MUTANTS OF PBS2

TEMPERATURE-SENSITIVE MUTANTS OF BACTERIOPHAGE PBS 2

BY

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SCOPE AND CONTENTS:

Temperature-sensitive mutants of the bacteriophage PBS 2 were isolated from lysates treated with various mutagens. Complementation tests assigned the mutants to 10 cistrons. The mutants were mapped by two factor crosses and formed a linear map approximately 50 recombination percent in length.

A method for phage transformation was developed. By the use of wild type DNA fragments fractionated according to their guanine + cytosine content in Hg-Cs_2SO_4 gradients, it was possible to determine the base composition of certain regions of the chromosomes.

PREFACE

The experiments described in this thesis were carried out in the Department of Biology, McMaster University, from July 1967 to July 1972. Except where others are specifically mentioned, this thesis consists only of original work. No similar thesis has been submitted at any other university.

I am grateful to my supervisor, Dr. I. Takahashi, for his guidance and helpful criticisms throughout this investigation. I would like to thank the Department of Biology of McMaster University, the National Research Council of Canada, and the Ontario Government for their financial support throughout this project. My thanks are also due to Dr. C. T. Chow for his suggestions, and Miss D. Danoff and Mrs. D. Bradford for their technical assistance. I would also like to thank my typist, Mrs. P. Hayward.

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CHAPTER I

INTRODUCTION

Genetics

The first demonstration of an inherited change in bacteriophage was made by Burnet and Lush (9) in 1936. Their mutants of the staphylococcal phage C were not exploited and so the development of phage genetics was delayed until Hershey (39) discovered rapid lysis mutants of the Escherichia coli phage T2 (40).

Since phage can be manipulated in many ways not possible in higher organisms, they quickly became a popular organism for geneticists. Most of the work, however, was concentrated on phages for E. coli, expecially T2 and T4. The earliest demonstration of recombination in a phage active on a Bacillus species was made by Murphy in 1953 (67). He looked at recombination between plaque morphology markers of a phage active on Bacillus megatherium. The earliest observations of recombination in phages active on B. subtilis were made by Okubo, Strauss and Stodolsky (71) and Green (34), who reported recombination between temperature-sensitive mutants of SP01 and SP82 respectively.

The first genetic map of T2 was based on crosses between rapid lysis and host range mutants. It consisted of three linkage groups (41). As more mutants were discovered, these three linkage groups were shown to be linked (4, 57, 88) to form a linear genetic map. This was true for both T2 and T4. Later, Streisinger, Edgar,

and Denhardt (89) found that end markers from this map appeared to be linked in a three factor cross, suggesting that the map is circular.

This conclusion was confirmed by Edgar and Lielausis (22) who isolated a large number of ts mutants of T4. These mutants were distributed over the entire map and in two-factor crosses they showed linkage around the circumference of the map.

Temperature-sensitive mutants are an example of conditional lethal mutants. Conditional lethal mutants are mutants which grow under certain conditions and do not grow under other conditions. Two types of conditional lethal mutants have been used extensively in phage genetics.

Temperature-sensitive mutants do not grow at a high temperature although the wild type phage does grow. Both mutant and wild type phage grow at a lower temperature. Suppressor-sensitive mutants can not grow in one strain of bacteria, but can grow in another strain which carries a suppressor mutation.

Campbell (10) isolated ts mutants, and suppressor-sensitive mutants of the phage λ in 1961. Two years later, Edgar and Lielausis (22) reported the isolation of ts mutants of T4 and Nishihara and Romig (70) reported their isolation in the B. subtilis phage SP3. In each case, complementation tests indicated that a variety of functions might be affected by the conditional lethal mutations (10, 22, 70). This was confirmed by physiological tests of the T4 mutants (25). Mapping of the conditional lethal mutants of λ

(10) and T4 (22) indicated that they were scattered over most of the genome.

Conditional lethal mutants are fairly easy to isolate, and appear to affect many functions. For these reasons, conditional lethal mutants were isolated in numerous phages which had not hitherto been studied genetically.

Three types of maps were found with these phages. The single-stranded DNA phage ϕX174 active on E. coli has a circular map (5) as well as a circular chromosome (27). Some other phages which have circular maps have linear chromosomes. However, these chromosomes are random circular permutations of the gene sequence (33, 99, 100). According to Streisinger, Edgar and Denhardt (89), when these molecules recombine they form longer molecules which then are broken to the normal size during maturation. As a result, all markers appear linked and a circular map is obtained. The E. coli phage T4 and Salmonella phage P22 have circular maps (22, 33), and circularly permuted chromosomes (33, 99, 100). The B. subtilis phage ϕ105 (79) and the Serratia phage Kappa (108) also have circular maps, but the structure of their DNA has not yet been determined.

Linear maps have been observed for the E. coli phages P2 (54) and λ (10), and the B. subtilis phages SP82 (46) and ϕ29 (37).

The chromosomes of the B. megatherium phage ∝ (50) and the two E. coli phages T5 (38) and BF23 (63) are shown to be linear molecules. The genetic maps of these phages are composed of several linear segments. Mutants within the segments are linked

in crosses while mutants in different segments are not linked. Hendrickson and McCorquodale (38) postulated that the T5 map is segmented because there is enhanced recombination at the nicks (8) in the DNA. This may also explain the maps of ∝ and BF23. However, since only a small number of mutants of ∝ and BF23 were mapped, the maps may appear segmented because they are incomplete.

Conditional lethal mutations are important for studying phage physiology. Epstein et al. (25) examined DNA synthesis, cell lysis and serum blocking antigen in cultures infected with ts or amber suppressor-sensitive mutants of T4 under non-permissive conditions. Lysates were also examined under the electron microscope for the presence of normal particles, contracted particles, heads, and tails. From these studies they were able to classify the mutants of T4 into DNA-defective mutants and maturation defective mutants.

Closer examination of some of the mutants has led to the identification of the actual enzymatic defect. For example, gene 43 codes a phage specific DNA polymerase (18, 104), while gene 56 codes for deoxycytidine triphosphatase (66). Other elegant experiments using maturation defective mutants have been carried out to determine how phage particles are assembled (53).

Phage PBS 1 for B. subtilis was isolated by Takahashi in 1961 (90). This phage is unusual in many respects, and thus warrants closer examination. It was one of the first generalized transducing phages for B. subtilis discovered. It mutates frequently to a form giving a clear plaque. This mutant was called PBS 2 (91) because it was isolated at the same time as PBS 1 and was considered to be distinct from PBS 1.

At first PBS 1 appeared to lysogenize cells of B. subtilis (90). However, cells which no longer carried PBS 1 could be obtained from cultures of apparently lysogenic cells by growing them in the presence of antiserum made against PBS 1. Therefore, PBS 1 has been described as pseudolysogenic (91).

The genome of PBS 1 can be incorporated into spores of B. subtilis, thereby becoming heat resistant. When DNA extracted from spores carrying PBS 1 was centrifuged in a CsCl density gradient, two bands of DNA were observed. The density of one band corresponded to that of the host DNA, while the density of the other band corresponded to that of the phage DNA. From the amount of the phage DNA present in the gradient, and the proportion of the spores which produced phage, Takahashi calculated that more than one copy of the phage genome was present in the phage carrying cells (92).

The buoyant density in CsCl of PBS 1 DNA is 1.722 g/cc (96), implying that the base composition of the DNA is 62% guanine + cytosine (G+C). The thermal denaturation temperature (Tm) is 76.5°C (96) suggesting that the base composition is 17.5% G+C. This discrepancy was resolved by chemical analysis which indicated that the DNA of PBS 1 contains no thymine, but instead contains uracil, and that the G+C content is 28% (96).

Morphologically, PBS 1 is similar to other phages in that it is tadpole shaped. However, it is morphologically more complex than other phages. The tail structure has two unusual features. The tail fibres are helical, and when the tail is contracted many fine fibres (contraction fibres) appear at right angles to the length of the tail.

PBS 1 is also much larger than most other phages. The volume of its head is 5×10^8 A^3 compared to the 2×10^8 A^3 volume of the T4 head (24).

The large size of PBS 2 is reflected in the size of its DNA. The molecular weight of PBS 2 DNA is estimated to be 1.9×10^8 (44) which is much larger than the molecular weight (1.3×10^8) of T2 DNA (78).

PBS 1 is one of the few phages known to adsorb on flagella (75). It requires active flagella and thus adsorption will not take place in the presence of metabolic inhibitors such as cyanide. The phage particles adsorb to the sides of the flagellum and appear to wrap their tail fibres around the flagellum. It is not known how the DNA is injected into the bacterium.

Because of the presence of uracil in the DNA, a number of new proteins appear in B. subtilis cells after infection with PBS 1. dCTP deaminase (102), dUMP kinase (47), TMPase (47), and dTTPase (Tomita and Takahashi, unpublished results) are involved in the synthesis of dUTP. There is a DNase present in uninfected cells which is specific for uracil containing DNA (101). An inhibitor of this DNase is produced after infection, and this may be partly responsible for creating the carrier state (or pseudolysogeny) (101). A new DNA polymerase which is distinct from the host enzyme appears after phage infection (73).

Phage transformation

Two methods have been developed to assay biological activity of phage DNA. The first of these, transfection, assays the activity of the whole molecules. For transfection, purified nucleic acid is taken up by cells and complete infective phage are produced. The second method, phage transformation (103) or marker rescue (34), assays the activity of a portion of the molecule. In this assay DNA extracted from phage of one genotype is added to cells infected with phage of another genotype, then the number of phages carrying the first genotype among progeny phages is determined.

Phage T2 disrupted either by osmotic shock (85) or urea (29) are infective for spheroplasts of E. coli. The "transfecting" activity of these phage preparations is sensitive to DNase but is also sensitive to trypsin and deproteinization by chloroform (82). Therefore, the infectivity of these subviral particles is dependent on the presence of some phage proteins.

Purified DNA from λ, T1, (7), and ϕX174 (36) can enter spheroplasts of E. coli and produce infective phage. This transfection can be prevented by DNase treatment and is not sensitive to proteases.

Free DNA from T4 was not shown to be infective for spheroplasts until recently. The DNA of T4 is infective at low magnesium concentration, or when spheroplasts prepared from a strain of E. coli lacking an endonuclease are used (3). This suggests that previous attempts to demonstrate the infectivity of T4 DNA were hampered by nuclease degradation of the relatively large T4 DNA molecule.

Recently, Mandel and Higa (59) showed that DNA from λ or P2

phage could infect cells when calcium was present. This is the first demonstration of transfection of intact E. coli cells.

Transfection of competent cells by DNA from various subtilis phages has been demonstrated. Purified DNA from SP3 (77), SP50 (28), SP82 (34), Ø1, Ø25, Ø29 (76), and SP01 (71) could transform competent cells of B. subtilis. When the DNA was treated with DNase no transfectants were observed.

The number of phage produced depends on the concentration of DNA used. The type of dose response curve obtained depends on the system used. For example, the dose response curve in transfection is linear for Ø1, Ø25, and Ø29 (76) and is non-linear for SP01 (71), and SP82 DNA (34). Okubo, Strauss and Stodolsky (71) suggested that a non-linear dose response curve represents a requirement for recombination between incoming molecules.

The study of transfection has been paralleled by the development of phage transformation systems. Unlike transfection, phage transformation allows us to determine the biological activity of DNA fragments. It assays the activity of a particular marker in the DNA rather than the formation of entire virus particles which requires the entire chromosome.

If spheroplasts of E. coli were infected with urea-disrupted phage T4 carrying the rII genotype and DNA from wild type phage was added, wild type phages were produced at a rate of 6×10^{-7}. The frequency of wild type phages in the absence of DNA was less than 5×10^{-9}. When the DNA was treated with DNase the number of wild

type phages produced was equivalent to that produced in the absence of DNA. Treatment with trypsin or heat denaturation of the DNA reduced the frequency of transformants slightly (103).

Kaiser and Hogness (49) developed a system which was later used for the transformation of λ. Cells of E. coli which could not utilize galactose (gal$^-$) were infected with λ helper phage and DNA from λ dg (a defective phage carrying the gal$^+$ gene from E. coli) was added. Some of the surviving cells were gal$^+$, indicating that they had incorporated the gal$^+$ marker from the λ dg DNA. They suggested that the helper phage facilitated the uptake of the DNA. The activity of the λ dg DNA was not affected by treatment with phage antiserum or trypsin but was sensitive to DNase treatment.

Later experiments (74, 48, 42) showed that the helper phage could be transformed by λ or λ dg DNA. In these experiments, cells were infected with helper phage genetically marked with two mutations and wild type DNA was added. They found transformants for individual markers as well as transformants for both markers, indicating that recombination occurs between the helper phage and the DNA added to the cells.

Green (34) was the first to demonstrate transformation of a phage active on B. subtilis. He added DNA from wild type SP82 to competent cells, then infected the cells with a ts mutant. When the infected cells were plated under non-permissive conditions a certain number of plaques were obtained. The number of wild type infective centres was directly proportional to the concentration

of DNA used. The activity of the DNA was sensitive to DNase but not to proteolytic enzymes.

Marsh, Breschkin, and Mosig (62) used transformation to determine the origin and direction of replication of the T4 chromosome. There is a class of T4 particles containing chromosomes only two-thirds the length of the normal chromosome. Since chromosomes of T4 are circular per mutations of the gene order, these incomplete chromosomes represent random segments of the T4 genomes (14, 64, 65, 72). If a cell of E. coli is infected with one of these small particles, the chromosome carries out a single cycle of replication. Small T4 particles labelled with heavy isotopes were used to infect cells growing in light medium. A low multiplicity of infection was used to ensure that infected cells received only one particle. After a period of incubation in light medium, DNA was extracted from these cells and parental and progeny DNA were separated by alkaline density gradient centrifugation. The denatured parental and progeny DNA were assayed by transformation to determine marker frequencies. As would be expected, markers were recovered from parental strands with equal frequencies. The frequency of markers in progeny strands was highest for markers near gene 43 and decreased with increasing distance from gene 43 in a clockwise direction on the genetic map. Thus, it appears that replication starts at a position near gene 43 and proceeds in a clockwise direction around the genetic map.

Fragments of DNA can be fractionated according to nucleotide composition in cesium sulphate density gradients containing mercury (68). The mercuric ions bind preferentially to regions of double-stranded DNA rich in adenine and thymine, thereby increasing the

density of the molecules. Therefore, in this system, DNA containing a relatively large amount of adenine and thymine shows a density higher than that of DNA containing less adenine and thymine (68).

Skalka, Burgi, and Hershey (84) used the Hg-Cs_2SO_4 density gradients to separate fragments of λ DNA produced by shearing. They compared the separations of λ DNA of different sizes and concluded that the λ molecule contained six segments of different base composition.

This heterogeneity of base composition is observed with half molecules of the λ DNA (84). When relatively large fragments of E. coli DNA were examined no heterogeneity of nucleotide composition was found (112). However, when E. coli DNA was broken into small fragments with a molecular weight of 1.5×10^6, a heterogeneous distribution of nucleotide composition was observed (112).

Small fragments of B. subtilis DNA also show a heterogeneous distribution of nucleotides (113). Since B. subtilis is transformable, Yamagishi and Takahashi (113) were able to assay the transforming activity of DNA fragments from different fractions obtained after Hg-Cs_2SO_4 density gradient centrifugation. The transforming activity for the methionine and tryptophan markers were in different fractions while the transforming activity for the tyrosine marker, which is linked to the tryptophan marker was found in the same fractions with that for the tryptophan marker.

Yamagishi (private communication) found that fragments of PBS 2 DNA could also be fractionated according to nucleotide composition by Hg-Cs_2SO_4 density gradient centrifugation. Therefore, if a system for transformation of PBS 2 could be developed it should be possible to determine the nucleotide composition of various regions of the PBS 2 chromosome.

This thesis describes the isolation of ts mutants of PBS 2. These mutants were induced by various mutagens, including a novel one, 5-fluorodeoxyuridine. The ts mutants were mapped and were assigned to cistrons by complementation tests.

The mutants were also used to develop a phage transformation system which was used in conjunction with Hg-Cs_2SO_4 density gradient centrifugation to determine the appropriate G + C content of different regions of the PBS 2 chromosome.

CHAPTER II

MATERIALS AND METHODS

1. Symbols

Symbols to designate the genotype of mutant bacteria are according to Demerec *et al.* (17).

The abbreviations used for amino acids, bases, nucleotides, and nucleic acids are thos recommended by the NAS-NRC ofice of Biochemical Nomenclature (45).

The abbreviations used were as follows: ts, temperature-sensitive; DNA, deoxyribonucleic acid; G+C, guanine + cytosine; PFU, plaque-forming units; DNase, deoxyribonuclease; RNase, ribonuclease; SSC, standard saline citrate (0.15 M NaCl + 0.015 M sodium citrate); FdUra, 5-fluorodeoxyuridine; nitrosoguanidine, N-methyl-N'-nitro-N-nitrosoguanidine; BGM, basic growth medium; CM, competence medium; VBS, Vogel and Bonner supplemented medium; Ad, adsorption medium; TBB, trytose blood agar base; PA, Difco antibiotic medium 3; *Str*^{r}, streptomycin resistance; *ery*^{r}, erythromycin resistance; *Su*$^+$, carrying a suppressor mutation; *AP*, mutant alkaline phosphatase; *gal*$^+$, galactose utilization; *his*, histidine requirement; *aro*, aromatic requirement; *trp*, tryptophan requirement; *tyr*, tyrosine requirement.

2. Bacterial strains

In most cases, *B. subtilis* SB19E (*str*^{r}, *ery*^{r}) (93) was the host bacterium for PBS 2. In a few instances, *B. subtilis* JBO-130 (*Su*$^+$, *his-5*, *met-5*, *AP-2*) obtained from Dr. S. Okubo was used to prepare lysates of PBS 2 for the isolation of mutants. *B. subtilis* SB202

(aro-2, trp-2, his-B2, tyr-1)(69) was used as host in PBS 2 transformation.

E. coli B was used as host for phage T4.

3. Phage strains

Phage PBS 1 (88) was from laboratory stocks. A single plaque isolate of the clear plaque mutant PBS 2 (91) was obtained from a PBS 1 stock and was used throughout this work. E. coli phage T4 was from laboratory stocks.

