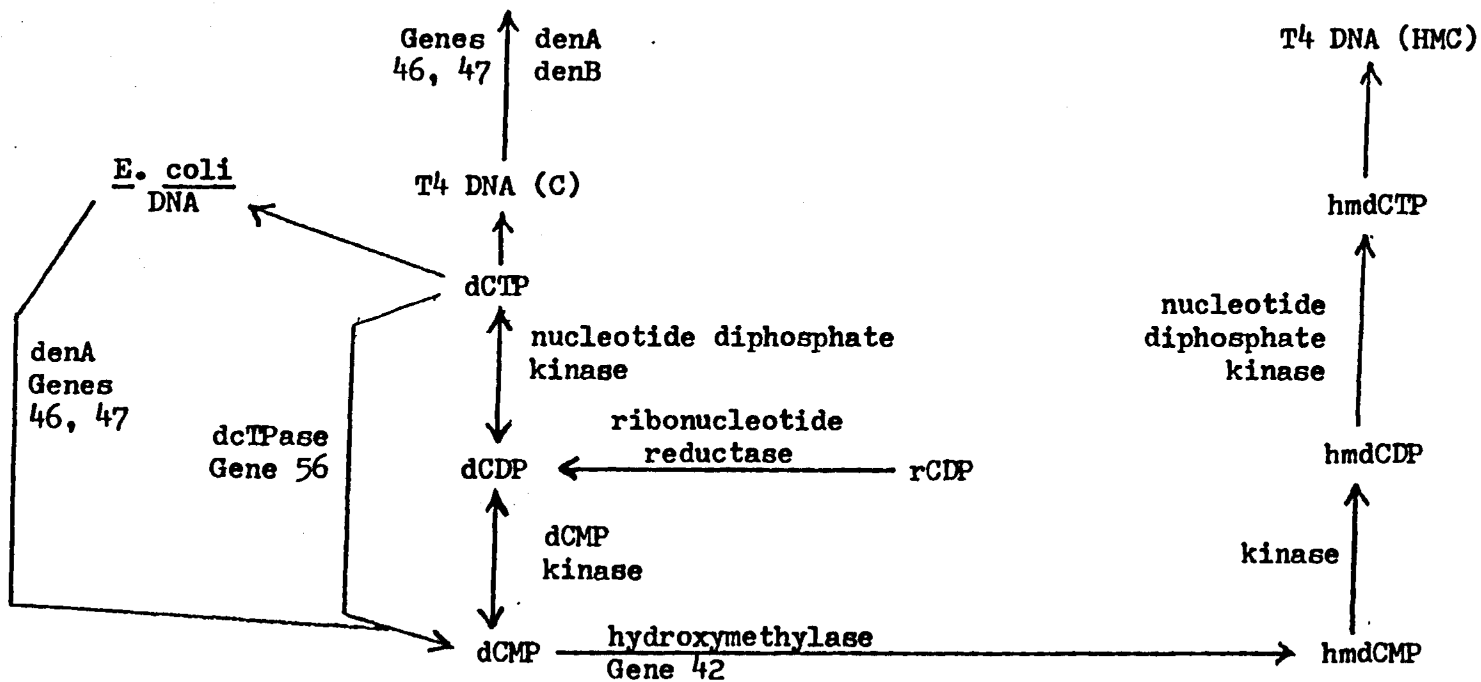


Figure 11. Pathways to dCMP production in T4 infected cells.

prevent the degradation of T4 C DNA thus formed (Kutter et al. 1975) and a mutation in alc allows late transcription of cytosine containing DNA and packaging to form infective particles. T4 DNA isolated from this mutant has been shown by thin layer chromatography to contain less than 5% HMC residues (data not shown).

Properties of the Plasmid, pBR322

With significant quantities (milligrams) of C DNA now obtainable a suitable plasmid vector had to be chosen for the cloning of T4 DNA. The Col El derivative, pBR322 was chosen; this plasmid was constructed in the laboratory of Herb Boyer at San Francisco (Bolivar et al. 1977). A restriction map of this plasmid is shown (Fig. 12). In addition to the property of self replication and amplification by chloramphenicol treatment this plasmid possesses three other very useful properties:

1. Five restriction enzymes cleave this plasmid once converting it from the covalently closed circular DNA form to linear DNA with no loss of genetic material. These enzymes are circled in Figure 12.

2. This plasmid codes for proteins which confer to any cell they reside in resistance to the antibiotics ampicillin (Ap) and tetracycline (Tc). These are extremely useful markers since any cell containing this plasmid will be able to grow in the presence of these antibiotics.