4. Media

a) Minimal medium (86)

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	1.0 g
$MgSO_4.7H_2O$	0.2 g
distilled water	1.0 l

The pH was adjusted to 7.0. After sterilization, 50 ml of 10% glucose is added. Minimal agar contains 15 g of Difco agar per litre of medium. Amino acids were added at a concentration of 25 µg/ml when required.

b) Transformation medium (94)

Minimal medium	100 ml
After autoclaving,	
glucose (10%)	5 ml
Difco casamino acids (1%)	1 ml
Difco yeast extract (1%)	1 ml

c) Basic growth medium (BGM) (modified from 58)

Minimal medium	100 ml

After autoclaving,

glucose (10%)	5 ml
tryptophan (0.5%)	1 ml
Difco yeast extract (10%)	1 ml
Difco casamino acids (1%)	2 ml
tyrosine (0.5%)	1 ml
phenylalanine (1.0%)	0.5 ml

Just before use the following supplement was added,

arginine (100 mg/ml)	1 ml

d) Competence medium (CM) (modified from 58)

Minimal medium	100 ml

After autoclaving,

glucose (10%)	5 ml
tryptophan (0.5%)	0.1 ml
Difco yeast extract (10%)	1 ml
Difco casamino acids (1%)	1 ml
tyrosine (0.5%)	0.5 ml
histidine (1%)	0.25 ml
shikimic acid (0.5%)	0.5 ml

Just before use, the following supplements were added,

0.125 M $CaCl_2$	2.5 ml
0.1 M $MgCl_2$	2.5 ml
0.05 M spermine	1.0 ml

e) Vogel and Bonner supplemented medium (VBS) (56)

$MgSO_4.7H_2O$	0.2 g
citric acid ($C_6H_8O_7.H_2O$)	2.0 g
K_2HPO_4	14.0 g
$NaNH_4HPO_4.4H_2O$	10.0 g
Difco casamino acids	1.0 g
tryptophan	10 mg
$FeCl_3.6H_2O$	10 mg
distilled water	1 l

The pH was adjusted to 7.0. After autoclaving glucose (10%) was added at a final concentration of 0.5%.

f) Spore agar (80)

Difco nutrient broth	8.0 g
$MgSO_4.7H_2O$	0.25 g
KCl	1.0 g
$MnCl_2$ (1.9%)	0.1 ml
Difco agar	15.0 g
distilled water	1.0 l

The pH was adjusted to 7.0 and after sterilization the following additions were made:

$FeSO_4$ (0.27%)	0.1 ml
$Ca(NO_3)_2.4H_2O$ (2.3%)	10 ml

g) Adsorption medium (Ad) (111)

h) Difco tryptose blood agar base (TBB)

i) 1% TBB

This medium contained the same ingredients as TBB except that 1% agar was added instead of 1.5%.

j) Difco antibiotic medium 3 (penassay, PA)

k) AT medium

AT was prepared by mixing 1 part Ad and 1 part 1% TBB before autoclaving.

5. Culture conditions

Bacterial stocks were maintained on TBB plates. Unless otherwise indicated, cultures were prepared by inoculating PA with less than 10^7 cells/ml from an overnight culture on TBB and shaking at 37 C for 4 h.

6. Phage techniques

The assay for PBS 2 was as described previously (91). In many cases, the volume of dilution fluid was adjusted so that 0.1 ml instead of 1 ml per plate was used. All dilutions were made in Ad. Phage T4 was assayed by the Adam's method (1) using TBB for base agar and AT for top agar.

To prepare lysates of PBS 2, a 4 h culture of host cells was diluted ten fold in PA and phage were added to give a multiplicity of infection of 0.1. After 1 h incubation with aeration, the infected culture was incubated further for 18 h without shaking (91). Incubation was at 37 C for the propagation of wild type phage and 30 C for ts mutants.

In some cases lysates were prepared by the plate method of Adams (1). After overnight incubation at 30 C agar plates with confluent lysis were flooded with 3 ml of PA. The PA was pipetted from the plates after they had sat for 1 h at room temperature with

occasional shaking.

Lysates were centrifuged at 3,000 X g for 15 min to remove cell debris and were stored at 4 C.

The velocity constant (K) of neutralization of rabbit serum prepared against PBS 2 was assayed as described by Adams (1). The K-value of the serum used was 500.

PBS 2 is quite stable in crude lysates (91). However, phage stocks can be maintained indefinitely in phage infected spores. A few drops of undiluted phage lysates were mixed with a few drops of a 4 h culture of SB19E. This mixture was streaked onto spore agar slants which were then incubated at 30 C for 2 days. To test the presence of the phage in spores, a loopful of infected cells from the slant was suspended in 1 ml of physiological saline. This suspension was heated at 85 C for 10 min. The heat treatment inactivates free phage and vegetative cells but does not inactivate spores. Thus, when samples of the heated suspension were plated, phage-infected spores gave rise to plaques. Plaques arising from spores infected with a ts mutant were tested for their ts character.

7. Preparation of 2-aminopurine treated phage

B. subtilis SB19E was grown to 5 x 10^8 cells/ml in minimal medium supplemented with 0.5% casamino acids. The bacteria were collected by centrifugation at 3,000 X g for 15 min, and were resuspended in the same volume of minimal medium containing 0.5% casamino acids and 1 mg/ml 2-aminopurine. Phage PBS 2 was added to

give a multiplicity of infection of 2. The infected culture was shaken for 1 h at 30 C, and incubated further for 18 h at 30 C, without shaking.

8. Preparation of hydroxylamine treated phage

Hydroxylamine treated phage was prepared by the method of Freese, Bautz, and Freese (32). In order to eliminate phage heteroxygotes (98) the phage was propagated at 30 C in the absence of hydroxylamine before testing for the presence of ts mutants.

9. Preparation of N-methyl-N'-nitro-N-nitrosoguanidine treated phage

Concentrated solutions of N-methyl-N'-nitro-N-nitrosoguanidine (nitrosoguanidine) were prepared according to Takahashi and Barnard (95), and diluted in minimal medium supplemented with 0.01% Difco nutrient broth (MNB). A 4 h culture of JBO-130 (approximately 5 x 10^8 cells/ml) in MNB was mixed with an equal volume of PBS 2 at 6 x 10^7 PFU/ml in Ad medium and the phage was allowed to adsorb for 5 min at room temperature. The mixture was then diluted 5-fold in MNB containing nitrosoguanidine. The final concentration of nitrosoguanidine was 20 µg/ml. After incubation with shaking for 1 h, the phage-infected culture was incubated further for 18 h without shaking at 30 C.

10. Preparation of 5-fluorodeoxyuridine treated phage

This method was based on that used by Lozeron and Szybalski (54) for preparation of PBS 2 DNA containing 5-fluorodeoxyuridine (FdUra). A culture of SB19E or JBO-130 grown for 4 h in PA was diluted 1:10 in VBS and grown to 10^8 cells/ml. The cells were collected by centrifugation

(3,000 X g, 15 min) and were resuspended in 0.5 volumes of VBS. The cells were infected with PBS 2 at a multiplicity of infection of approximately 0.1. The infected culture was incubated at 30 C with shaking for 20 min and then FdUra dissolved in VBS was added to give a final concentration of 5 μg/ml. The treated culture was incubated at 30 C with aeration for 1 h and further incubated without aeration for 18 h.

11. Isolation and purification of ts mutants

Samples of mutagen treated phage were plated to obtain about 100 plaques per plate. The plates were incubated for 18 h at 30 C. Individual plaques were picked with sterile toothpicks and transferred onto a pair of TBB plates which had been overlaid with 2.5 ml of AT containing 10^7 cells/ml of SB19E. One plate of each pair was incubated at 30 C and the other was incubated at 45 C for 18 h.

Most of the plaques transferred gave lysis on both plates. Some of them, however, showed lysis at 30 C, but not at 45 C. In this case a small amount of the lysed area was suspended in PA, and plated after appropriate dilution. A plaque from this plating was used to prepare mutant phage lysates. At all steps, phage suspensions were tested for the ts character by spotting a small amount on pairs of plates prepared as described above and incubating at the two temperatures.

12. Mutant nomenclature

Temperature-sensitive mutants are identified by the prefix T followed by two letters referring to the mutagen employed and two

or three numbers representing the order of isolation. Thus mutants induced with 2-aminopurine are prefixed TAP; with FdUra, TFU; with nitrosoguanidine; TNG; and with hydroxylamine, THA.

13. Crosses

Cells of SB19E were grown in PA to early stationary phase. Growth was followed by measuring the turbidity of the culture by means of a Klett-Summerson colorimeter equipped with a #59 (green) filter. When the culture reached early stationary phase (3-4 h) it was diluted 1:10 in PA and grown until it reached a cell density, as measured by turbidity, of 1×10^8 cells/ml. The crossing mixture contained 0.2 ml of the culture diluted to 2×10^7 cells/ml in PA and 0.1 ml of each phage (2×10^8 PFU/ml) to be crossed. These were equilibrated at 30 C before mixing. After a 20 min adsorption period, antiserum was added to inactivate the unadsorbed phage. Five min later this mixture was diluted 5,000 fold in PA and incubated further for 95 min. A few drops of chloroform were added and samples were diluted before the total yield was assayed by incubating plates at 30 C for 18 h. Samples were also plated and incubated at 45 C for 4-8 h to determine the number of wild type recombinants. At least 400 plaques were counted for total yield and 200 recombinants were counted. The percentage recombination is

$$\frac{\text{the number of wild type recombinants X 2}}{\text{total yield}} \quad \text{X 100.}$$

The factor 2 is used to account for the double mutant type which is not scored.

14. Complementation tests

Although several attempts were made to develop a spot test for complementation, none of the techniques used were satisfactory for this system. For this reason, complementation was determined by measuring the burst size of the mixed infections at the non-permissive temperature.

In each set of experiments the burst size of the wild type and each mutant alone was determined as well as the burst size of the mixture. Cells and phage were prepared as for the crosses and were equilibrated at 45 C. Equal volumes of cells and phage were mixed and adsorption was allowed to proceed for 10 min. Antiserum was added to inactivate unadsorbed phage. Six minutes later the mixture was diluted 10,000 fold in PA and infective centres (the number of infected cells producing progeny phages) were assayed. After a total incubation period of 45 min the final yield of phage was measured. The burst size is obtained by dividing the final phage yield by the number of infected cells.

15. Bacterial transformation

Bacterial transformation was carried out as described by Takahashi (94).

16. Phage transformation

To a competent culture of SB202, a ts mutant (multiplicity of infection of 1-2) and PBS 2 DNA were added. This mixture was incubated at 30 C with shaking for 100 min at which time lysis

was complete. The lysate was then diluted and wild type transformants were determined by incubating assay plates at 45 C for 4-8 h. Controls were routinely made by plating samples to which either no DNA or no phage had been added.

17. Induction of competence

a) Method I

Competent cells were prepared by the method of Takahashi (94). A four hour culture of SB202 in PA was diluted 10 fold in transformation medium. This was incubated with shaing at 37 C for 60 min for phage transformation and for 90 min for bacterial transformation.

b) Method II

Competent cells prepared by a modification of Mahler's method (58) gave higher levels of transformation than did competent cells prepared by Method I. Cultures of SB202 in BGM were grown to the end of the exponential phase. Growth was followed by measuring the turbidity with a Klett-Summerson colorimeter. The cells were then diluted 10 fold in CM and incubated with shaking for 90 min.

18. Phage purification

Phage lysates were centrifuged at 3,000 X g for 15 min to remove bacterial debris. The supernatant was centrifuged at 40,000 X g for 1 h to sediment the phage particles. After resuspension in a small volume of a buffer (0.15 M NaCl and 0.1 M phosphate, pH 7.0), the phage particles were treated with lysozyme (100 µg/ml), DNase (10 µg/ml),

and RNase (10 µg/ml) at 37 C for 30 min. The phage particles were subjected to two more cycles of differential centrifugation, and were finally resuspended in 1 x SSC (0.15 M NaCl + 0.015 M sodium citrate).

19. DNA extraction

Purified phage particles in 1 x SSC were shaken for 10 min with an equal volume of phenol saturated with 1 x SSC. This was centrifuged at 3,000 X g for 10 min and the aqueous layer was dialysed against 1 x SSC to remove dissolved phenol. In some cases, phenol was removed by ether extraction (61). The DNA was precipitated by adding 2 volumes of 95% ethanol and was stored in 75% ethanol for 3 days to sterilize it. Precipitated DNA was dissolved in 0.1 x SSC then the SSC concentration was adjusted to 1 x SSC by adding 20 x SSC. Solutions of DNA were stored at -10 C. The concentration of DNA was estimated by measuring the absorbance at 260 nm. An A_{260} of 20 was taken as a DNA concentration of 1 mg/ml.

20. DNA shearing

Solutions of DNA in SSC were sheared by stirring at 33,000 rpm for 20 min in a Virtis homogenizer. The sheared DNA was then dialysed extensively against 0.1 M Na_2SO_4 to remove chloride ions.

21. Hg-Cs_2SO_4 density gradients

Gradients were prepared as described by Yamagishi and Takahashi (113). The ingredients were added directly to a 12 ml nitrocellulose

centrifuge tube in the following order: Cs_2SO_4, 2.52 g; DNA (in 0.1 M Na_2SO_4), 2.5 ml; 0.1 M sodium borate solution, 0.125 ml; $HgCl_2$ (100 µg/ml), volume determined according to the rf value used in each experiment; 0.1 M Na_2SO_4-0.005 M sodium borate, to make a total weight of 6.04 g. The amount of mercuric chloride used depended on the rf values (mole Hg/mole nucleotide) used, and was calculated from the following formula: the amount of mercuric chloride in µg = amount of DNA in µg X 0.7882 (a constant factor) X rf value. The mixture was overlaid with mineral oil and centrifuged for 48 h at 4 C and 36,000 rpm in a 50 Ti rotor in a Beckman L2-65 ultracentrifuge.

After centrifugation, 50 fractions (8 drops/fraction) were collected from the bottom of the tube. For use in transformation, the fractions were collected in 1 ml of 1.0 M NaCl. The amount of DNA in the fractions was estimated by measuring the absorbance at 260 nm in a Unicam spectrophotometer fitted with 0.2 ml cuvettes (1 cm path length). Generally, sixteen fractions contained significant amounts of DNA. For use in transformation, the fractions containing DNA were pooled to obtain eight fractions as indicated in Figs. 12-14. The pooled DNA samples were dialysed against 1.0 M NaCl, and then against 1 x SSC. The dialysed samples were stored at -10 C.

22. Materials

Common chemicals were obtained from Fisher Scientific Co., and were reagent grade. Optical grade Cs_2SO_4 was obtained from Kawecki Chemical Co. Nitrosoguanidine and spermine were obtained

from Aldrich Chemical Co., and 2-aminopurine was from Sigma Chemical Co. Fluorodeoxyuridine was a gift from Dr. W. Szybalski, and Dr. R. J. Horsley. Prepared media, casamino acids, and yeast extract were obtained from Difco. Amino acids were obtained from Calbiochem. Enzymes were obtained from Worthington Biochemical Corp.

CHAPTER III

INDUCTION OF MUTATIONS IN PBS 2

The effect of various common mutagens on PBS 2 has been examined and the results are summarized in Table 1.

It has been reported that the base analogue FdUra can be incorporated into PBS 2 DNA in place of deoxyuridine (56), and that fluorouracil is mutagenic for RNA viruses (52, 97). The effect of various concentrations of FdUra on the yield of PBS 2 is shown in Fig. 1. When uninfected cells were treated with FdUra at 5 μg/ml, approximately 30% of the treated cells were killed. This amount of killing was not great enough to account for the decrease in the yield of phage when infected cells were treated with FdUra at 5 μg/ml. Lysates prepared in the presence of FdUra at 5 μg/ml gave a relatively high yield of phage and so were used to isolate ts mutants. As shown in Table 1, FdUra induced ts mutants of PBS 2 more frequently than did other mutagens tested.

Lozeron and Szybalski (56) observed an increase in the buoyant density in CsCl of DNA extracted from PBS 2 which had been grown in the presence of 50 μg/ml FdUra. According to their data an increase in density of $0.0012 g/cm^3$ corresponds to a replacement of 2 mole per cent of deoxyuridine by FdUra. This amount of density increase is detectable in analytical centrifugation of DNA samples in CsCl density gradients.

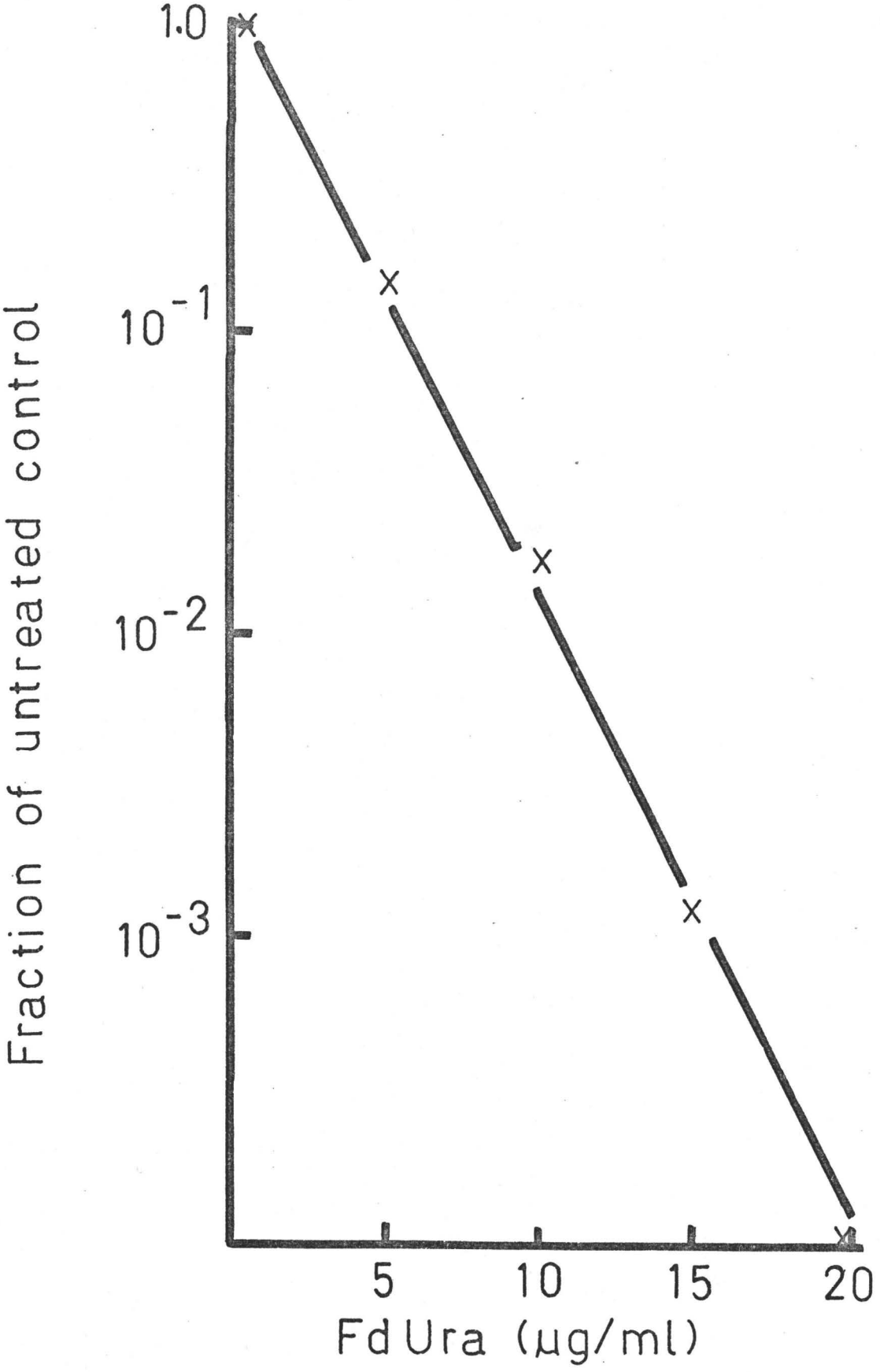
Table 1

Mutagenic effect of various agents on PBS 2

Mutagen	Concentration of mutagen	Treatment	Survival or viability (%)	% ts mutants
FdUra	5 µg/ml	in vivo	14	0.6
Nitrosoguanidine	1 mg/ml	in vitro	100	0
	20 µg/ml	in vivo	4	0.3
Hydroxylamine	87 mg/ml	in vitro	50	0.1
2-Aminopurine	1 mg/ml	in vivo	10	0.1

The conditions of treatment are described in Chapter II.

Figure 1: The effect of 5-fluorodeoxyuridine on PBS 2. Lysates were prepared in the presence of different concentrations of FdUra as described in Chapter II. Titres of treated lysates were compared to the titre of an untreated lysate.


1.0
10^{-1}
10^{-2}
10^{-3}
Fraction of untreated control
5
10
15
20
Fd Ura (µg/ml)

In order to determine the amount of FdUra incorporated under our conditions, DNA was extracted from PBS 2 grown in the presence of 5 μg/ml of FdUra and was centrifuged in CsCl in a Model E analytical centrifuge. No increase in the buoyant density of the DNA was detectable. It appears, therefore, that under the conditions used to induce ts mutants, less than 2 mole per cent of deoxyuridine are replaced by FdUra.

Nitrosoguanidine neither inactivated PBS 2 nor induced mutations in PBS 2 when the phage was treated in vitro (Table 1). This has been observed with other phages (114), and is not unexpected since nitrosoguanidine affects regions near the replication point of the chromosome (12). When infected cells were treated with nitrosoguanidine the yield of phage was greatly affected (Fig. 2). In order to induce ts mutants, infected cells were treated with nitrosoguanidine at 20 μg/ml. Nitrosoguanidine induced ts mutants at a higher frequency than did hydroxylamine and aminopurine.

Hydroxylamine exhibited a greater killing effect on PBS 2 than on the E. coli phage T4 (Fig. 3). This could be a lethal effect produced by a reaction between hydroxylamine and uracil in the DNA (32). Freese, Bautz, and Freese (32) showed that bromouracil and uracil react with hydroxylamine in the same way, and that T4 containing bromouracil is much more sensitive to the lethal action of hydroxylamine than T4 particles containing only thymine. The rate of reaction of hydroxylamine with uracil increases as the pH increases (32). Thus, if the lethal action of hydroxylamine on

Figure 2: The effect of nitrosoguanidine on PBS 2. Nitrosoguanidine was added at various concentrations to cultures of JBO-130 which had been infected with PBS 2 at a multiplicity of infection of about 2. Lysates were prepared as described in Chapter II and titres of treated lysates were compared to the titre of an untreated lysate.

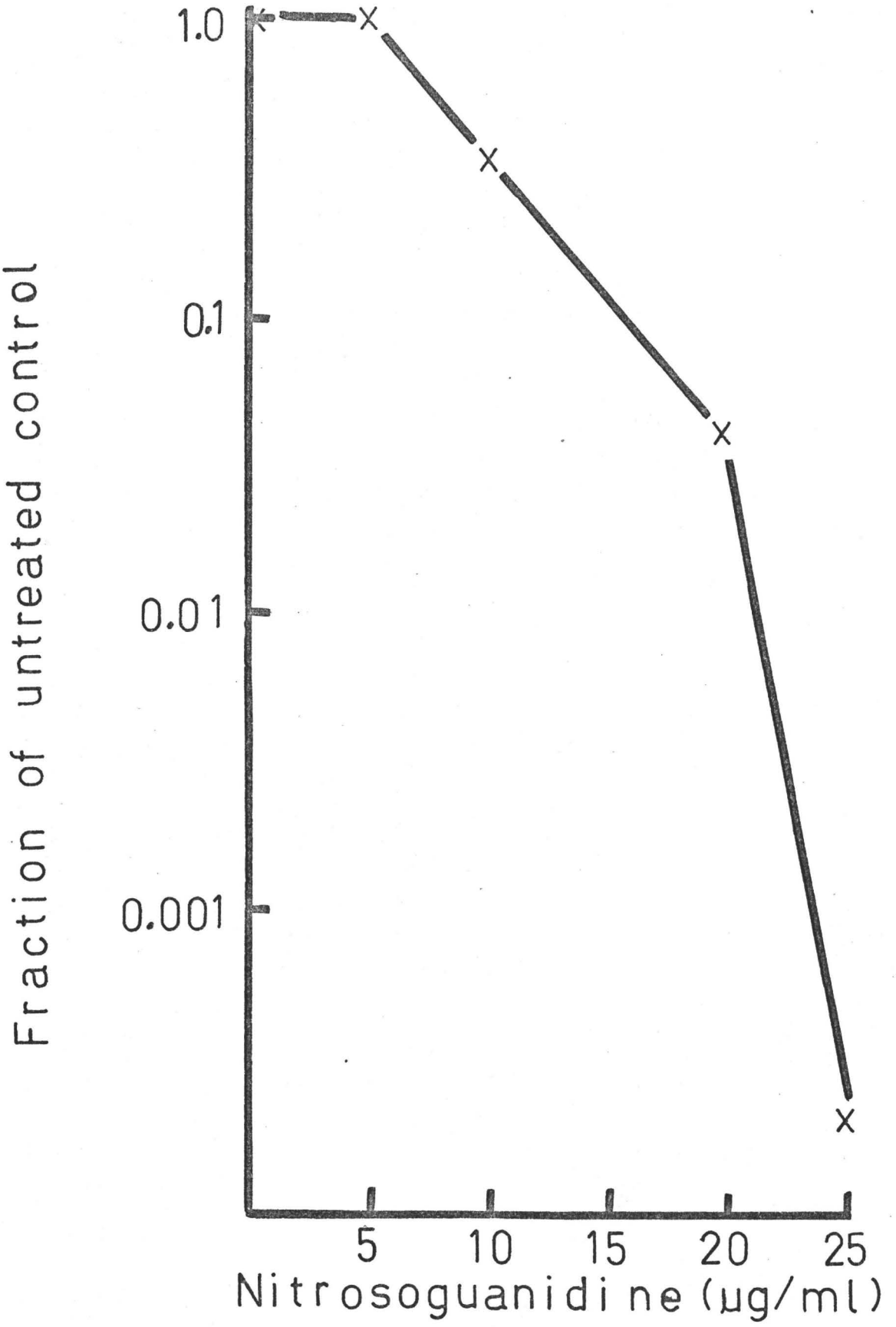
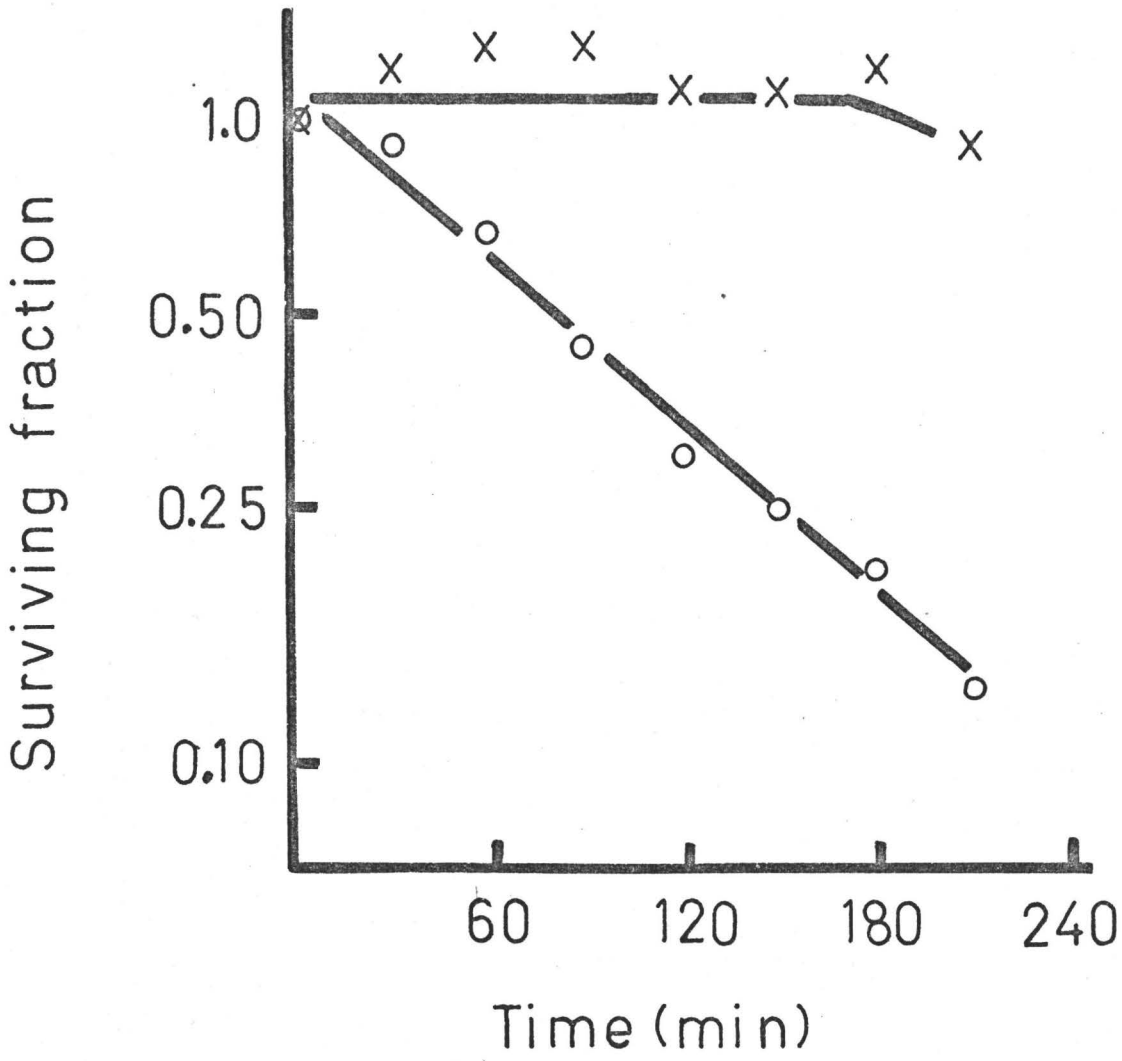

1.0
0.1
0.01
0.001
Fraction of untreated control
5
10
15
20
25
Nitrosoguanidine (µg/ml)

Figure 3: The effect of hydroxylamine on PBS 2 and T4.

X———X Surviving fraction of T4

0———0 Surviving fraction of PBS 2

Hydroxylamine treatment was carried out as described in Chapter II.


Surviving fraction
1.0
0.50
0.25
0.10
60
120
180
240
Time (min)

PBS 2 is due to the reaction with uracil, the inactivation of PBS 2 should be greater at pH 9.0 than at pH 6.0. Table 2 gives the inactivation of PBS 2 and T4 at different pH values. The hydroxylamine treatment had practically no affect on the viability of phage T4 under the conditions used. The inactivation of PBS 2, on the other hand, was greatest at pH 6.0 and least at pH 7.5. In fact, PBS 2 was inactivated within a few seconds of contact with hydroxylamine at pH 6.0. This inactivation was not observed in buffer at pH 6.0 in the absence of hydroxylamine.

Hydroxylamine also reacts lethally with the tail of T-even phages after a long period of contact (51). According to Rima (unpublished results) no gross morphological changes in hydroxylamine-treated PBS 2 were detectable under the electron microscope. Therefore, the mechanism of inactivation of PBS 2 by hydroxylamine is not known at present.

The effect of 2-aminopurine on PBS 2 was not examined in detail.

Table 2

Inactivation of PBS 2 and T4 by hydroxylamine at different pH values.

pH	Fraction of phage surviving after 60 min	
	PBS 2	T4
6.0	0	1.0
7.5	0.67	1.3
9.0	0.34	1.1

Phages were treated with hydroxylamine as described in Chapter II.

CHAPTER IV

GENETICS OF PBS 2

A set of mutants can be genetically characterized in two ways. First, they can be assigned to groups according to the functions that are affected, by complementation tests (6). Then, their chromosomal locations can be determined from their frequency of recombination obtained in phage crosses. Both crosses and complementation tests are carried out by co-infecting host cells with two different mutants, and allowing a single cycle of phage growth to proceed under the appropriate conditions.

To measure complementation, infected cells are incubated at the non-permissive temperature for a length of time equivalent to one cycle of growth of the wild type phage. The number of infective centres present in the mixture is determined by plating samples at the permissive temperature. The burst size is the ratio of the number of infective centres at the end of the incubation to the number of infective centres at the beginning of the experiment. Mutants are unable to grow at the non-permissive temperature, and so their (apparent) burst sizes are small (in fact, often less than 1). If two mutants, both lacking the same function, co-infect a cell, neither will be able to grow, and so this mixture will have a low burst size. These mutants, then, do not complement. Conversely, if two mutants lack different functions, they may be able to grow when they co-infect a cell, since each mutant supplies the function lacking in the other. This results in a burst size which is larger than that obtained with either mutant alone.

Therefore, the mutants complement. This, of course, is more complicated if the protein affected is composed of several identical polypeptides. In this case, the protein formed by combining polypeptides of each mutant type may be active, and the mutants may appear to complement. This is intracistronic complementation (11).

In phage crosses cells are co-infected with two mutants and are incubated at the permissive temperature which allows both mutants to replicate. During growth, recombination takes place and some wild type phages are produced. The proportion of wild type phage present in the final yield of the mixed infection is a measure of the genetic distance between the two mutations. Both crosses and complementation tests have been carried out with the PBS 2 ts mutants, and the results are presented here.

Preliminary experiments

The burst size and efficiency of plating of some mutants were determined at three temperatures (Table 3). All mutants had low burst sizes and impaired efficiency of plating at both the non-permissive temperature, 45 C, and an intermediate temperature, 37 C. The degree of impairment at 37 C varied among the mutants. Most mutants showed burst sizes lower than that of wild type PBS 2 at the permissive temperature, 30 C.

The growth of PBS 2 was followed at 30 C and 45 C to determine the period of incubation required for crosses and

Table 3

Burst size and efficiency of plating of ts mutants.

Mutant	Burst size			Efficiency of plating *	
	30 C	37 C	45 C	37 C	45 C
Wild type	50	100	9.2	1.30	0.61
TAP11	70	14	0.05	0.81	3.2×10^{-6}
TFU03	17	8.3	0.02	0.13	1.4×10^{-4}
TFU11	24	11	0.30	0.14	$<3.4 \times 10^{-6}$
TFU62	27	20	0.31	0.60	2.7×10^{-6}
TFU91	38	23	0.30	0.65	$<5.7 \times 10^{-7}$
TFU172	42	7.9	0.65	0.44	2.1×10^{-6}
TFU32	14	8.5	1.7	0.53	7.0×10^{-7}
TFU01	32	21	1.5	0.51	3.9×10^{-6}
TFU31	22	14	0.19	0.59	9.3×10^{-6}

* Effiency of plating is the ratio of PFU at the given temperature to that at 30 C.

complementation tests. The growth curves are shown in Fig. 4. In this experiment, the burst size at 30 C was 38, and at 45 C was 31. The burst size at 45 C was larger than generally observed, since in a large number of determinations it ranged from 2.5 to 32 with an average of 9.2. The latent period was 26 min at 45 C and 78 min at 30 C.

Complementation

For complementation tests the burst size was determined for the wild type, each mutant alone, and various pairs of mutants. Infected cells were incubated at 45 C for 45 min. By this time, cells infected with wild type had lysed. It was not determined whether cells infected with mutants lysed under these conditions.

The number of possible pairwise combinations of the mutants is quite large. The number of complementation tests required was reduced in two ways. First, pairs of mutants which appeared to be relatively loosely linked from recombination data were not tested. Secondly, when a pair of mutants obviously failed to complement (i.e. gave a burst size less than or equal to that of the individual mutants) one of the pair was excluded from further tests.

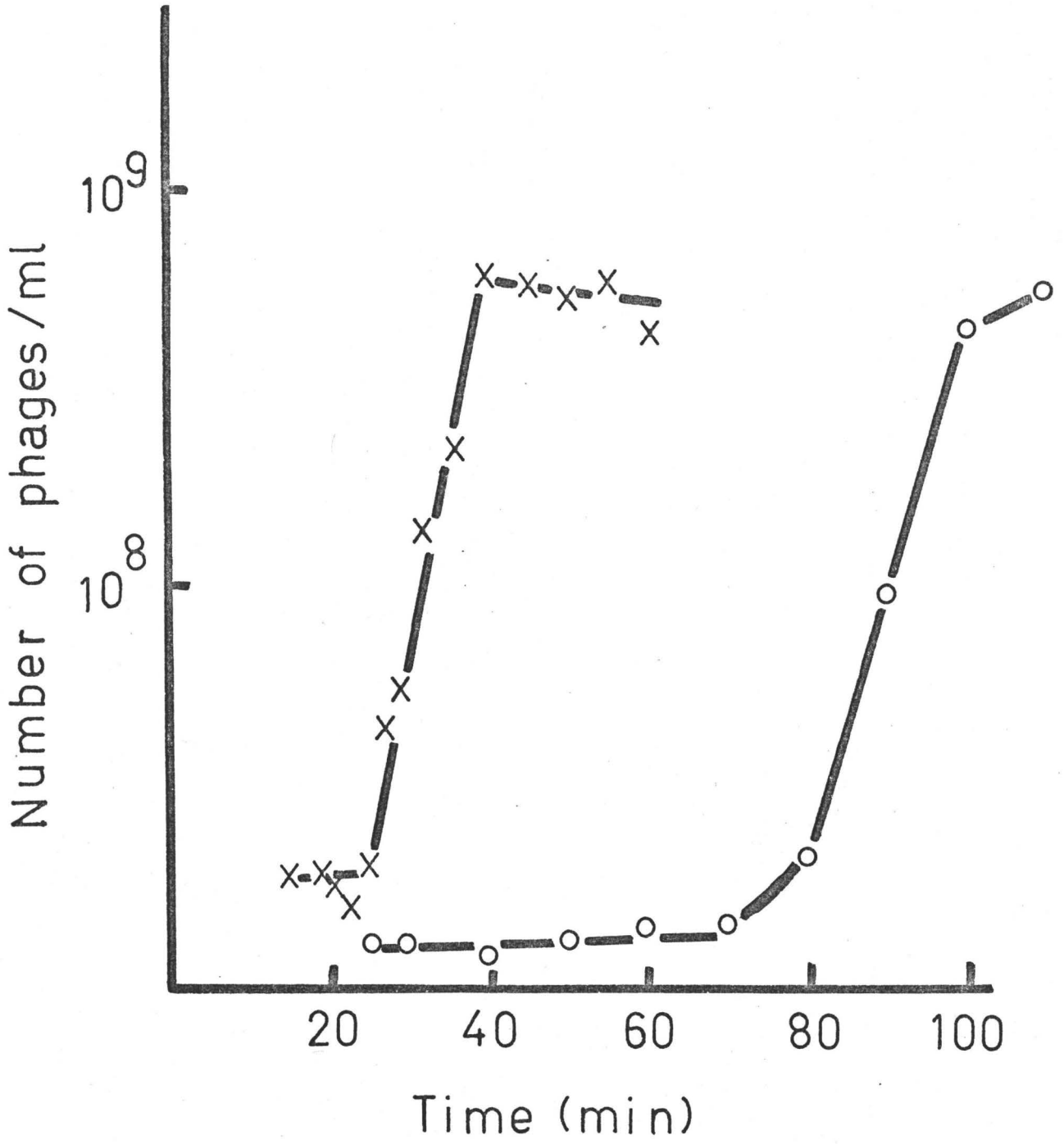
The burst sizes of the mutants at 45 C ranged from 0.01 to 1.8. The burst sizes of the mixed infections varied from 0.05 to 11. Approximately one-third of the possible combinations of mutants were tested. After consideration of the available data, it was arbitrarily decided that if the burst size of the mixed infection was more than 1.5 times the larger burst size of the 2 mutants alone, the pair was said to complement.

Figure 4: Growth of PBS 2 at 30 C and 45 C.

Strain SB19E grown as for crosses (Chapter II) was infected with PBS 2 at a multiplicity of infection of 10. The phage was allowed to adsorb 20 min at 30°C or 10 min at 45°C. Antiserum was added to inactivate free phage. After incubating for 5 min with antiserum, the infected cells were diluted in prewarmed PA and were incubated. Samples were taken at different times to determine the number of infective centres.