3. The cleavage sites for four of the five circled restriction enzymes lie in an area responsible for antibiotic resistance. This means that any foreign DNA inserted (cloned) in these sites will

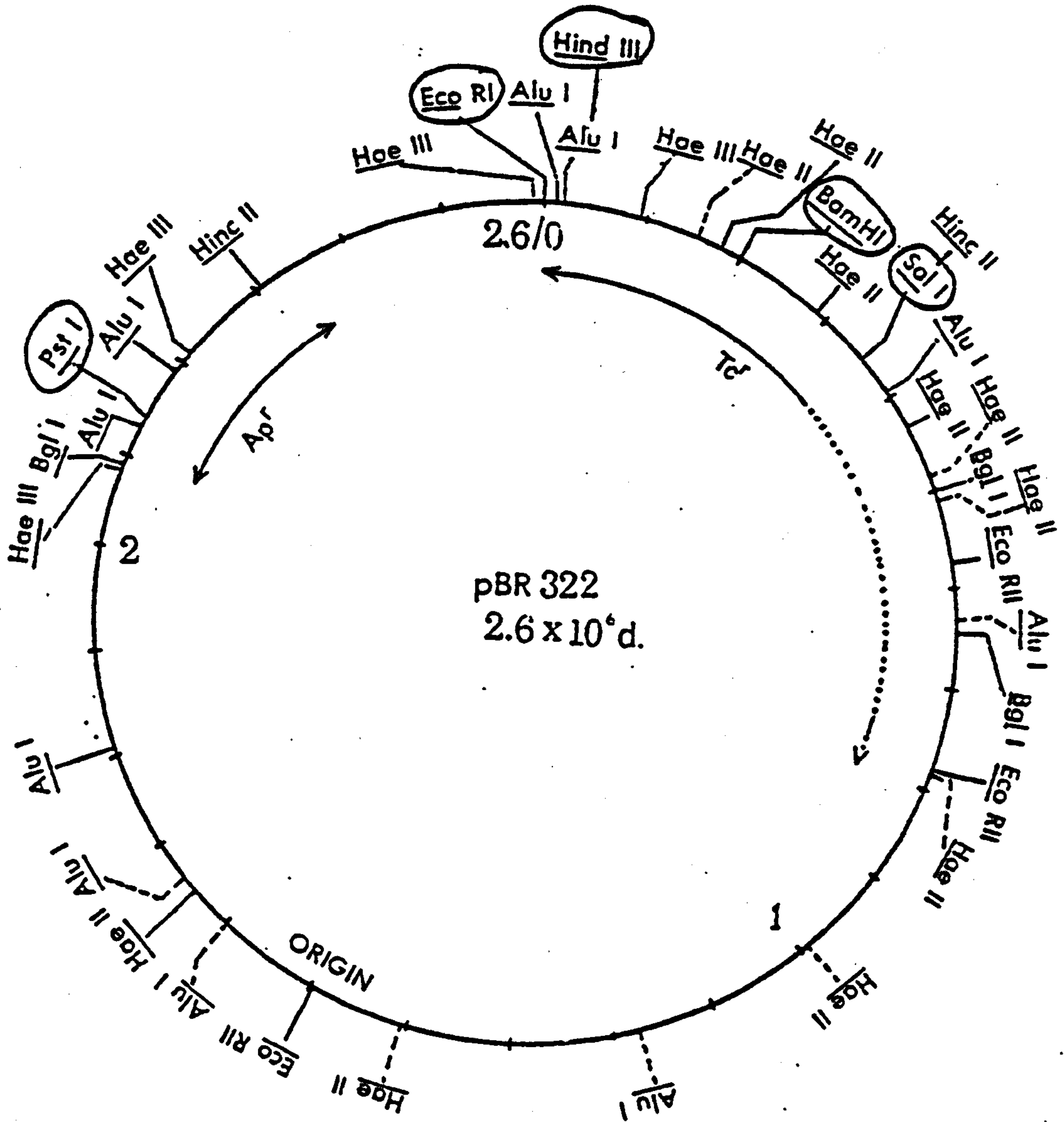


Figure 12. Restriction map of pBR322.

inactivate resistance. For example, Pst 1 cleaves pBR322 in the area responsible for resistance to ampicillin. Cleaving DNA with this enzyme and then inserting it into the Pst 1 site of pBR322 will produce a recombinant molecule that is ampicillin sensitive (Ap^s), but tetracycline resistant (Tc^r) (Fig. 12). This is very useful for it enables one to distinguish upon transformation whether a cell has taken up a recircularized plasmid (Ap^rTc^r) or a recombinant (Ap^sTc^r).

Restriction of C DNA

Restriction studies were initiated on dec8 DNA with the four enzymes cleaving pBR322 once and inactivating resistance to one of the antibiotics. The hope was that one of these enzymes would cleave dec8DNA into roughly 25-30 fragments (to give an average size of $4\text{-}5\times10^6$ daltons) such that most would be large enough to code for td and dfr but not too large to significantly reduce the efficiency of ligation with pBR322 and subsequent transformation into a suitable host cell. Hind 111 gave too many fragments, whereas Bam H1 and Sal 1 gave too few (data not shown).

A recently developed double enzyme digestion technique (D. Kaplan, personal communication 1978) was then developed using Sal 1 and Bam H1 to increase the number of fragments produced. Unfortunately neither simultaneous nor sequential digestion gave anything more than about ten fragments with several being too large in size (Fig. 13).

The three enzymes discussed so far all inactivate resistance to tetracycline and most cloning to date with pBR322 involved the insertion of foreign DNA into these sites. Pst 1 was the last

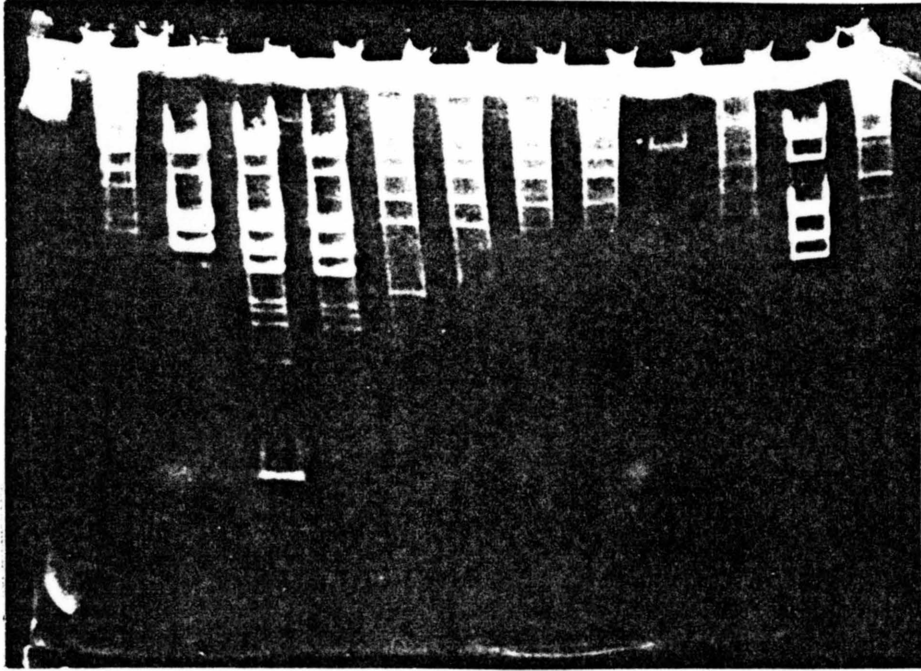


Figure 13. Double enzyme restrictions.

Well No.	DNA	Restriction Enzyme	[$MgCl_2$]
1	dec8	-	
[a]12,3,4,5	λ	Sal I, Bam HI	3,6,9,15 mM
[a]6,7,8,9	dec8	Sal I, Bam HI	3,6,9,15 mM
[b]10	λ	Sal I, Bam HI	
[b]11	dec8	Sal I, Bam HI	
[c]2	λ	Sal I	
[c]13	dec8	Sal I	

[a]Both enzymes added simultaneously.
[b]Sal I added initially, Bam HI added after one hour.
[c]Aliquot of Well No. taken before Bam HI addition.

possibility and seemed very attractive when it was learned (John Wilson personal communication 1978) that the Pst 1 cleavage site in pBR322 was very close to the promoter for the ampicillin resistance proteins(s). This could increase the chances of obtaining transcription of any cloned fragments especially if the T4 promoter for td and dfr was somehow inactivated, not recognized by RNA polymerase, or actually cleaved by Pst 1 and not present in any potential recombinants. Hence, dec8 DNA was restricted with Pst 1 (Smith et al. 1976) to give approximately thirty fragments (Fig. 14). This pattern was extremely reproducible.

Isolation of a Thy$^-$ Host for Transformation

One final step remained before the actual cloning was to be performed -- finding a suitable host for transformation. A logical choice was B834, one of the strains dec8 was originally grown in (Snyder et al. 1976). However, this strain had a functional bacterial thymidylate synthetase; since a shotgun experiment was to be conducted there would be no way of telling on which restriction fragment the DNA coding for phage td or dfr would reside. For this reason a selection procedure was utilized to isolate a B834 strain with an inactive bacterial td gene. The same situation existed for dihydrofolate reductase but no procedure existed for constructing and isolating strains mutated in the bacterial dfr gene.