O———O growth at 30 C

X———X growth at 45 C


Number of phages/ml
10^9
10^8
20
40
60
80
100
Time (min)

This arbitrary criterion of complementation was used to assign 47 mutants to 10 groups. A mutant was assigned to a cistron if it did not complement with a mutant already assigned to that cistron. Burst sizes of different mixed infections are given in Table 4. The mutants are listed in the order in which they appear on the genetic map, and tests within cistrons are listed separately.

Two mutants (TFU75 and TFU123) appeared to complement all the mutants with which they were tested and so were assigned to cistron 5 on the basis of mapping data. Complementation tests involving TAP01 were not used for assigning mutants to cistrons for two reasons. First, the burst size of the mutant alone was high (average 1.8) and varied considerably (range 0.89 - 4.0). In addition, it failed to show complementation in most tests suggesting that it might be dominant. For these reasons, TAP01 was assigned to cistron 7 from mapping data.

Several mutants, including TAP01, were tested for dominance. This was done by co-infecting cells with wild-type and the mutant to be tested. Burst sizes were determined in the same fashion as for complementation tests. A dominant mutant would probably depress the burst size of the wild type phage in such a test. The mutant TFU33 affected the wild type burst size slightly and so may be dominant (Table 5). The other mutants tested were not dominant (Table 5).

Table 4

Complementation tests

Burst sizes were determined as described in Chapter II. Burst sizes of individual mutants and of mixtures are given. Those of individual mutants are averages of 2 or more determinations. Superscripts on the burst sizes of the mixtures indicate if the test was done more than once. The ratio is the burst size of the mixture divided by the largest of the mutant burst sizes. A ratio greater than 1.5 indicates complementation. The mutants are arranged in the order they are found in the map and particular pairs of mutants are given only once.

A. Tests between cistron 1 and other cistrons.

B. Tests between cistron 2 mutants.

C. Tests between cistron 2 and other mutants.

D. Tests between cistron 3 and other mutants.

E. Tests between cistron 4 and other mutants.

F. Tests between cistron 5 mutants.

G. Tests between cistron 5 mutants and other mutants.

H. Tests between cistron 6 mutants and other mutants.

I. Tests between cistron 7 mutants.

J. Tests between cistron 7 mutants and other mutants.

K. Tests between cistron 8, 9, and 10 mutants.

L. Tests between cistron 10 mutants.

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
A.					
THA31	TNG04	0.04	0.08	13	163
	THA01	0.04	0.04	6.2	155
	TFU172	0.04	0.65	11	17
	TFU101	0.04	0.04	11	275
	TFU41	0.04	0.08	2.7	34
	TFU22	0.04	0.03	3.9	98
	TFU01	0.04	1.5	3.7	2.5
	TNG01	0.04	0.95	5.6	5.9
	TFU104	0.04	0.15	3.3	22
	TFU06	0.04	1.1	4.8	4.4
	TFU62	0.04	0.31	6.1	20
	TAP01	0.04	1.8	1.4	0.78
	TNG03	0.04	1.3	7.0	5.4
	TFU73	0.04	0.18	9.8	54
	TFU103	0.04	0.20	8.9	45
	TFU91	0.04	0.30	5.8	19
	TFU11	0.04	0.30	3.4	11
	TAP02	0.04	0.07	12	171
B.					
TNG04	TFU61	0.08	0.11	0.14	1.3
TFU122	TFU61	0.47	0.11	0.21	0.44
C.					
TNG04	THA01	0.08	0.04	12	150
	TFU172	0.08	0.65	11	17
	TFU101	0.08	0.04	6.1	76
	TFU41	0.08	0.08	6.1	76
	TFU01	0.08	1.5	5.7	3.8
	TFU104	0.08	0.15	5.9	39
	TFU06	0.08	1.1	7.9	7.2
	TFU62	0.08	0.31	10	32
	TAP01	0.08	1.8	1.1	0.61
	TFU73	0.08	0.18	11	61
	TFU103	0.08	0.20	5.3	27
	TFU91	0.08	0.30	5.2	17
	TFU11	0.08	0.30	2.5	8.3
	TAP02	0.08	0.07	12	150
TFU122	TFU22	0.47	0.03	6.2	13
TFU61	THA01	0.11	0.04	3.5	32
	TFU41	0.11	0.08	3.3	30
	TFU01	0.11	1.5	3.9	2.6
	TFU12	0.11	0.07	5.3	48
	TFU02	0.11	0.06	4.2	38

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
TFU61	TFU03	0.11	0.02	4.3	39
	TFU103	0.11	0.20	5.8	29
	TAP13	0.11	0.54	3.9	7.2
D.					
THA01	TFU172	0.04	0.65	7.8	12
	TFU101	0.04	0.04	7.6	190
	TFU22	0.04	0.03	0.65	16
	TFU01	0.04	1.5	4.4	2.9
	TAP11	0.04	0.05	4.5	90
	TFU104	0.04	0.15	9.9	66
	TFU06	0.04	1.1	5.2	4.7
	TFU62	0.04	0.31	7.8	25
	TAP01	0104	1.8	3.2	1.8
	TAP12	0.04	0.54	8.9	16
	TFU73	0.04	0.18	6.8	38
	TFU03	0.04	0.02	0.27	6.8
	TFU103	0.04	0.20	9.6	48
	TFU91	0.04	0.30	8.7	29
	TFU11	0.04	0.30	3.4	11
	TAP02	0.04	0.07	8.8	125
	TAP13	0.04	0.54	3.5	6.5
E.					
TFU172	TFU101	0.65	0.04	1.7	2.6
	TFU01	0.65	1.5	3.8	2.5
	TFU010	0.65	0.14	6.6	10
	TFU06	0.65	1.1	3.5	3.2
	TFU62	0.65	0.31	15	23
	TAP01	0.65	1.8	4.4	2.4
	TFU171	0.65	0.27	3.2	4.9
	TFU73	0.65	0.18	11	17
	TFU11	0.65	0.30	3.4	5.2
	TAP02	0.65	0.07	7.7	12
F.					
TFU101	TFU105	0.04	0.45	0.37	0.82
	TFU111	0.04	1.0	2.8	2.8
	TFU01	0.04	1.5	3.4	2.3
	TNG01	0.04	0.95	2.6	2.7
	TFU75	0.04	0.71	2.8	3.9
	TFU123	0.04	0.33	1.3	3.9
	TFU04	0.04	0.51	3.2	6.2
	TFU010	0.04	0.14	2.7[3]	19
	TFU74	0.04	0.92	4.1	4.4
	TFU174	0.04	0.16	2.0	13
	TFU121	0.04	0.02	1.8	45

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
TFU101	TFU104	0.04	0.15	4.7	31
	TFU106	0.04	0.65	5.2	8.1
	TFU06	0.04	1.1	5.1	4.6
TFU41	TFU22	0.08	0.03	0.12	1.5
	TFU01	0.08	1.5	1.4	0.93
TFU105	TFU111	0.45	1.0	0.97	0.97
	TNG01	0.45	0.95	1.1[2]	1.2
	TFU75	0.45	0.71	2.4	3.4
	TFU123	0.45	0.33	0.82	1.8
	TFU04	0.45	0.51	0.50	0.98
	TFU010	0.45	0.14	0.98	2.2
	TFU74	0.45	0.92	1.0	1.1
	TFU71	0.45	0.23	0.71	1.6
	TFU174	0.45	0.16	0.38	0.84
	TFU106	0.45	0.64	0.90	1.4
TFU111	TNG01	1.0	0.95	4.9	4.9
	TFU75	1.0	0.71	11	11
	TFU123	1.0	0.33	3.6	2.4
	TFU04	1.0	0.51	1.0	1.0
	TFU010	1.0	0.14	1.2	1.2
	TFU74	1.0	0.92	5.0	5.0
	TFU71	1.0	0.23	2.7	2.7
	TFU174	1.0	0.16	1.8	1.8
	TFU106	1.0	0.64	2.2	2.2
TFU22	TFU01	0.03	1.5	3.7	2.5
	TFU12	0.03	0.07	2.1	30
	TFU02	0.03	0.06	1.3	22
	TFU104	0.03	0.15	2.3	15
	TFU06	0.03	1.1	2.1	1.9
TFU01	TFU12	1.5	0.07	2.1	1.4
	TFU02	1.5	0.06	1.3	0.87
	TFU104	1.5	0.15	2.3	1.5
	TFU06	1.5	1.1	2.1	1.4
TNG01	TFU75	0.95	0.71	3.0	3.2
	TFU123	0.95	0.33	2.4	2.5
	TFU04	0.95	0.51	4.3[2]	4.5
	TFU010	0.95	0.14	2.6	2.7
	TFU74	0.95	0.92	7.2	7.6
	TFU71	0.95	0.23	0.10	0.11
	TFU174	0.95	0.16	0.04	0.04
	TFU106	0.95	0.64	0.56	0.59

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
TFU75	TFU123	0.71	0.33	4.5	6.3
	TFU04	0.71	0.51	2.0	2.8
	TFU010	0.71	0.14	2.7	3.8
	TFU74	0.71	0.92	7.4	8.0
	TFU71	0.71	0.23	6.2	8.7
	TFU174	0.71	0.16	3.8	5.3
	TFU106	0.71	0.64	5.8[2]	8.2
TFU123	TFU04	0.33	0.51	0.89	1.7
	TFU010	0.33	0.14	1.5	4.5
	TFU74	0.33	0.92	2.6	2.8
	TFU71	0.33	0.23	3.8	12
	TFU174	0.33	0.16	2.5	7.6
	TFU106	0.33	0.64	1.4	2.2
TFU04	TFU74	0.51	0.92	4.4	4.8
	TFU71	0.51	0.23	2.7	5.3
	TFU106	0.51	0.64	6.5	10
TFU010	TFU74	0.14	0.92	6.8	7.4
	TFU71	0.14	0.23	3.8	17
	TFU174	0.14	0.16	2.1	13
	TFU121	0.14	0.02	1.8	13
	TAP11	0.14	0.05	3.9	28
	TFU12	0.14	0.07	2.7	19
	TFU106	0.14	0.64	0.83	1.3
	TFU06	0.14	1.1	5.2	4.7
TFU74	TFU174	0.92	0.16	3.3	3.6
	TFU121	0.92	0.02	4.6	5.0
	TAP11	0.92	0.05	8.5	9.2
	TFU12	0.92	0.07	3.9	4.2
	TFU106	0.92	0.64	4.1	4.4
TFU71	TFU174	0.23	0.16	1.3	5.7
	TFU121	0.23	0.02	0.06[2]	0.26
	TAP11	0.23	0.05	0.07	0.30
	TFU12	0.23	0.07	0.13	0.57
	TFU81	0.23	0.09	6.1	27
	TFU21	0.23	0.08	4.9	21
	TFU02	0.23	0.06	6.0	26
	TFU106	0.23	0.64	3.3	5.1
TFU174	TFU121	0.16	0.02	0.08	0.50
	TAP11	0.16	0.05	0.08	0.50
	TFU12	0.16	0.07	0.08	0.50
	TFU106	0.16	0.64	3.5	5.5

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
TFU121	TAP11	0.02	0.05	0.07	1.4
	TFU12	0.02	0.07	0.12	1.7
	TFU81	0.02	0.09	4.6	51
	TFU21	0.02	0.08	1.6	20
	TFU02	0.02	0.06	2.0	33
TAP11	TFU12	0.05	0.07	0.04	0.57
	TFU81	0.05	0.09	3.2	36
	TFU21	0.05	0.08	2.9	36
	TFU02	0.05	0.06	2.6	43
TFU12	TFU05	0.07	1.1	0.37	0.34
	TFU81	0.07	0.09	4.9	54
	TFU21	0.07	0.08	2.1	26
	TFU02	0.07	0.06	3.0	43
TFU05	TFU02	1.1	0.06	1.6[2]	1.5
TFU81	TFU21	0.09	0.08	0.11	1.2
	TFU02	0.09	0.06	0.10	1.1
	TFU06	0.09	1.1	5.3	4.8
TFU21	TFU02	0.08	0.06	0.09	1.1
	TFU06	0.08	1.1	3.3	3.0
TFU02	TFU06	0.06	1.1	1.9	1.7
TFU104	TFU06	0.15	1.1	3.2[3]	2.9
G.					
TFU101	TFU62	0.04	0.31	7.3	24
	TAP01	0.04	1.8	2.4	1.3
	TFU171	0.04	0.27	1.3[2]	4.8
	TFU73	0.04	0.18	5.9	33
	TFU103	0.04	0.20	6.1	31
	TFU91	0.04	0.30	5.4	18
	TFU11	0.04	0.30	2.7	9.0
	TAP02	0.04	0.07	7.6	110
TFU41	TAP13	0.08	1.4	8.0	5.7
FU01	TFU62	1.5	0.31	4.4	2.9
	TAP01	1.5	1.8	0.75	0.42
	TFU73	1.5	0.18	4.3	2.9
	TFU103	1.5	0.20	4.3	2.9

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
FU01	TFU91	1.5	0.30	3.1	2.1
	TFU11	1.5	0.30	2.9	1.9
	TAP02	1.5	0.07	6.6	4.4
TNG01	TNG03	0.95	1.3	4.0	3.1
TFU010	TFU62	0.14	0.31	7.9	25
TFU174	TFU03	0.16	0.02	16	100
	TAP02	0.16	0.07	12	75
TFU12	TFU103	0.07	0.20	3.3	17
TFU02	TFU103	0.06	0.20	3.3	17
TFU104	TFU62	0.15	0.31	7.3	24
	TAP01	0.15	1.8	1.1	0.61
	TFU73	0.15	0.18	3.8	21
	TFU103	0.15	0.20	4.9	25
	TFU91	0.15	0.30	3.7	12
	TFU11	0.15	0.30	0.97	3.2
	TAP02	0.15	0.07	8.0	53
TFU06	TFU62	1.1	0.31	6.8	6.2
	TAP01	1.1	1.8	2.3	1.3
	TFU73	1.1	0.18	5.2	4.7
	TFU103	1.1	0.20	5.2	4.7
	TFU91	1.1	0.30	4.6	4.2
	TFU11	1.1	0.30	3.6	3.3
	TAP02	1.1	0.07	5.7	5.2
H.					
TFU62	TFU73	0.31	0.18	6.4	21
	TFU103	0.31	0.20	11	35
	TFU91	0.31	0.30	11	35
	TFU11	0.31	0.30	2.5	8.1
I.					
TFU34	TFU32	1.3	1.7	2.6	1.5
	TAP01	1.3	1.8	1.8[2]	1.0
	TNG03	1.3	1.3	4.0	3.1
	TFU31	1.3	0.19	8.2	6.3
	TFU72	1.3	0.24	1.8	1.4
	TFU171	1.3	0.27	5.9[2]	4.5
	TFU33	1.3	0.09	0.90	0.69

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
TFU32	TAP01	1.7	1.8	1.6	0.89
	TNG03	1.7	1.3	5.0[2]	2.9
	TFU31	1.7	0.19	5.0[2]	2.9
	TFU72	1.7	0.24	1.7	1.0
	TFU171	1.7	0.27	4.5	2.6
	TFU33	1.7	0.09	1.0	0.59
	TFU103	1.7	0.20	11[2]	6.5
TAP01	TNG03	1.8	1.3	0.80	0.44
	TFU31	1.8	0.19	1.3[2]	0.72
	TFU72	1.8	0.24	0.65	0.36
	TFU171	1.8	0.27	0.21	0.12
	TFU73	1.8	0.18	0.67	0.37
	TFU102	1.8	0.02	0.03	0.02
	TFU33	1.8	0.09	0.04	0.02
	TFU103	1.8	0.20	0.62	0.34
TNG03	TFU31	1.3	0.19	1.2	0.92
	TFU72	1.3	0.24	0.77	0.59
	TFU171	1.3	0.27	1.1	0.85
	TAP03	1.3	0.01	1.2	0.92
TFU31	TFU72	0.19	0.24	2.0	8.3
	TFU171	0.19	0.27	3.8	14
TFU72	TFU171	0.24	0.27	0.32	1.2
	TFU33	0.24	0.09	0.42	1.8
TFU171	TAP03	0.27	0.01	1.2	4.4
TAP12	TFU73	0.54	0.18	0.20	0.37
	TFU03	0.54	0.02	0.54	1.0
	TFU173	0.54	0.05	0.35[2]	0.65
TFU73	TFU03	0.18	0.02	18	100
	TFU173	0.18	0.05	0.97	5.4
	TFU103	0.18	0.20	0.08	0.40
TFU03	TFU173	0.02	0.05	5.8	120
TFU173	TAP03	0.05	0.01	0.02	0.40
	TFU102	0.05	0.02	0.04	0.80
	TFU33	0.05	0.09	0.17	1.9
TAP03	TFU102	0.01	0.02	0.03	1.5
	TFU33	0.01	0.09	0.09	1.0

Mutant (1)	Mutant (2)	Burst size of (1)	Burst size of (2)	Burst size from mixed infection	Ratio
TFU102	TFU33	0.02	0.09	0.02	0.22
TFU33	TFU103	0.09	0.20	1.1	5.5
J.					
TFU32	TFU91	1.7	0.30	4.2	2.5
	TFU11	1.7	0.30	3.3	1.9
TAP01	TFU91	1.8	0.30	5.1	2.8
	TFU11	1.8	0.30	0.74	0.41
	TAP02	1.8	0.07	0.34	0.21
TAP12	TAP02	0.54	0.07	1.0	1.9
TFU73	TFU91	0.18	0.30	5.1	2.8
	TFU11	0.18	0.30	0.74	0.41
	TAP02	0.18	0.07	0.37	0.21
TFU03	TAP02	0.02	0.07	3.7	53
	TAP13	0.02	1.4	9.2	6.6
TFU173	TAP02	0.05	0.07	1.9	27
TFU33	TFU91	0.09	0.30	1.8	6.0
	TFU11	0.09	0.30	0.80	2.7
TFU103	TFU91	0.20	0.30	1.8	6.0
	TFU11	0.20	0.30	8.2[2]	27
	TAP02	0.20	0.07	6.5	33
K.					
TFU91	TFU11	0.30	0.30	7.3	24
	TAP02	0.30	0.07	7.1	24
TFU11	TAP02	0.30	0.07	7.0	23
L.					
TAP02	TAP13	0.07	1.4	1.5	1.1

Table 5

Burst sizes in mixed infections at 45 C with wild type and mutants of PBS 2.

Mutant	Burst size of mutant alone	Burst size of mixture (mutant + wild type)
TFU02	0.06	16
TFU12	0.07	4.4
TFU33	0.09	1.6
TFU105	0.45	5.5
TAP01	1.8	15
TFU01	1.5	8.7
TNG04	0.08	12
THA01	0.04	14

Burst sizes were determined as described in Chapter II. Burst sizes of individual mutants are averages of two or more determinations. The burst size of wild type varied from 2.5 to 32 with an average from 47 determinations of 9.2.

Two-factor crosses

Recombination percentages in two-factor crosses were measured with the PBS 2 ts mutants. The values of the recombination percentage obtained for a particular cross varied considerably in different experiments. The ratio of the standard deviation to the mean of the recombination percentage varied from 0.19 to 0.64 (Table 6). As a comparison, when host range and rII mutants of T4 were crossed, this ratio ranged from 0.12 to 0.25 (20). In retrospect, part of the variation with PBS 2 may have been caused by daily variation in the plating efficiency at 45 C.

The ts mutants are ordered according to the recombination percentages obtained from two-factor crosses. The map thus obtained appears linear. Figure 6 gives the data and order obtained. Some recombination percentages between distant markers have been omitted for reason of clarity. These were generally consistent with the order given. In ordering mutants, smaller recombination percentages were given more weight than larger percentages, and mutants in the same cistron were kept together.

The frequency of recombinants varied from 0.01 to 23% and the total map length was approximately 50%. Both cistron 5 (18%) and 7 (21%) were very long. Additivity in the crosses was poor. According to Edgar and Lielausis (22) variation in the size of the elemental intervals, and poor additivity combine to introduce uncertainty in the ordering of T4 mutants. The map of PBS 2 shares these features with T4, and this, combined with the variability in the PBS 2 crosses introduces a fairly high degree of uncertainty in the ordering of the mutants.

Figure 5: Correlation between burst size in complementation tests and recombination percentages.

The percent recombination between pairs of mutants in either cistron 5 or cistron 7 is plotted against the burst size in complementation tests of the mutants.

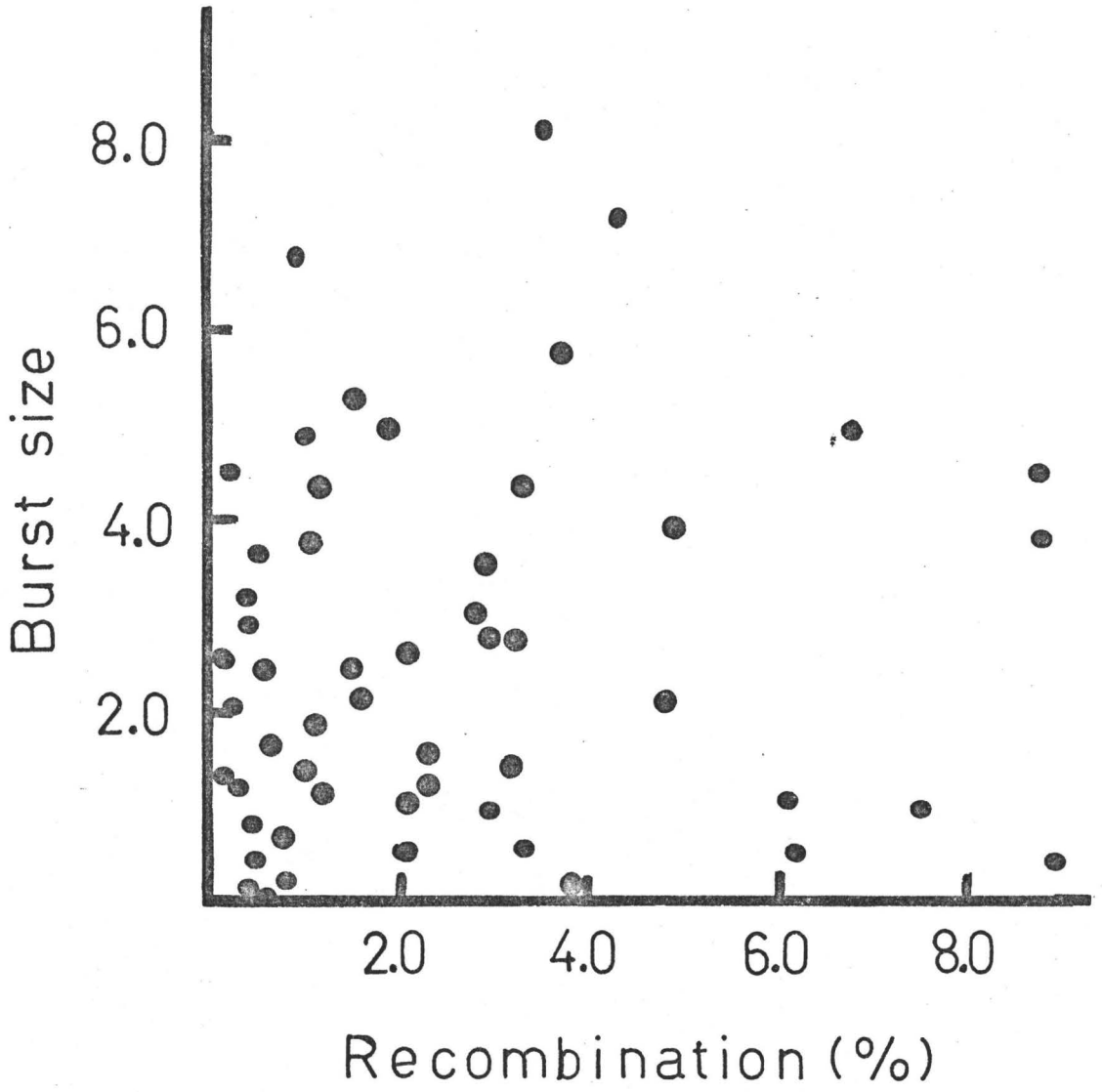

8.0
6.0
4.0
2.0
Burst size
2.0
4.0
6.0
8.0
Recombination (%)

Table 6

Variation in crosses

Cross	Number of experiments	Mean % recombination	Standard deviation	Ratio *
TFU12 X TFU01	10	4.8	1.9	0.40
TFU12 X TAP02	4	10	2.8	0.28
TFU12 X TFU73	3	11	6.9	0.64
TFU12 X TAP01	3	9.1	3.3	0.36
TFU12 X TFU33	3	13	4.7	0.36
TFU12 X TFU81	3	5.7	1.2	0.21
TFU12 X TFU23	3	5.2	2.1	0.40
TFU12 X TFU131	3	7.9	2.7	0.34
TFU12 X TFU34	3	11	2.1	0.19
TFU12 X TFU75	3	3,8	1.3	0.50

* The ratio of the standard deviation to the mean % recombination.

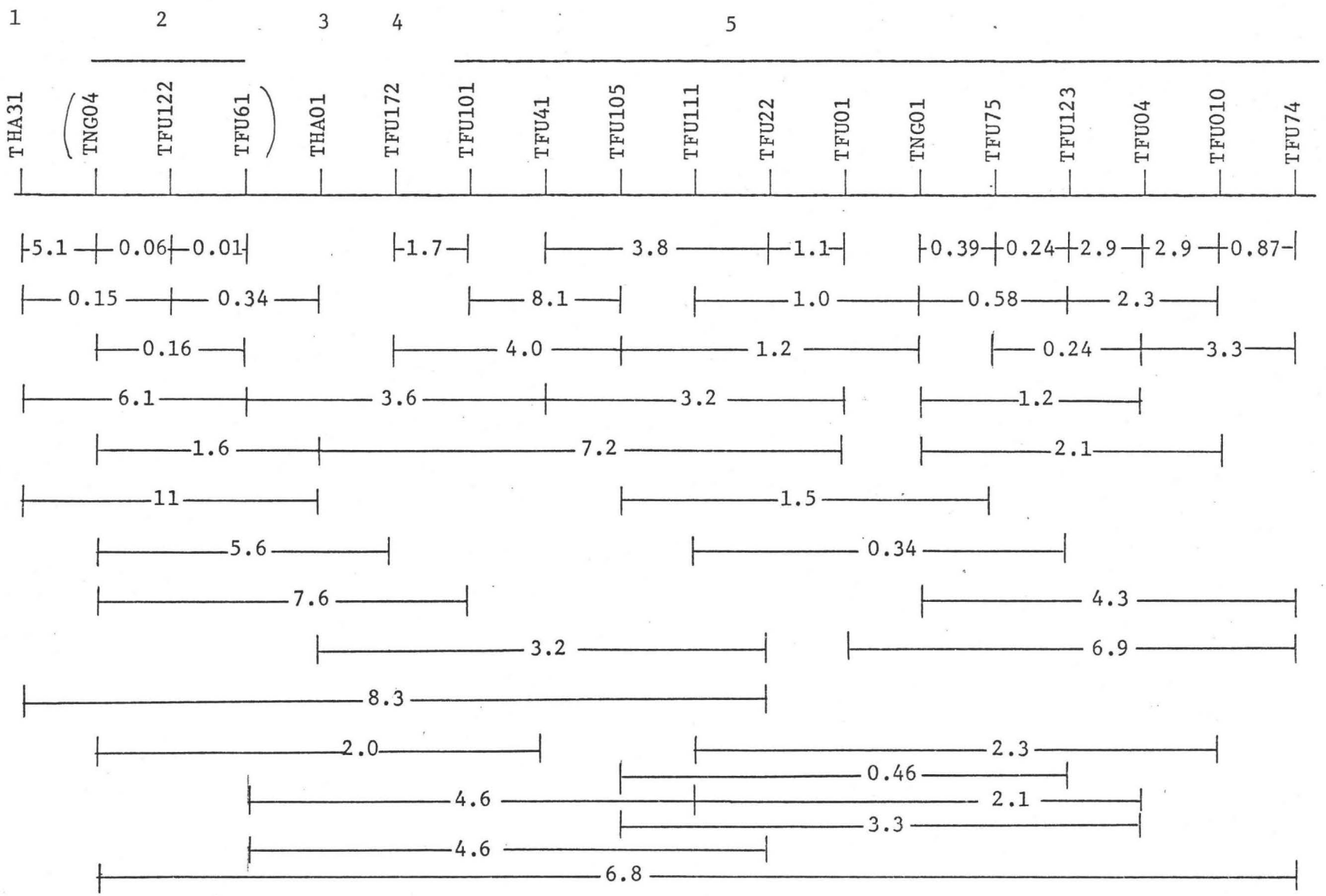
Figure 6: Map of PBS 2 ts mutants. Recombination percentages are given. Mutants which are in parentheses could not be ordered with respect to the rest of the map. Horizontal bars above the mutants indicate mutants in the same cistron. Cistrons are identified by numbers above the bars or above individual mutants.

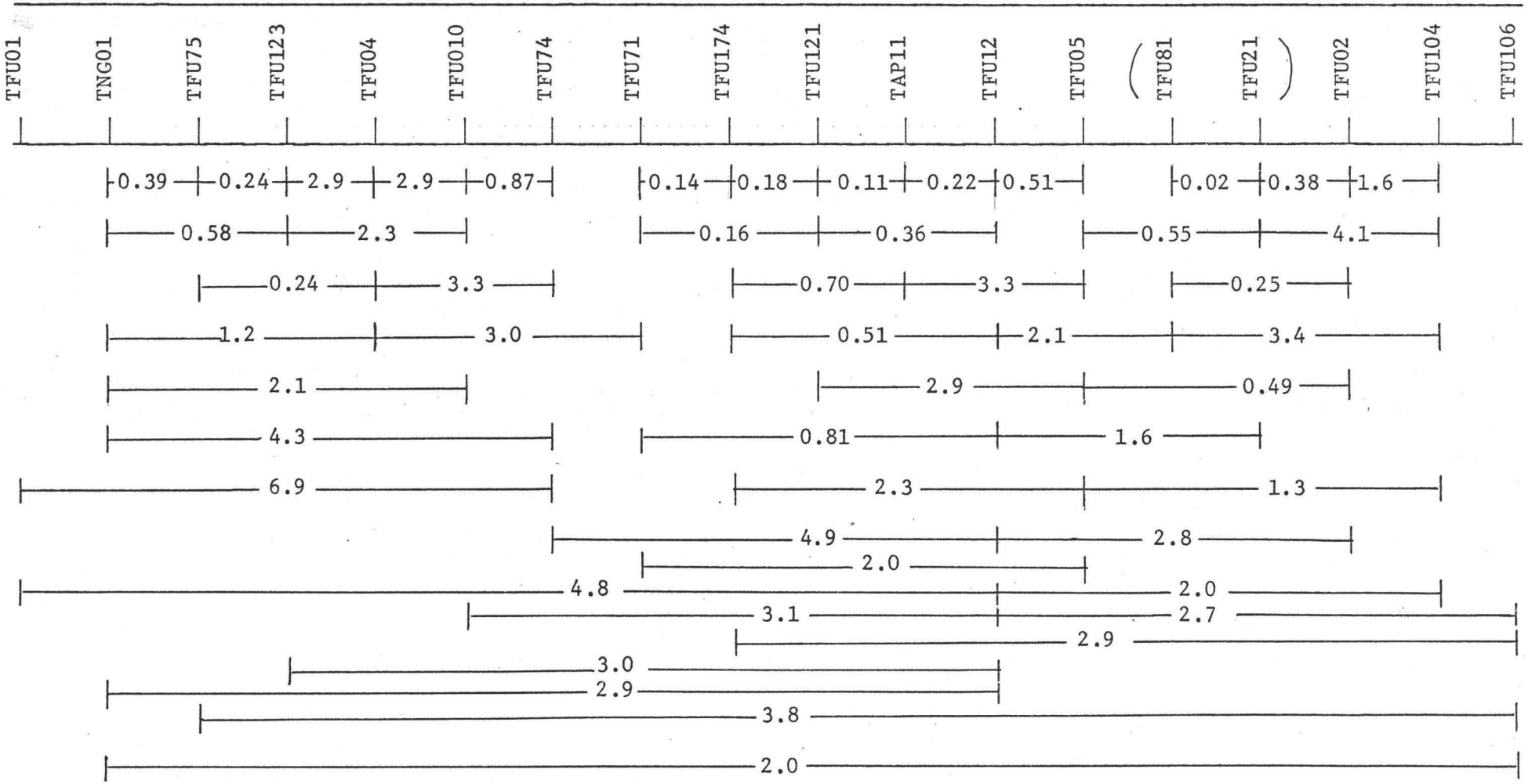
A. Map of segment THA31--TFU01--TFU74

B. Map of segment TFU01--TFU74--TFU106

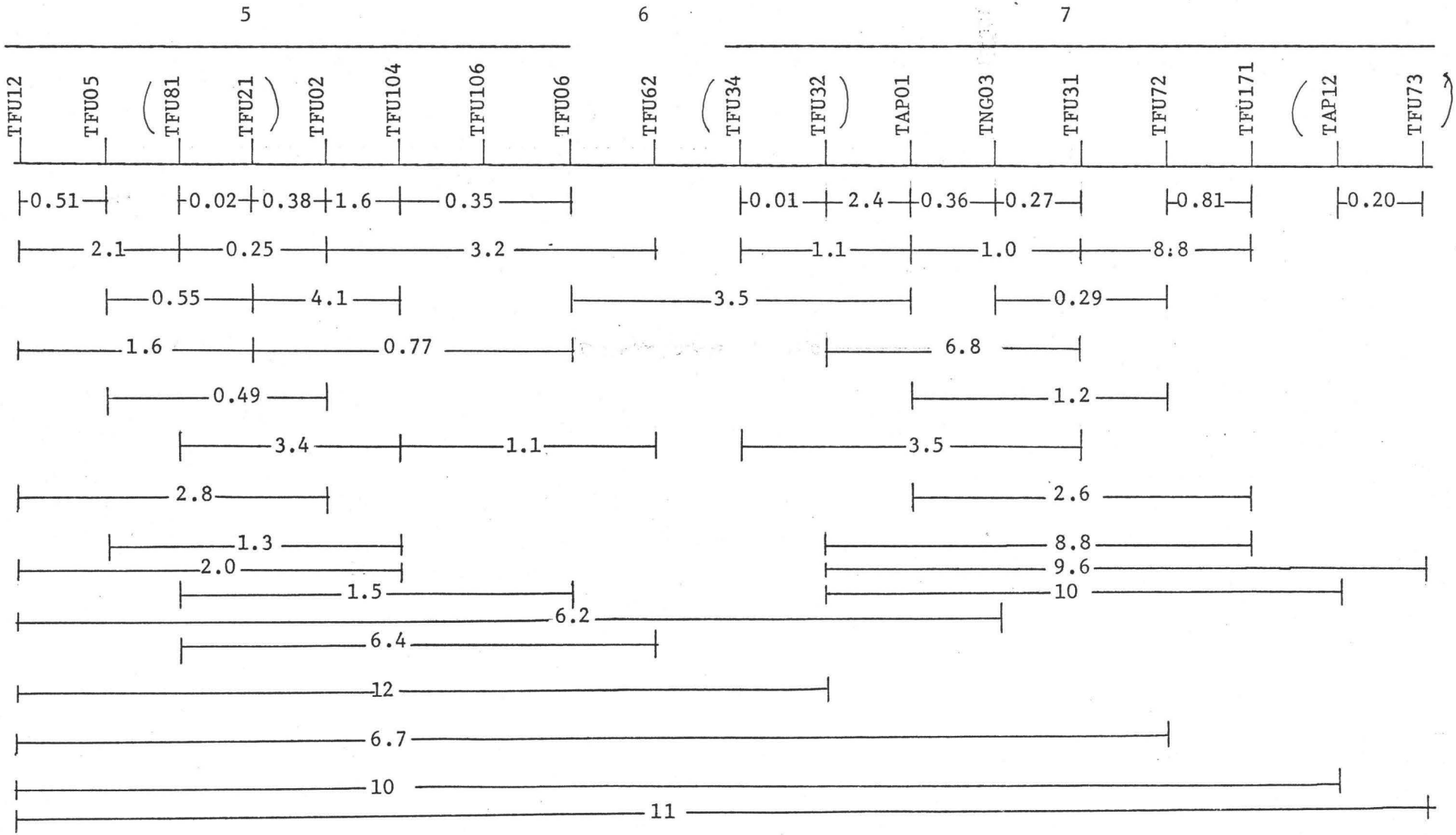
C. Map of segment TFU12--TFU106--TFU73

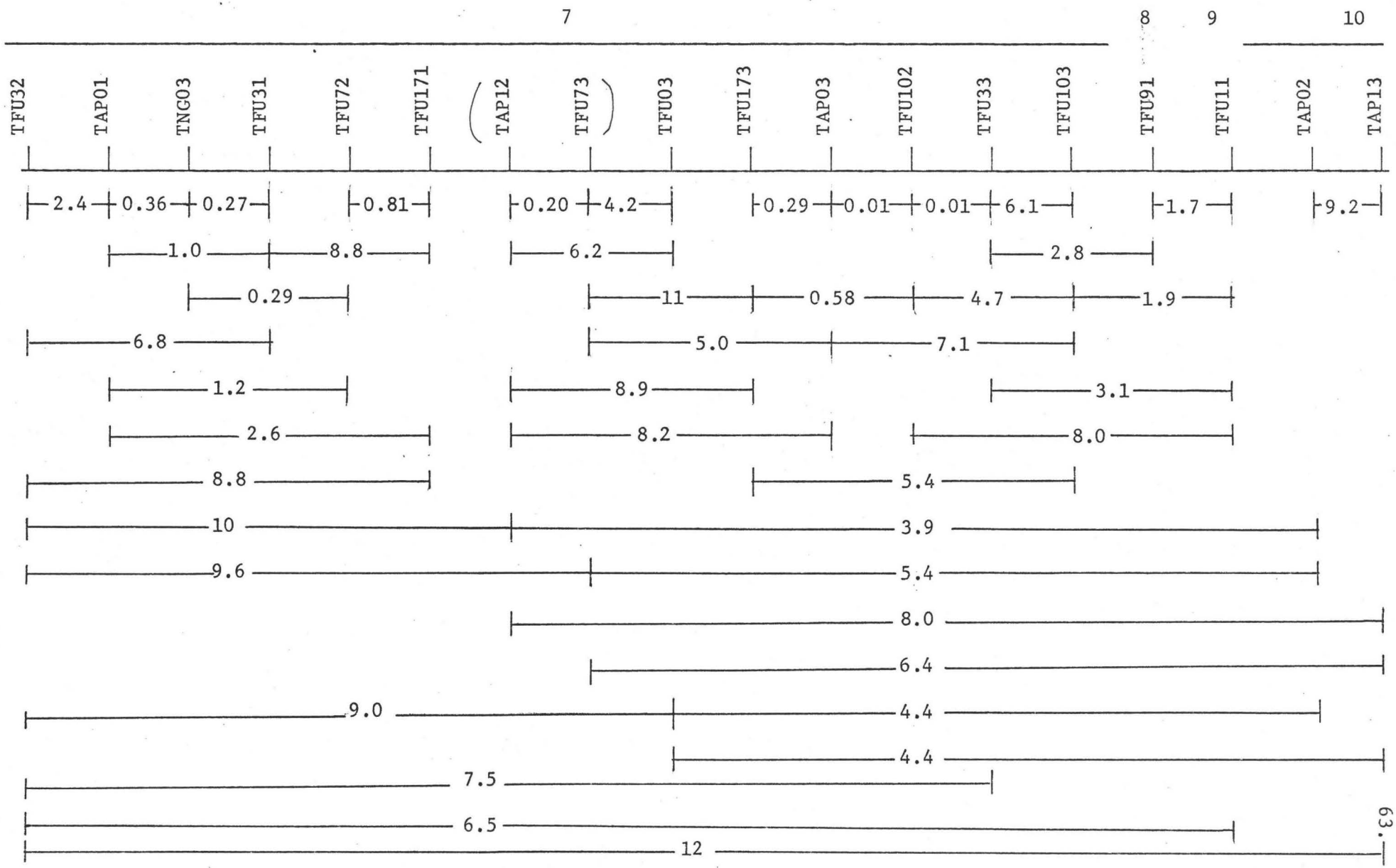
D. Map of segment TFU32--TFU73--TAP13


1
2
3
4
5
THA31
TNG04
TFU122
TFU61
THA01
TFU172
TFU101
TFU41
TFU105
TFU111
TFU22
TFU01
TNG01
TFU75
TFU123
TFU04
TFU010
TFU74
5.1
0.06
0.01
1.7
3.8
1.1
0.39
0.24
2.9
2.9
0.87
0.15
0.34
8.1
1.0
0.58
2.3
0.16
4.0
1.2
0.24
3.3
6.1
3.6
3.2
1.2
1.6
7.2
2.1
11
1.5
5.6
0.34
7.6
4.3
3.2
6.9
8.3
2.0
2.3
0.46
4.6
2.1
3.3
4.6
6.8