The rationale for transforming into a thymine requiring cell was as follows: *E. coli* cells can use T4 td so as a selection procedure for clones containing the T4 region coding for td, cells would have either bypassed the need for exogenous thymine (i.e., reverted)

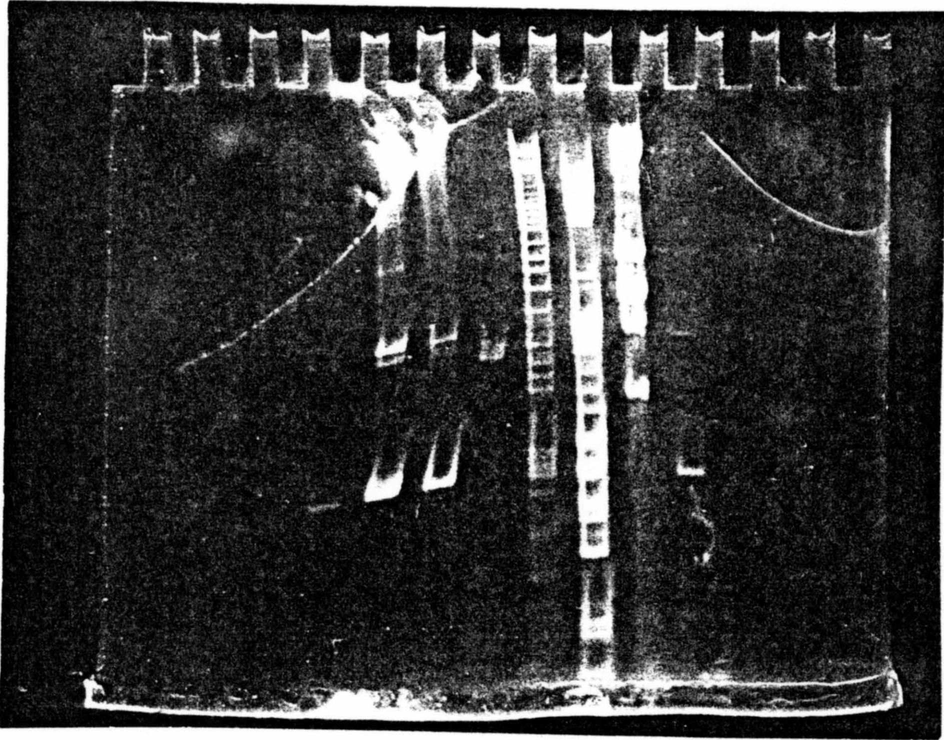


Figure 14. Restriction of dec8 and pBR322.

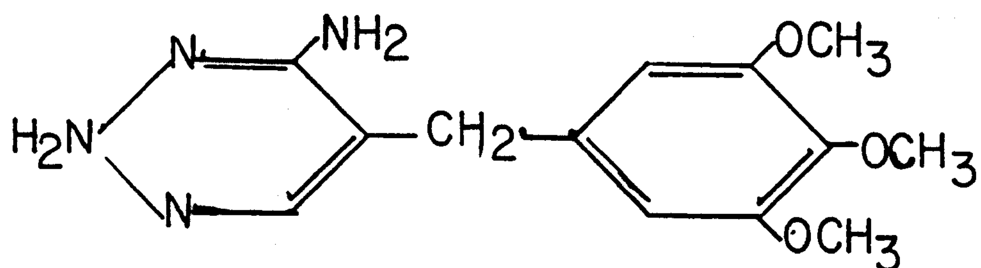
Well No.	DNA	Restriction Enzyme
1	pBR322	-
2	pBR322	-
3	pBR322	Pst I
4	dec8	Pst I
5	λ	Pst I
6	λ	EcoR I
7	pBR322	-

or contained a recombinant plasmid with the DNA sequence coding for T4 td being transcribed and translated producing functional enzyme.

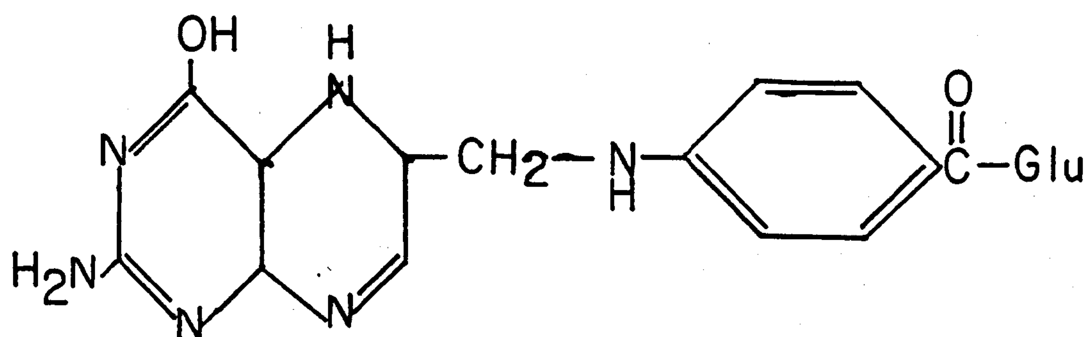
A published procedure existed for the isolation of thymine requiring hosts (referred to as thy$^-$ bacteria) (Stacey and Simson 1965). Basically this procedure involved growing up thy$^+$ bacteria in the presence of trimethoprim and thymine. Trimethoprim is a substrate analog of dihydrofolic acid and a competitive inhibitor of dfr (Fig. 15). Cells that have a functional td gene will deplete all of the tetrahydrofolic acid in the course of producing dTMP. Since dfr is inhibited by the trimethoprim this supply will not be replenished. With no tetrahydrofolic acid synthesis of purines and f-met-tRNA will eventually drop below the minimal level required for cell growth. On the other hand, td mutants will not deplete tetrahydrofolic acid so completely and cells will be able to grow, although at a reduced rate. When conducted this protocol was successful and a thymine requiring B834 strain was isolated (B834T$^-$). As a check, several tests for known markers in B834T$^+$ were performed with B834T$^-$. As shown (Table 8) both strains were isogenic in all respects except for thymine dependency.