TFU01
TNG01
TFU75
TFU123
TFU04
TFU010
TFU74
TFU71
TFU174
TFU121
TAP11
TFU12
TFU05
(TFU81
TFU21)
TFU02
TFU104
TFU106
0.39
0.24
2.9
2.9
0.87
0.14
0.18
0.11
0.22
0.51
0.02
0.38
1.6
0.58
2.3
0.16
0.36
0.55
4.1
0.24
3.3
0.70
3.3
0.25
1.2
3.0
0.51
2.1
3.4
2.1
2.9
0.49
4.3
0.81
1.6
6.9
2.3
1.3
4.9
2.8
2.0
4.8
2.0
3.1
2.7
2.9
3.0
2.9
3.8
2.0

B.
61.


5
6
7
TFU12
TFU05
TFU81
TFU21
TFU02
TFU104
TFU106
TFU06
TFU62
TFU34
TFU32
TAP01
TNG03
TFU31
TFU72
TFU171
TAP12
TFU73
0.51
0.02
0.38
1.6
0.35
0.01
2.4
0.36
0.27
0.81
0.20
2.1
0.25
3.2
1.1
1.0
8.8
0.55
4.1
3.5
0.29
1.6
0.77
6.8
0.49
1.2
3.4
1.1
3.5
2.8
2.6
1.3
8.8
2.0
9.6
1.5
10
6.2
6.4
12
6.7
10
11


7
8
9
10
TFU32
TAP01
TNG03
TFU31
TFU72
TFU171
TAP12
TFU73
TFU03
TFU173
TAP03
TFU102
TFU33
TFU103
TFU91
TFU11
TAP02
TAP13
2.4
0.36
0.27
0.81
0.20
4.2
0.29
0.01
0.01
6.1
1.7
9.2
1.0
8.8
6.2
2.8
0.29
11
0.58
4.7
1.9
6.8
5.0
7.1
1.2
8.9
3.1
2.6
8.2
8.0
8.8
5.4
10
3.9
9.6
5.4
8.0
6.4
9.0
4.4
4.4
7.5
6.5
12

An estimate of the maximal recombination frequency in PBS 2 can be made by examining the recombination between distant markers. This frequency appears to be approximately 10%. Thus, mutants which show a frequency of recombination of 10% or more appear unlinked. They are shown to be linked by crosses to mutants between them or by being in the same cistron. For example, in Fig. 6C, TFU32 and TAP12 are not linked according to the recombination data available. However, they are in the same cistron and so must be linked.

CHAPTER V

PHAGE TRANSFORMATION AND PHYSICAL MAPPING

Under appropriate conditions bacterial cells can take up DNA from the medium. If cells, under these conditions, are infected with a phage mutant and exposed to DNA from wild type phage, some of the phage produced are wild type. This phage transformation (103) or marker rescue (34) provides a useful technique for determining the genetic activity of DNA.

Yamagishi (unpublished results) has shown that fragments of PBS 2 DNA can be fractionated in a Hg-Cs_2SO_4 density gradient according to their nucleotide composition. The G + C content of the fragments varied from 25 to 35%. If PBS 2 could be transformed, then an estimate of the nucleotide composition of different genes could be made.

Characterization of PBS 2 transformation

Competence, or the ability to take up DNA can be induced in B. subtilis by numerous methods (69, 86, 94). Preliminary experiments showed that PBS 2 transformation was as efficient when competence was induced by the method described by Takahashi (94) (Method I), as with other methods tested at that time. However, the efficiency of transformation with DNA sheared to a molecular weight of 1.5×10^6 was extremely low. Since DNA of this size could be fractionated in the Hg-Cs_2SO_4 density gradients (Yamagishi, unpublished results),

it was desirable to obtain a higher efficiency of transformation with it. Therefore, several other methods of inducing competency were tested. Cells made competent by Mahler's method (58) gave a high efficiency of transformation with both unsheared and sheared DNA's. This method was further modified as described in Chapter II (Method II) to give a higher efficiency of transformation. Bacterial transformation obtained with cells made competent by Method I was compared with that obtained by cells made competent by Method II. Cells of SB202 (aro-2, trp-2, his-2, tyr-1) were made competent by either Method I or Method II, then were transformed with DNA from a wild type strain at 0.02 μg/ml (within the proportional range). Cells made competent by Method I were co-transformed for the four markers at a frequency of 1.6×10^{-5}, while those made competent by Method II gave a frequency of 3.2×10^{-4}.

PBS 2 was transformed in the following way: To a competent culture of SB202, a ts mutant and DNA from wild type phage were added at the same time and the culture was incubated at 30 C, the permissive temperature, until lysis occurred. The lysate was then assayed for wild type phage by plating suitable dilutions at 45 C, the non-permissive temperature.

Figure 7 shows transformation of the mutant TAP02 carried out in cells made competent by Methods I and II. In both cases, the number of wild type transformants was linearly dependent on the DNA concentration up to concentrations of 1 μg/ml.

The following experiments were done to determine the optimal conditions for PBS 2 transformation. In the first experiment competent cells were mixed with DNA and TAP02 was added at different times (Fig. 8). Wild type transformants were assayed after the cells lysed. The amount of transformation decreased rapidly in the first 10 min, indicating that the DNA might be degraded in the absence of phage. A deoxyribonuclease which degrades specifically native PBS 2 DNA can be extracted from uninfected cells (101). This DNase may be responsible for the inactivation of PBS 2 DNA in vivo, thereby causing the reduction in the amount of transformation. In the second experiment, DNA was added to competent cells at different times after infection. Transformation could be observed for at least 45 min but the amount of transformation obtained decreased slowly (Table 7).

The following experiments show that the phage transformation is mediated by free DNA molecules. Low concentrations of pancreatic deoxyribonuclease I inactivated the transforming activity of DNA (Table 8). Treatment of the DNA with trypsin, pronase, and ribonuclease reduced its transforming activity slightly (Table 9). This may be due to an inhibitory effect of protein, since addition of bovine serum albumin (0.5 μg/ml) directly to the transformation mixtures also reduced the amount of transformation (Table 9). Alternatively, there may be contamination of these proteins by DNase.

Figure 7: Comparison of PBS 2 transformation with cells made competent by Methods I and II. Competent SB202 was infected with TAP02 at a multiplicity of infection of 1-2 and DNA was added at different concentrations. The number of wild type transformants were measured after 100 min incubation at 30 C.

X———X Transformants with competent cells prepared by Method I.

0———0 Transformants with competent cells prepared by Method II.

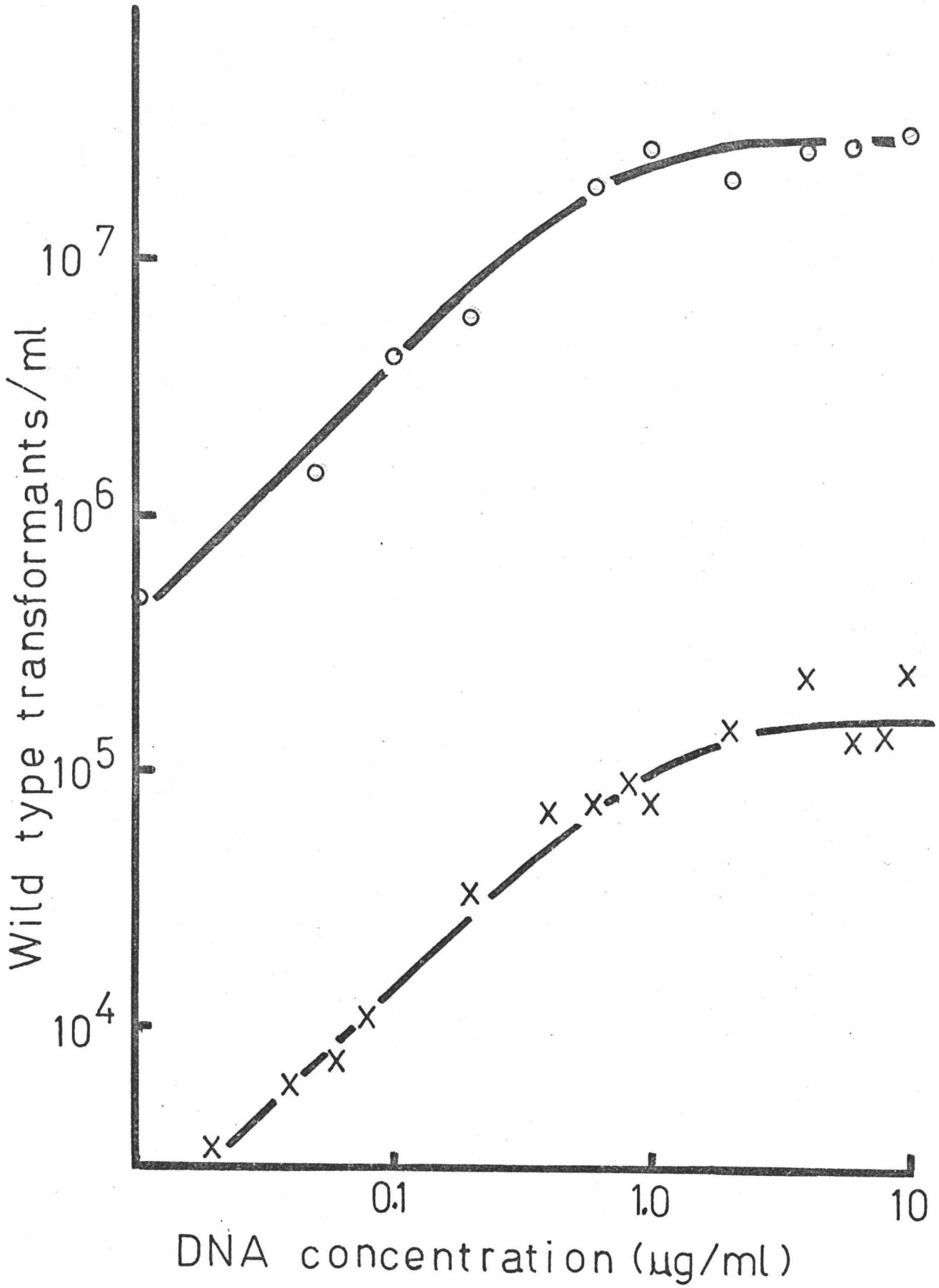

Wild type transformants/ml
10 7
10 6
10 5
10 4
0.1
1.0
10
DNA concentration (µg/ml)

Figure 8: Dependence of transformation on the time of infection. Competent cells (Method II) were treated with DNA (2 μg/ml). TAP02 was added at a multiplicity of infection of 1-2 at different times after the addition of DNA. Cultures were incubated at 30 C with shaking for 100 min from the time of DNA addition. The values are the average of two determinations.

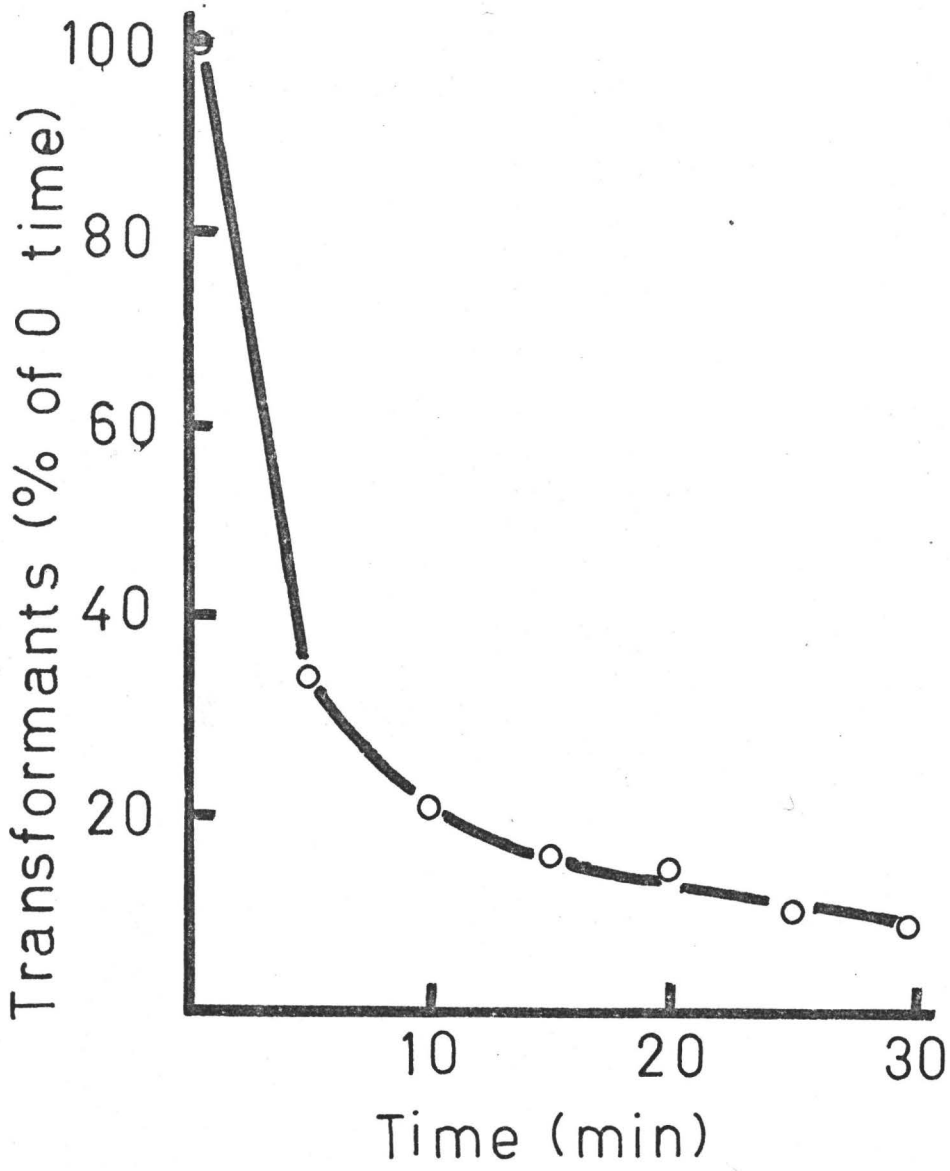

Transformants (% of 0 time)
100
80
60
40
20
10
20
30
Time (min)

Table 7

Dependence of transformation on time of DNA addition.

Time of DNA addition	Wild type transformants/ml	
	Experiment 1	Experiment 2
0	2.2×10^6	1.6×10^7
5	1.8×10^6	1.9×10^7
10	1.5×10^6	3.3×10^7
15	1.8×10^6	2.3×10^7
20	1.1×10^6	
25	1.1×10^6	
30		1.0×10^7
35	4.3×10^5	8.0×10^6
40	3.6×10^5	8.7×10^6
45	3.4×10^5	

Competent cells (Method II) were infected with TAP02 at a multiplicity of infection of 1-2, and were incubated at 30 C with shaking. At various times after infection 2 μg/ml of PBS 2 was added. The mixture was assayed for wild type transformants 100 min after infection.

Heat denaturation of the DNA reduced its transforming activity to 2% of that of native DNA. As shown in Table 10, mutant phages were transformed by DNA from wild type phage or by DNA from phages carrying different mutations but not by homologous DNA. The variation in the level of transformation obtained with various mutants (Table 10) may be due to different efficiencies of transformation, or small variations in the multiplicity of infection used as well as from the day to day variation which was observed when cells were made competent by Method I.

Further experiments were carried out to characterize the process of transformation. As shown in Table 11, the number of transformants obtained varied with the multiplicity of infection. Multiplicities of 0.5 - 1.0 gave the largest number of transformants. Therefore, transformation was carried out routinely at a multiplicity of about 1.

The time course of the transformation process was determined by assaying the number of infective centres present in a mixture for transformation at different times. Figure 9 shows the total number of infective centres (assayed at 30 C) and the number of wild type infective centres (assayed at 45 C) at different times after infection. The total infective centres followed a typical single-step growth curve with a latent period of 63 min, a rise period of 22 min, and a burst size of 7.7. The production of wild type infective centres was more complex. Wild type infective centres appeared rapidly during the first 15 min, then appeared at

Table 8

The effect of DNase on transforming DNA.

DNase concentration (μg/ml)	Percentage transformation
0	100
0.0001	100
0.001	78
0.01	1.4
0.1	1.2
1.0	0.7

DNA (100 μg/ml) in 0.02 M tris-HCl, 0.1 M NaCl, 0.01 M $MgSO_4$, pH 7.5, was treated with different concentrations of pancreatic DNase at 37 C for 30 min. Samples were diluted 10 fold in SSC and transformation of TAP02 was assayed. Competent (Method II) cells of SB202 were infected at a multiplicity of infection of 1 with TAP02 and 1.0 μg/ml of treated DNA was added.

Table 9

Treatment of transforming DNA with different proteins.

Protein	Transformation as a percentage of the untreated control
Trypsin	100
Pronase	75
RNase	91
Bovine serum albumin	78

Conditions of treatment:

Trypsin: DNA (50 µg/ml) was treated with trypsin (50 µg/ml) in 0.05 M tris pH 8.0 + 0.02 $MCaCl_2$ at 37 C for 60 min. This was diluted 10 fold in SSC at 0°C and was assayed for ability to transform.