Cloning and Transformation of This Host with T4 C DNA

Initial transformation studies were attempted using pBR322 alone on both hosts. This was done to ascertain that these hosts were in fact transformable. The results are shown (Tables 8, 9) (Meade et al. 1979). The actual cloning was next. pBR322 and dec8 DNA were restricted with Pst 1 separately, brought together for ligation with T4 DNA ligase, and then used in the transformation of B834T$^+$ and B834$^-$.



Trimethoprim



Dihydrofolic acid

Figure 15. Dihydrofolic acid and its substrate analog, trimethoprim.

Table 8. Properties of hosts for transformation.

Traits	B834T$^+$	B834T$^-$	B834T$^+$ (pBR322)	B834T$^-$ (pBR322)
methionine	-	-	-	-
lactose	+	+	+	+
$r^- m^-$	+	+	+	+
ampicillin	S	S	R	R
tetracycline	S	S	R	R
td	+	-	+	-
reversion		4×10^{-7}		

Table 9. Transformation results. -- Refer to Materials and Methods for transformation procedure.

Host DNA	B834T$^+$ pBR322	B834T$^-$ pBR322	B834T$^+$ pBR322 dec8	B834$^-$ pBR322 dec8
Total Cells	4.5×10^8	2.5×10^9	5.5×10^8	6×10^6
Transformants	5×10^1	3.4×10^3	2.6×10^2	7×10^1
Trans./ug DNA	-	540	71	10
Trans. Efficiency	1.1×10^{-7}	1.4×10^{-6}	5×10^{-7}	1.2×10^{-5}
Total Transformants	-	-	496	145
Total Recombinants	-	-	10	13
Cloning Efficiency (%)	-	-	2.02	8.97

The transformation mixtures were then spread on TYE plates containing tetracycline since all transformants should be resistant to this antibiotic. All colonies formed were then spotted on an additional TYE-Tc plate and also a TYE-Ap plate. Recombinants would be those restricted on the Ap plate.

Screening for Recombinants

The results are presented in Table 7 indicating 23 recombinants as judged by this selection procedure, 13 of which were in a $B834T^-$ host. Subsequently, two of these 13 now permitted the host to grow without thymine (pLTR5, pLTR7) (Meade et al. 1979). All recombinants were then grown in liquid media prior to preparing cell extracts for enzymatic assays. All were initially grown in minimal media but several recombinants did not grow and rich media had to be used. This was unexpected (the host and host with pBR322 grow fine in minimal media) and indicates some possible deficiency caused by the introduction of recombinant DNA. However, all recombinants grew slower than the corresponding parent and have a much longer lag time (data not shown).

Enzymatic Assays of Recombinants

Cell extracts were then prepared and sonicated. Difficulty was encountered at this step also. Sonication for extended periods of time even with the addition of lysozyme resulted in partial lysis (as incicated by turbidity) and protein concentrations of recombinant extracts were routinely one half to two thirds that of host alone or T4 infected host. The results of td and dfr assays are shown (Tables 10, 11). As

Table 10. Td assays of recombinants. -- Assays performed as described in Materials and Methods.

Strain[d]	td Activity[a]	Strain[e]	td Activity[a]
T4D(B834T$^-$)	1.00	T4D(B834T$^+$)	1.00
B834T$^-$	0.00	B834T$^+$	0.15
pLTR5M[b]	1.56	pLTR10	0.15
pLTR5R[c]	0.78	pLTR12	0.36
pLTR7R[c]	0.43	pLTR17	0.47
pLTR26	0.00	pLTR19	0.19
pBR322	0.00	pLTR23	0.15

[a]Values normalized with respect to T4D.

[b]Cells grown in minimal media.

[c]Cells grown in rich media.

[d]Recombinants in Thy$^-$ host.

[e]Recombinants in Thy$^+$ host.

Table 11. Dfr assays of recombinants.