Pronase: DNA (50 µg/ml) was treated with pronase (200 µg/ml) in 0.01 M potassium phosphate buffer pH 7.0 at 37 C for 45 min then was diluted 10 fold in SSC at 0°C and was assayed for transformation.

RNase: DNA (5 µg/ml) was treated with RNase (10 µg/ml) in 0.1 M acetate buffer pH 7.0. This was incubated at 37 C for 30 min and assayed for transformation.

Bovine serum albumin: Bovine serum albumin (0.5 µg/ml)was added directly to the transformation mixture.

Transformation: Competent SB202 (Method II) was infected with TAP02 at a multiplicity of 1 and DNA was added to give a final concentration of 0.5 µg/ml. DNA used for controls was incubated under the same conditions as the treated samples except that enzymes were not added.

Table 10

Transformation with DNA from different mutants

Phage	PBS 2	DNA TAP02	TFU01	TFU12	TFU06
TAP02	100	21	320	260	170
TFU01	100	410	0	1700	370
TFU12	100	170	240	0	190
TFU06	100	300	1000	440	0

Transformation is expressed as the percentage of transformation of the mutant by PBS 2 DNA. Cells (SB202) were made competent by Method I. The multiplicity of infection was 1, and the DNA concentration was 0.1 μg/ml.

Table 11

Effect of the multiplicity of infection on PBS 2 transformation.

Multiplicity of infection	Wild type transformants/ml
2	3.5×10^4
1	1.6×10^5
0.5	1.4×10^5
0.3	8.4×10^4
0.02	4.0×10^4

Competence of SB202 was induced by Method I. The DNA concentration was 0.1 µg/ml.

a lower rate throughout the remainder of the latent period and the rise period.

The time required for uptake of DNA by competent cells was determined by adding DNase to the transformation mixture at different times, and assaying for transformants after the cells had lysed (Fig. 10). No transformants were obtained when the cells were in contact with DNA for less than 5 min. As the time of contact with DNA increased, the amount of transformation increased sharply until the 40th min. There was an unexplained decrease in transformation thereafter. These results indicated that the uptake of DNA from the time of infection to 5 min after infection was inadequate to cause transformation, and that the uptake of DNA was complete 40 min after infection.

The mutant TAP02 was used in most of the experiments described. Other mutants were transformable although the frequency of transformants varied from 4.1×10^6/ml to 6.0×10^4/ml with unsheared DNA and from 4.4×10^4/ml to 1.1×10^4/ml with sheared DNA (Table 12).

Transformation of temperature-sensitive mutants by fractionated DNA

Before fractionation, fragments of DNA were prepared by stirring a solution of DNA at high speeds with a thin flat blade mounted horizontally on the stirring motor of a Virtis Homogenizer, as described in Chapter II. According to Yamagishi (unpublished results) this shears the DNA to 1.5×10^6 molecular weight. The

Figure 9: Single-step growth curve in transformation. Competent cells (Method II) were infected with TAP02 and 1 μg/ml wild type DNA was added. Samples were plated at 30 C and 45 C to determine the total PFU and the wild type PFU respectively.

X———X total PFU/ml

0———0 wild type PFU/ml

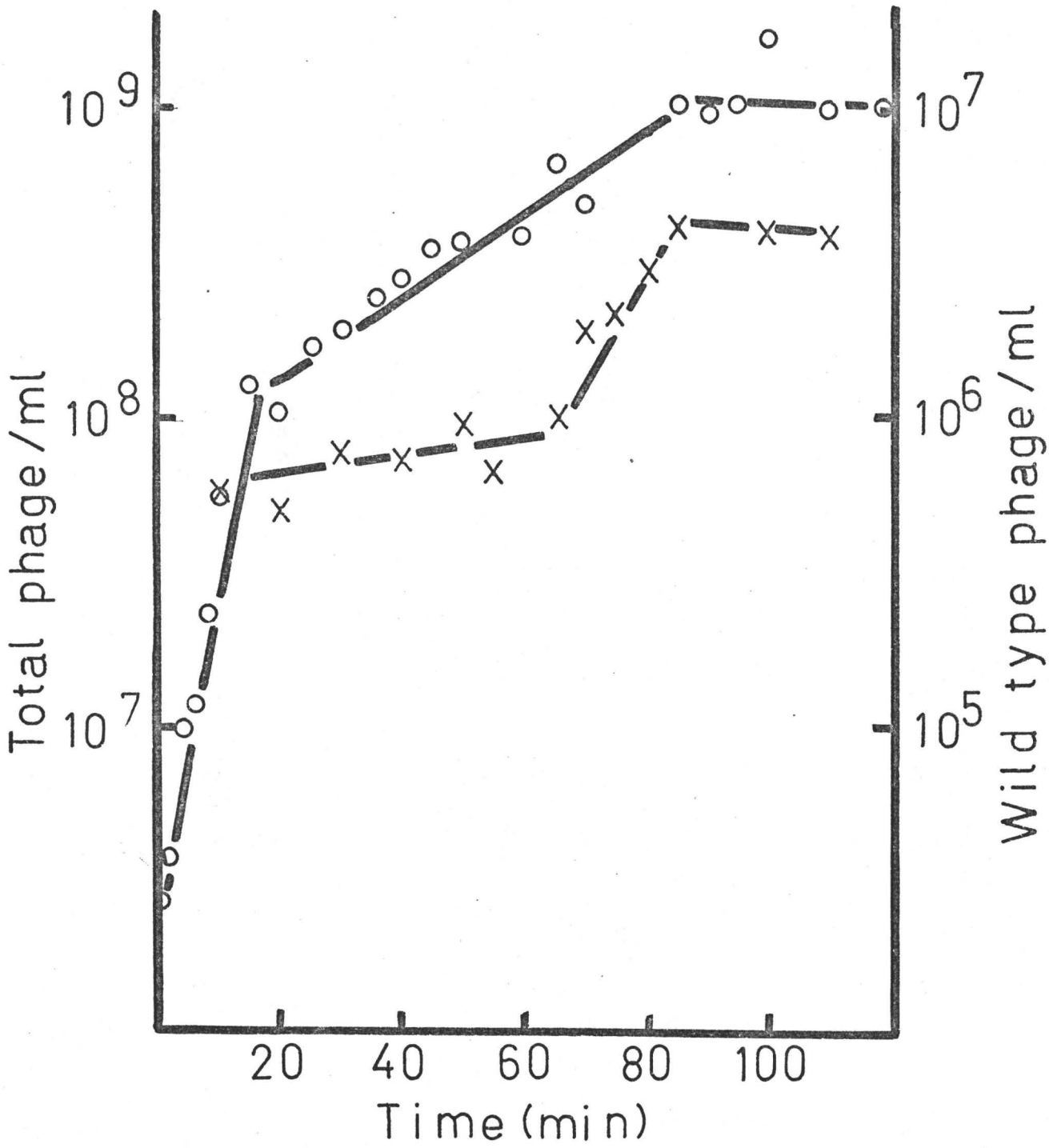
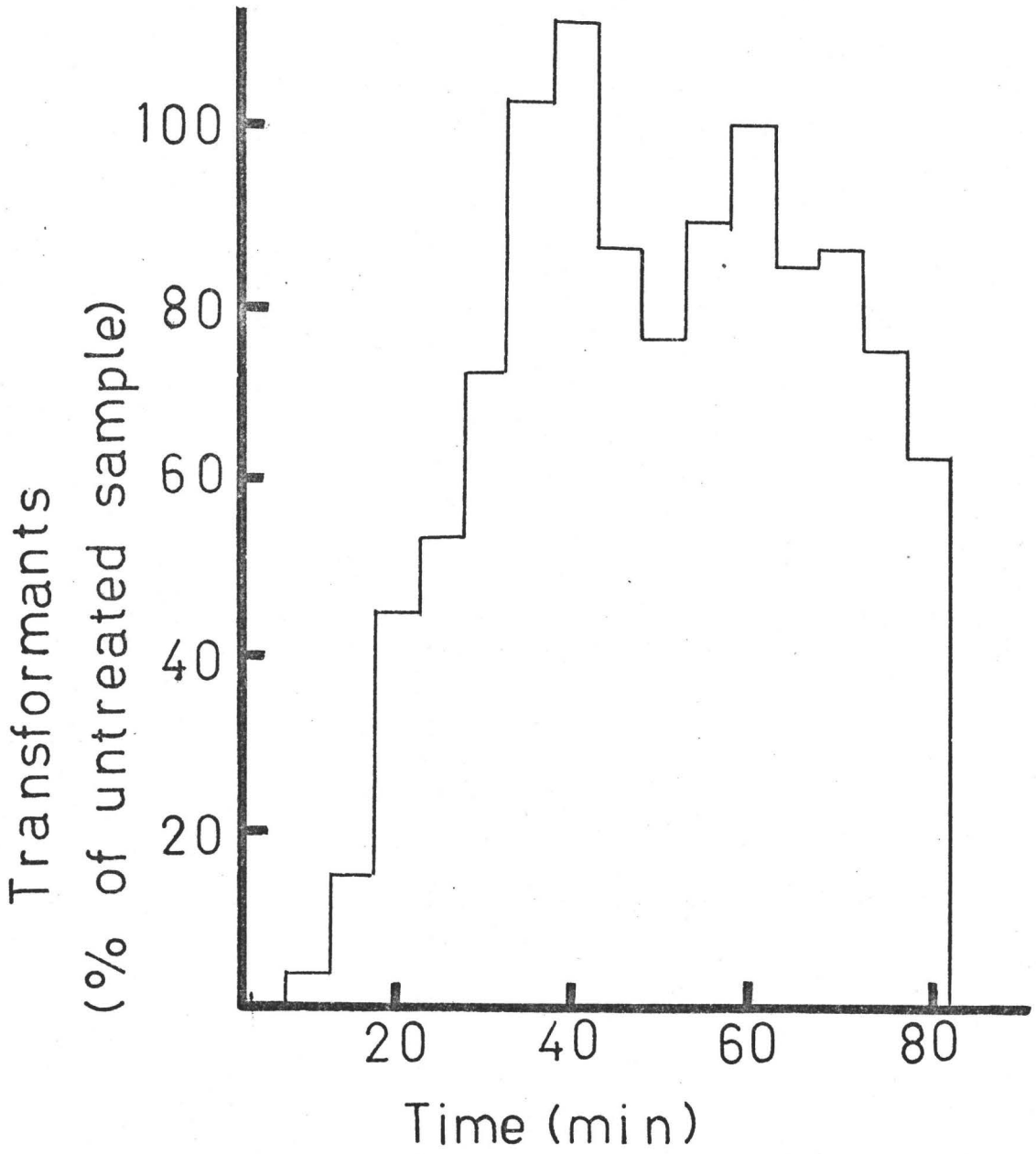

Total phage/ml
10^9
10^8
10^7
Wild type phage/ml
10^7
10^6
10^5
20
40
60
80
100
Time (min)

Figure 10: Kinetics of transformation by PBS 2 DNA. Competent cells (Method II) were infected with TAP02 at a multiplicity of infection of 1-2, and unsheared wild type PBS 2 DNA was added at a concentration of 2 μg/ml. The mixtures were incubated at 30 C with shaking. At different times 10 μg/ml of pancreatic DNase was added. Mixtures were assayed for wild type phage 100 min after infection.


Transformants
(% of untreated sample)
100
80
60
40
20
20
40
60
80
Time (min)

transforming activities of different concentrations of sheared and unsheared DNA are shown in Fig. 11. In both cases, the frequency of wild type transformants was directly proportional to the concentration of DNA. The proportional range for unsheared DNA was 0.01 - 1 μg/ml, and for sheared DNA was 0.05-6 μg/ml. The specific transforming activity of unsheared DNA was 120 times that of sheared DNA.

To obtain fractionated DNA for transformation a relatively large amount of DNA (220 μg) was centrifuged as described in Chapter II. The fractions were collected in 1 M NaCl, and the absorbance at 260 nm was measured. Fractions containing DNA were pooled as indicated in Fig. 12-14, and were dialysed extensively against 1 M NaCl, then against 1 X SSC.

Figures 12, 13, and 14 show the results obtained. Histograms in these figures indicate the absorbance at 260 nm of each fraction and horizontal bars indicate the fractions pooled for the transformation assays. The results for the pooled samples were expressed as a percentage of the total number of transformants. From these figures, it can be seen that the transforming activity for different mutants was concentrated in different fractions. For example, in Fig. 12 B, the activity for TFU91 is quite separate from the activity for TFU103, while the activity for TAP11 overlaps both TFU91 and TFU103. Six of the eleven mutants tested were transformed by fractions from the central region of the gradient, while three of the mutants were transformed by DNA of higher G + C content and two were transformed

Table 12

Transformation of different mutants

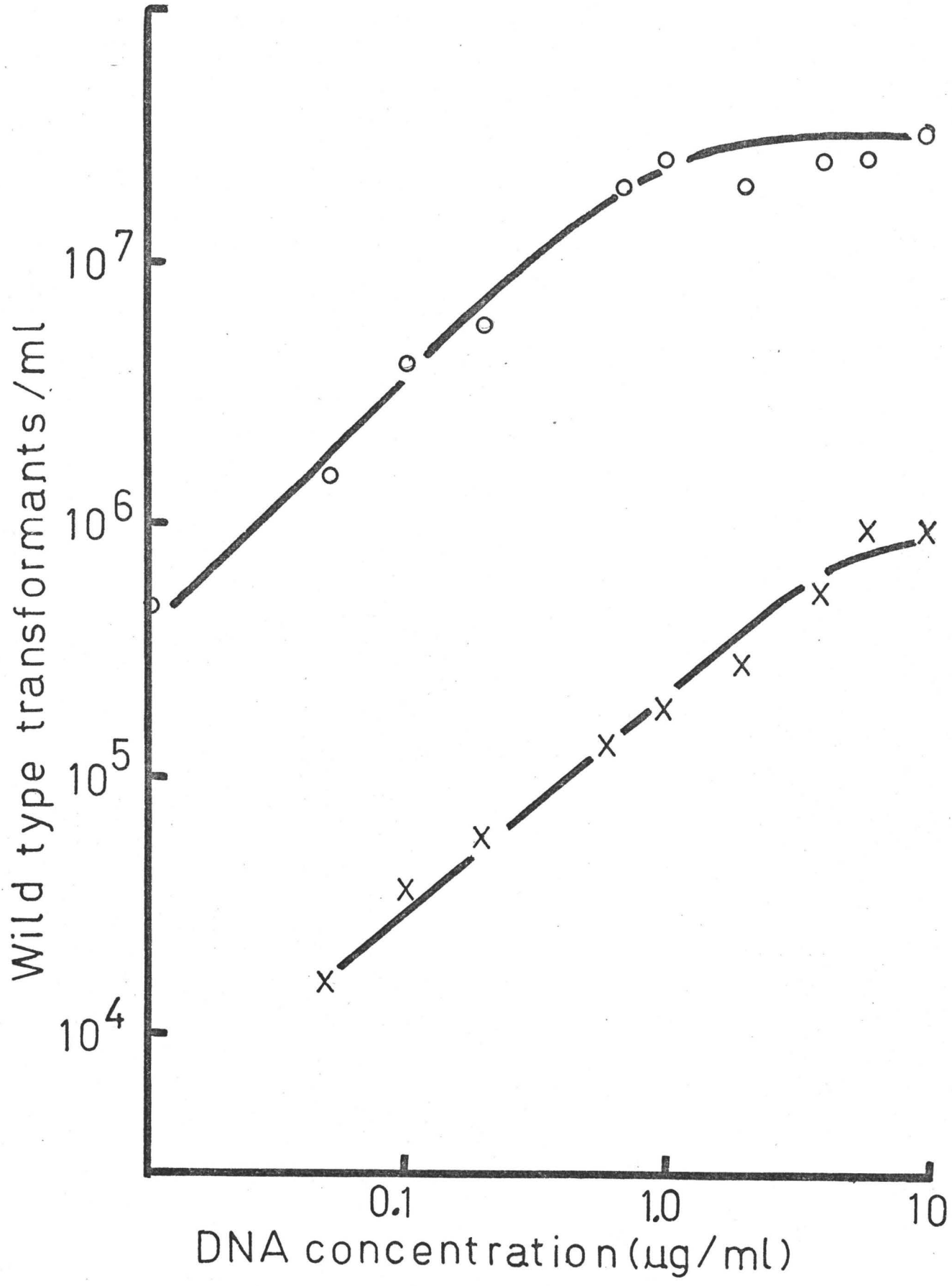
Mutant	Transformants/ml with Unsheared DNA	Sheared DNA
TAP02	4.1×10^6	3.7×10^4
TFU121	8.8×10^5	4.4×10^4
TNG04	6.0×10^4	1.4×10^4
TFU34	1.7×10^6	1.3×10^4
TFU91	7.6×10^5	1.1×10^4

Strain SB202 was made competent by Method II. Cells were infected at a multiplicity of infection of 1 and the concentration of DNA was 0.1 µg/ml.

Figure 11: Dependence of PBS 2 transformation on the concentration of DNA. Cells of SB202 were made competent by Method II and were infected with TAP02 at a multiplicity of infection of 1-2. DNA was added and the mixtures were incubated 100 min at 30 C, then wild type transformants were measured.

0———0 Transformation with unsheared DNA

X———X Transformation with DNA sheared as described in Chapter II.


Wild type transformants/ml
10^7
10^6
10^5
10^4
0.1
1.0
10
DNA concentration (μg/ml)

by DNA of lower G + C content. The transforming activity for a particular mutant was found in the same region in different gradients.

The transforming activity for some of the mutants (e.g. TFU73, Fig. 13 B; TFU102, Fig. 14 A) was concentrated in a few fractions while the activity for other mutants (e.g. TFU11, Fig. 12 A) was more diffuse. This may be a reflection of the degree of heterogeneity of the region of the chromosome involved and will be discussed later.

Figure 12: Transformation of temperature-sensitive mutants by fractionated DNA, (I).

Competent (Method II) SB202 (0.4 ml) was mixed with 0.05 ml of DNA from a fraction, and 0.05 ml of a mutant (1×10^9 phages/ml) and was incubated with shaking for 100 min. Samples were diluted and plated. Transformation is expressed as the percentage of the total transformants in the fractions measured.

The gradient contained 220 μg of DNA. Mercuric chloride was added to give an rf of 0.08.

A. Percentage transformants in the gradient.

●——● Transformation of THA01 (100% = 3573)

X——X Transformation of TFU11 (100% = 1921)

B. Percentage transformation in the gradient.

●——● Transformation of TFU103 (100% = 1860)

X——X Transformation of TFU91 (100% = 863)

▲——▲ Transformation of TAP11 (100% = 252)

C. Distribution of DNA in the gradient.

Histogram: The absorbance at 260 nm.

Horizontal bars: Pooled fractions.