Strain	dfr Activity[a]	Strain	dfr Activity[a]
T4D	1.00	pLTR12	0.05
B834T$^-$	0.05	pLTR13	0.00
B834T$^+$	0.06	pLTR14	0.16
pLTR1	0.11	pLTR15	0.04
pLTR2	0.02	pLTR16	0.01
pLTR3	0.02	pLTR17	0.02
pLTR4	0.02	pLTR18	0.03
pLTR5	0.23	pLTR19	0.02
pLTR7	0.17	pLTR20	0.02
pLTR8	0.05	pLTR21	0.03
pLTR9	0.13	pLTR22	0.04
pLTR10	0.02	pLTR23	0.00
pLTR11	0.04	pBR322	0.08

[a]Values normalized with respect to T4D.

indicated both pLTR5 and pLTR7 make significant quantities of td especially when considering that the host they are in makes none (Table 10) (Meade et al. 1979). Also, more activity was observed from cell extracts prepared in minimal media than in rich media. This stands to reason since by having thymine present the enzyme would not be needed in as high a concentration.

The results obtained with the dfr assays were more ambiguous. Clones pLTR5 and pLTR7 had two to three times the activity of the uninfected host and significantly more than other clones but still only about one fifth of the phage infected cells. Two experiments were performed to try to unequivocably demonstrate that the activity seen was either host or phage encoded. Antisera specific for phage dfr (Mosher et al. 1977) was added to extracts of pLTR5, pLTR7, T4D (positive control), and B834T$^-$ (negative control). Unfortunately, even with an excess of antisera T4D was not totally inactivated -- so no conclusions could be drawn from this experiment (data not shown).

Host versus phage enzyme can be separated by ammonium sulphate fractionation. With a 50% cut phage enzyme precipitates whereas the host enzyme remains soluble. The results of assays on both precipitate and supernatant are shown (Table 12). Both recombinants have more activity than host but much less than originally seen when compared to extracts of T4 infected cells. Thus the question still remains as to the presence or absence of DNA coding for phage dfr in pLTR5 and pLTR7, but the presence of phage td seems fairly certain.

Table 12. Dfr assays. -- Cell extracts were precipitated by a 50% $(NH_4)_2SO_4$ cut, resuspended in boffer and assayed.[a]

Strain	dfr Activity[a]
T4D	1.00
B834T$^-$	0.01
pLTR5	0.05
pLTR7	0.10

DISCUSSION

Deletion Mutants

The data presented in this paper concerning the deletion mutants are contradictory to the original results reported by Homyk and Weil (1974). However, some discrepancies remain both in the original work and the work reported here. Both mutants tested are reported to contain long deletions extending some 4000 to 5000 nucleotide pairs, at least five times the length of the frd gene (Erickson and Mathews 1971). If mapped correctly these mutants could easily span the entire frd-td region and would be expected to possess no dfr or td activity. There is some uncertainty concerning the endpoints of these deletions. Figure 16 shows the endpoints as reported from the heteroduplex mapping data of Homyk and Weil (1974). This is juxtaposed with the physical map of the 63-32 region, as taken from Wood's (1974) T4 linkage map. This juxtaposition shows both del7 and del9 to extend through gene 32, a situation clearly incompatible with the viability of the phage. Del1 is shown to cover frd, even though this mutant does induce normal levels of dihydrofolate reductase (Mosher et al. 1977). Moreover, the figure shows that del7 and del9 do not cover the td gene but it has been shown that they are td^-. These anomalies might be resolved if all three deletions are placed closer to the rII A/rIIB cistron divide.

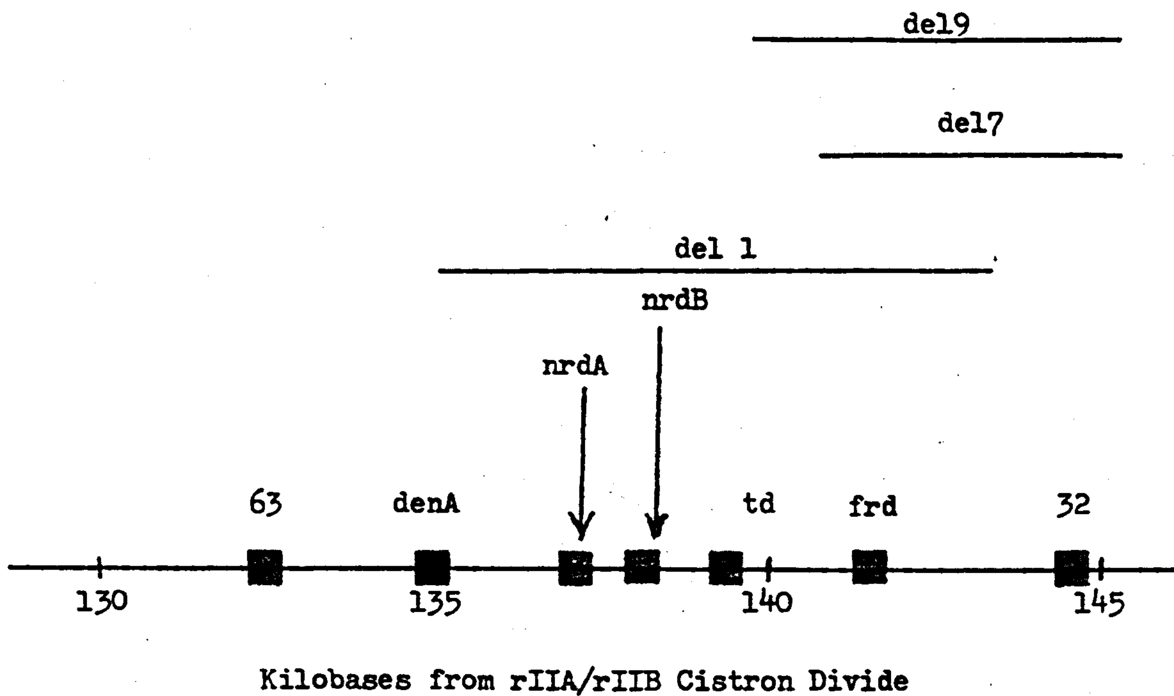


Figure 16. Position of deletions as reported by Homyk and Weil when juxtaposed over genetic map of T4 in 63-32 region as reported by Wood.

Additional uncertainty regarding the positions of frd and td relative to the deletions comes from the finding reported here that the capacity to synthesize functional dfr is regained in recombinants between these deletions and T4D (Table 3), even though the recombinants still contain the original deletion, as shown by failure to induce td activity and restriction by E. coli CT526. Since the same results were seen with five independent recombinants, it seems impossible to explain this in terms of a rare illegitimate recombination event occurring within the deleted segment. In addition, crossing back in the original rII deletion or an rII point mutant did not result in the loss of dfr activity, ruling out an involvement of the DNA from this region in the control of dfr production (Table 4).

It seems far more likely that del7 and del9 each contains an additional mutation affecting the expression of the frd gene, and that this mutation maps at a site distinct from that of the 63-32 deletion, such that it is not recovered in the del7 or del9-T4D recombinant. That such a mutation would be present in both strains, in addition to the 63-32 and rII deletions, is not implausible for two reasons: (1) construction of these strains involved heavy uv irradiation before the deletion bearing strains were crossed into a T4D-r1589 background (Homyk and Weil 1974); this could have introduced new point mutations that were not removed in subsequent backcrossing; (2) there is ample reason for believing that del7 and del9 are identical (Homyk and Weil 1974), thus the idea that they contain the same additional mutation is not surprising.

No success was achieved in trying to isolate this purported regulatory mutant. One problem already mentioned was the fine tuning needed in setting up the halo plating technique and also in interpreting plaque formation. It is also known that other mutations in conjunction with td and dfr will suppress the halo phenotype due to limiting the buildup of dUMP (Hall 1967). Amont these are deficiencies in the cd gene (cytidine deaminase which blocks dCMP going to dUMP) and in any of the three nucleotide reductase genes. As shown in Figure 16, del7 and del9 could very well extend through nrdA when considering that they cannot extend through 32. This would suppress halo formation; in fact del7 and del9 have been shown to lack nrdA activity (Mosher 1978).

Another problem in the work reported here is the lack of an efficient and well characterized host strain restrictive for the deletion mutants. The fact that deletions affecting the expression of the frd gene restrict growth in several of the wild type Cal Tech strains (7 out of 26) tested indicates that some gene product(s) missing in these deletions is essential in some natural environments. However, none of the restrictions have been shown to be a specific effect of inactivation of the frd gene. Also the routine reversion seen (i.e., growth of CT526) is a further complication when working with these strains.

Restriction and Cloning of T4 DNA

Properties of Recombinants

The results reported here confirm the findings of others (D. Kaplan personal communication 1978; H. Revel personal

communication 1978) that restriction of nonglucosylated HMC containing T4 DNA gives irreproducible patterns when restriction occurs at all. This could be the result of steric hindrance caused by the hydroxymethyl group such that restriction is inhibited to certain degrees. All restriction enzymes used in this study contain at least one cytosine residue in their sequence recognition site.

Whatever the inhibitory factor in HMC containing DNA is, it is removed when using cytosine containing DNA. Because of this the molecular cloning of T4 C DNA was successful. A common and very useful cloning vector was used (pBR322) which confers resistance to ampicillin and tetracycline to any cell harboring it. Of the five restriction enzymes which cleave it once, Pst 1 was chosen for two reasons: (1) this enzyme cleaves T4 C DNA to give approximately 30 fragments whose average size is both clonable and large enough to code for both td and dfr; and (2) since the Pst 1 cleavage of pBR322 occurs in the locus responsible for ampicillin resistance this serves as a built in selection procedure to distinguish between recombinants and plasmid alone upon transformation of ligated DNA.

After transformation, 23 colonies arose with the properties expected of recombinants (i.e., Ap^s, Tc^r). These were designated pLTR1, pLTR2, ... pLTR23 respectively. Thirteen recombinants were in a $B834T^-$ background of which two conferred upon their host thymine independence, namely pLTR5 and pLTR7.

Some peculiarities arose in working with these recombinants which were probably related to the inserted T4 DNA since the host alone did not exhibit these properties.

1. All recombinants grew slower than the host alone with a much longer lag time. This slower growth was probably due to the fact that they were always grown in tetracycline. The host with pBR322 alone also had a long lag time but in contrast to the recombinants its doubling time was similar to the host alone once it started growing.

2. Some of the recombinants were unable to grow in minimal media (pLTR7 was one of them) even after prolonged incubation. The addition of Casamino acids to this media produced growth with some, but not all, of the recombinants. Apparently some deficiencies are introduced by these recombinants; maybe through utilization of precursors or essential nutrients by the inserted T4 DNA resulting in a drain of material necessary for cell growth.

3. Unusual appearances were often seen with some of these clones. Clumping of debris (cells?) was seen and occasionally a green color of one of the recombinants (pLTR11).

4. As mentioned previously, they were extremely hard to sonicate for preparation of cell extracts. This was and still is a mystery especially since it occurred with all of the recombinants but not host alone, host with pBR322, or T4 infected host. Preliminary experiments were performed where the cells were incubated with chloramphenicol, washed, incubated for two hours, and then extracts were prepared to see if there was any amplification of protein in these cells. pBR322 is a multicopy plasmid that will replicate in the presence of chloramphenicol, whereas the host chromosome will not., thus greatly increasing the number of copies present per cell. The expectation was that the clones would also increase in copy and upon removal of

chloramphenicol produce elevated levels of any proteins encoded by this DNA. With chloramphenicol treated cells sonication went smoothly and easily resulting in extracts with normal levels (unfortunately there was no amplification) of protein when compared to controls. This implies that maybe a protein made from the recombinant is responsible for this difficulty in sonicating cells with the addition of chloramphenicol blocking its synthesis and allowing cell lysis to take place.

Unfortunately pLTR5 and pLTR7 gave no clear cut indication of possessing the DNA coding for phage dfr. Both the experiments with phage specific antisera and attempts to separate host and phage enzyme by ammonium sulfate fractionation were unsuccessful. In light of the work presented with the deletion mutants, the actual cloning of the dfr region could have occurred but if there is a distinct region involved in the regulation of functional dfr production and exclusion of this region could prevent transcription and/or translation of the DNA into active dfr.

Significance of Cloning

The cloning of this region of T4 is significant for several regions.

1. The area immediately surrounding td and frd contain genes nonessential for T4 growth. Hence, there is no procedure available to select for mutants in this area due to loss of phage viability. With specific clones of this region it will become possible to study this area especially with regards to regulation of transcription and translation by making use of *in vitro* systems as well as *in vivo* studies.

2. Both td and dfr appear to possess both a structural and an enzymatic role in the production of mature phage particles. These two roles are utilized at two different stages of the phage life cycle. Enzymatically both proteins are involved in DNA precursor metabolism and as such their messages appear as soon as three minutes after infection. Structurally, however, their products are not needed until sometime after DNA synthesis begins (after 12 minutes). They are the only structural proteins whose messages are classified as early (made before the onset of DNA synthesis). Cloning of this region will enable one to get a handle, hopefully, on the dual role of these proteins, especially with regards to their structural function.

3. Two recent communications have appeared describing the cloning of T4 (Mattson et al. 1977; Wilson, Tanyashin and Murray 1978). In both cases clones were constructed using a different restriction enzyme than reported here. Analysis showed that none of the clones contained the region described here. Mattson et al. (1977) cloned C DNA using a different plasmid (pCR1) and a different restriction enzyme (Eco R1). Clones were identified by hybridization to early and late T4 RNA, then complementation with ts and amber T4 mutants in various genes. Wilson et al. (1978) used partial Eco R1 digests of nonglucosylated HMC DNA and cloned onto a lambda phage. The hybrids produced were identified by marker rescue of certain amber T4 mutants. The failure of these authors to obtain recombinants in the area reported here might be due to the use of a different restriction enzyme. The proposal has been set forth that the cloning of DNA coding for gene 32 protein would be lethal to the cell (L. Gold, personal communication 1978). Gene 32

codes for an unwinding protein which recognizes and binds to single stranded regions of DNA. Large quantities of this in the cell might cause large regions of DNA to be unwound interfering with replication. Since gene 32 maps close to the td-frd region, an Eco R1 fragment might be large enough to contain both of these regions and be lethal. Also, there are no essential genes in this region so complementation tests would be inapplicable here.

Summary

In summary, T4 HMC DNA did not prove to be a suitable substrate for reproducible restriction. T4 C DNA did fulfill the necessary criteria and was used in cloning experiments in conjunction with pBR322 to produce recombinant molecules. Two of these recombinants were shown to contain the DNA coding for phage td as judged by enzymatic assays and ability to eliminate the need for exogenous thymine in td$^-$ host cells. In addition, evidence was presented contradicting the work of Homyk and Weil (1974) which reported the existence of deletion mutants spanning the td-dfr region of the T4 genome.

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