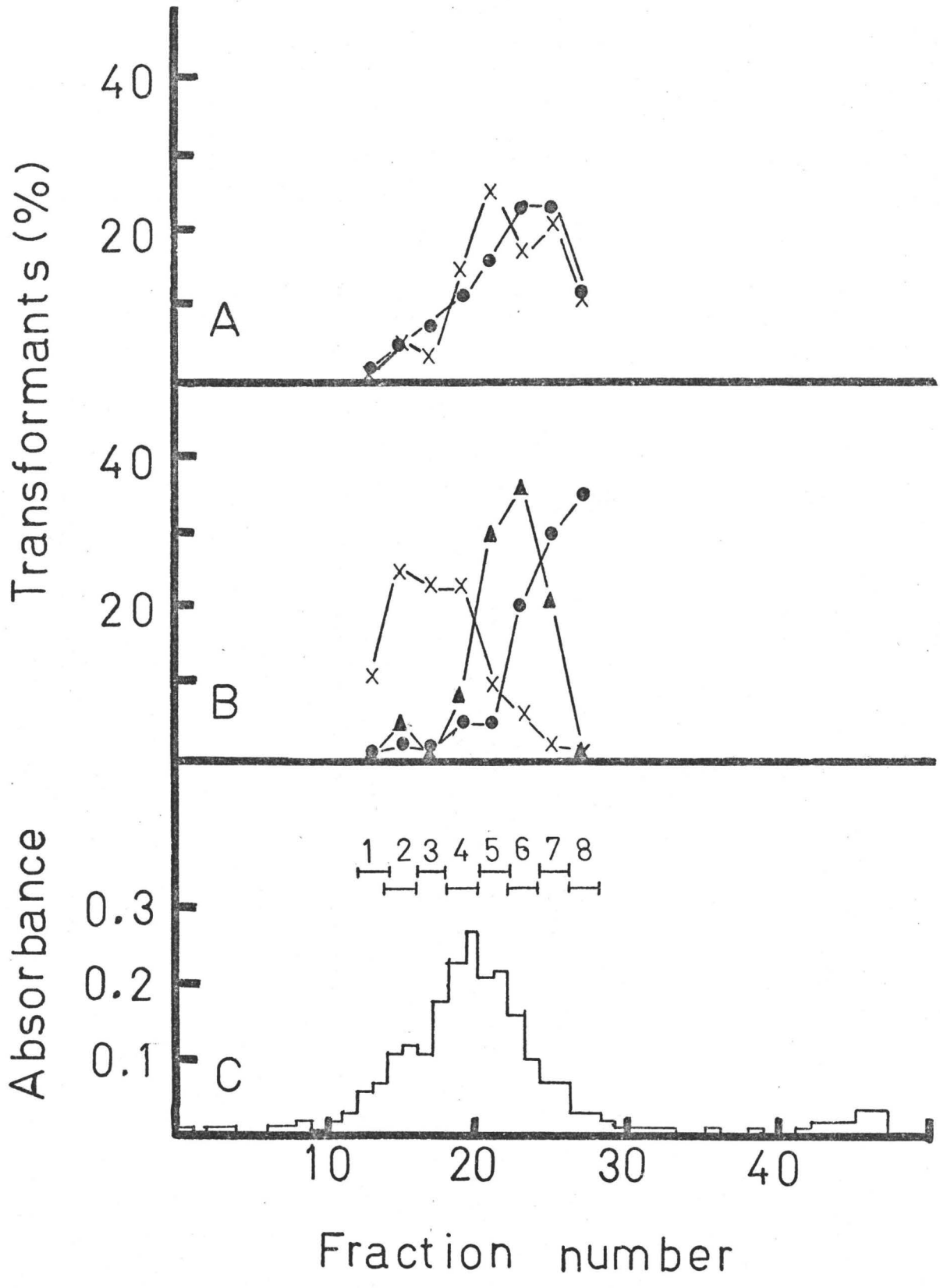

Transformants (%)
40
20
A
40
20
B
1 2 3 4 5 6 7 8
Absorbance
0.3
0.2
0.1
C
10
20
30
40
Fraction number

Figure 13: Transformation of temperature-sensitive mutants by fractionated DNA, (II).

The method is described in Fig. 12.

A. Percentage transformation in the gradient.

●———● Transformation of TFU34 (100% = 113)

X———X Transformation of TFU12 (100% = 984)

B. Percentage transformation in the gradient.

●———● Transformation of TAP02 (100% = 301)

X———X Transformation of TFU73 (100% = 807)

C. Distribution of DNA in the gradient.

Histogram: The absorbance of 260 nm.

Horizontal bars: pooled fractions.

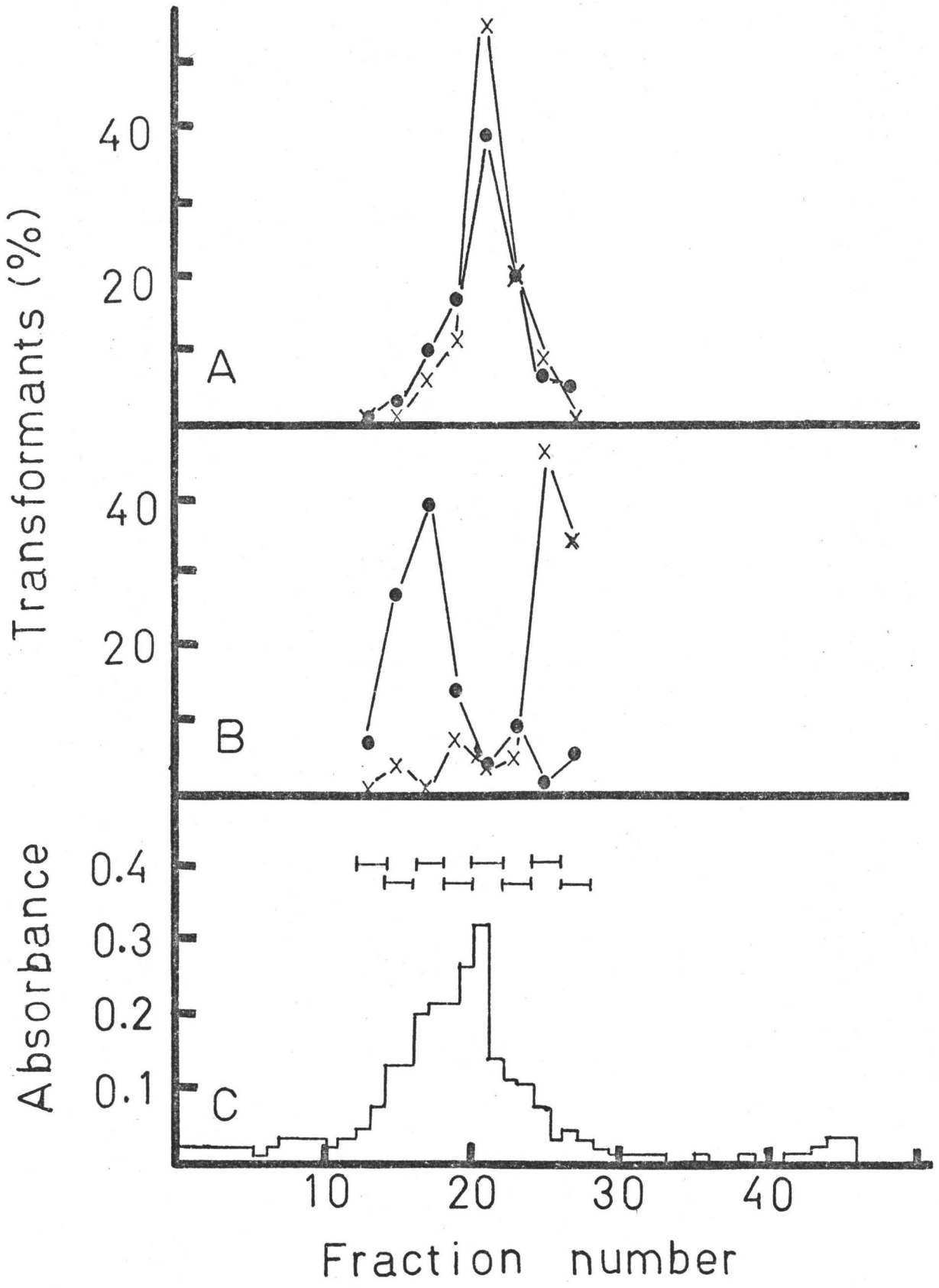

Transformants (%)
40
20
A
40
20
B
Absorbance
0.4
0.3
0.2
0.1
C
10
20
30
40
Fraction number

Figure 14: Transformation of temperature-sensitive mutants by fractionated DNA, (III).

The method is described in Fig. 12.

A. Percentage transformation in the gradient.

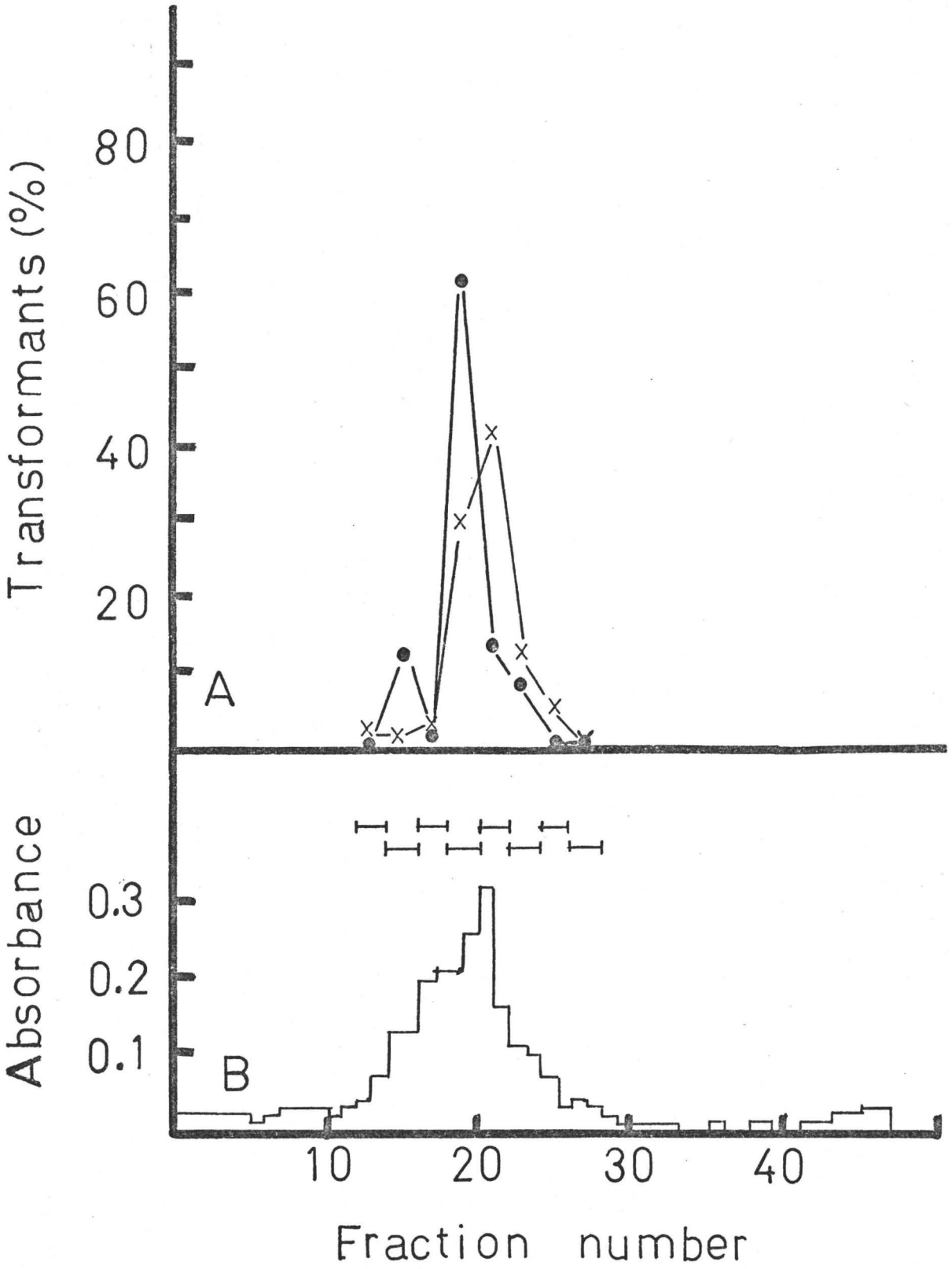
●——● Transformation of TFU171 (100% = 111)

X——X Transformation of TFU102 (100% = 736)

B. Distribution of DNA in the gradient.

Histogram: The absorbance at 260 nm.

Horizontal bars: pooled fractions.


Transformants (%)
80
60
40
20
A
Absorbance
0.3
0.2
0.1
B
10
20
30
40
Fraction number

CHAPTER VI

DISCUSSION

Mutagenesis

In this study, representatives of different types of chemical mutagens were tested for their ability to mutate PBS 2. The mutagens tested were 2-aminopurine, 5-fluorodeoxyuridine, nitrosoguanidine, and hydroxylamine.

Purine and pyrimidine bases exhibit tautomeric forms, some of which are more probable than others. The formation of the specific hydrogen bonds between adenine and thymine, and between guanine and cytosine described by Watson and Crick (106) requires that these bases be in their most probable tautomeric form. Occasionally, a base may take up a rarer tautomeric form, enabling it to form hydrogen bonds with a base other than its normal partner. Changes in base sequence arise either when the base is incorporated into DNA in its rarer form, or when the incorporated base assumes its rarer form during replication. Base analogues, because of their substituted group, may exist in the rarer tautomeric form more frequently than the normal base. As a result, the probability of an error occurring during replication is enhanced, and therefore, the frequency of mutation is increased (87).

The purine analogue, 2-aminopurine, is an effective mutagen for phage T4 (30). When PBS 2 was grown in the presence of aminopurine, we found ts mutants at a frequency of 0.1% (Table 1). This frequency was comparable to that observed with another B. subtilis phage, SP82 (46).

The thymine analogue, 5-bromouracil, is mutagenic for T2 when the phage is grown in its presence (55). Bromouracil is incorporated into the phage DNA in place of thymine (19). Preliminary experiments carried out by Takahashi (unpublished results) show that DNA extracted from PBS 1 grown in the presence of 5-bromouracil has the buoyant density identical with that of control PBS 1 DNA. It appears that this analogue is not incorporated into PBS 1 DNA as readily as in the case of the T-even phages. The mutagenic effect of 5-bromouracil on PBS 2 has not been investigated.

The uracil analogue, 5-fluorouracil, is incorporated into the RNA of E. coli (43). It can induce mutations in tobacco mosaic virus, a plant virus containing RNA as its genetic material (52). Fluorouracil normally forms hydrogen bonds with adenine. Occasionally, it can pair with guanine (13). The changes observed in the amino acid composition of the coat protein from fluorouracil induced mutants of tobacco mosaic virus are consistent with a uracil to cytosine or a cytosine to uracil change in the RNA (109).

Lozeron and Szybalski (56) have shown that FdUra is incorporated into PBS 2 DNA in place of deoxyuridine. For this reason, FdUra may be mutagenic for PBS 2. In fact, this analogue induced ts mutants of PBS 2 at a relatively high frequency (Table 1). Suppressor-sensitive mutants of PBS 2 were induced at the same frequency as ts mutants (M. Levine, personal communication).

Nitrosoguanidine is a potent mutagen for E. coli (2, 60) and Salmonella typhimurium (23). This mutagen appears to act at the replicating point of the DNA in E. coli (12). Although nitrosoguanidine induces a high frequency of mutations, it should be used with caution since it appears to frequently induce multiple mutations (35). In this study, when PBS 2 was grown in the presence of nitrosoguanidine, ts mutants were induced at a frequency of 0.3 % (Table 1). Suppressor-sensitive mutants of PBS 2 were induced at a frequency of 1% (M. Levine, personal communication). Nitrosoguanidine induced ts mutants of B. subtilis phage SP82 at a frequency of 1% (46).

Hydroxylamine deaminates cytosine giving rise to uracil (31). This event is mutagenic. Hydroxylamine also reacts lethally with the DNA (32) and with the tail of the T-even phages (51). The present study showed that PBS 2 was inactivated more rapidly than T4 by hydroxylamine. The reaction of hydroxylamine with uracil (32) may cause the greater sensitivity of PBS 2 to hydroxylamine. Alternatively, the reaction of hydroxylamine with the phage tail (51) may result in a higher rate of inactivation with PBS 2 than with T4. However, the present data do not support either of these hypotheses. The frequency of ts mutants induced in PBS 2 by hydroxylamine (0.1%, Table 1) was much lower than that of SP82 (1%) reported by Kahan (46).

Genetics

The most sensitive measure of complementation in bacteriophage may be the burst size determined in cultures infected with

two mutants under non-permissive conditions. In some studies (21, 26, 54), the burst size of the mixture was expressed as a percentage of the burst size of the wild type. Other studies (46, 50) compared the burst size at the non-permissive temperature with that at the permissive temperature. In both cases, the results could be separated into two groups which indicated the presence or absence of complementation.

Since the burst size of PBS 2 at 45 C was low (approximately 9) and variable, and since several of the mutants had burst sizes of 1-2 phage/cell, we felt that comparing the burst size obtained in mixed infection directly with the mutant burst sizes was a more reasonable criterion of complementation than either of those mentioned above. Therefore, a burst size of a mixture that was greater than 1.5 times the largest of the burst size of the individual mutants was taken to indicate complementation. The foregoing results showed that the 47 mutants used can be grouped into 10 cistrons. This assignment will have to be verified from other evidence, such as physiological or morphological characterization of the mutants.

The molecular weight of PBS 2 DNA is 1.9×10^8 (44). This is adequate to code for 190 genes if we assume that the average molecular weight of a gene is 10^6 (105). Obviously, the ten cistrons identified above are much less than expected from the molecular weight of the DNA.

The number of genes that can be discovered by isolating ts mutants may be affected by several factors. First, the protein

affected by a mutation must be essential for growth under the conditions used. In the T-even phages, several enzymes produced by the phage are dispensible for the growth of the phage (15, 16). Since PBS 2 is even larger than these phages, the PBS 2 chromosome might contain non-essential genes.

The intrinsic sensitivity of different genes to particular mutagens will affect the number of genes that can be discovered. For example, a gene with a high G + C content will be more readily mutated by hydroxylamine. Most of the ts mutants of PBS 2 were isolated from lysates treated with FdUra. This may be one of the reasons for the limited number of genes discovered in the present study.

Another factor which will affect the number of genes to be discovered is the structure of the proteins. Some proteins may have numerous sites where a change in the amino acid can result in temperature-sensitivity while other proteins have few such sites. As a result, mutants affecting genes with the first type of protein as a product will be more frequent than mutants affecting the second type.

Finally, the method used to select ts mutants might affect the number of genes to be discovered. Suspected mutants of T4 were tested for temperature-sensitivity during the isolation procedure by measuring the efficiency of plating (22). The temperature-sensitivity of putative mutants of PBS 2 was confirmed by spot tests at the high temperature, rather than by determining the

plating efficiency. Because of this, mutants having a relatively high plating efficiency at this stage may have been discarded. This, then, not only may have reduced the total number of mutants isolated, but also may have selected against genes in which only "leaky" mutations occurred.

Suppressor-sensitive mutants of PBS 2 were assigned to 39 cistrons (M. Levine, personal communication). When representatives from each cistron defined by the ts mutants were tested for complementation with the suppressor-sensitive mutants using spot tests devised by M. Levine (personal communication), only two of the cistrons appeared to be common to both the ts and suppressor-sensitive mutants. These results suggest that the 10 cistrons identified here were only a small fraction of the phage genome.

A large number of the ts mutants of PBS 2 occur in two cistrons (5 and 7). These genes may either be very large, or extremely sensitive to mutagens. Alternatively, the protein products may be more susceptible to alterations than the average protein.

The possibility exists that cistrons 5 and 7 are not single cistrons. If a mutant were partially or totally dominant, it would fail to complement many or all of the mutants with which it was tested. Therefore, all of these mutants could be erroneously assigned to the same cistron.

When complementation was measured by burst size determinations with ts mutants of T4 (21), many mutant pairs within cistrons gave

burst sizes significantly higher than the mutants alone. This intragenic complementation may be due to the formation of protein aggregates containing polypeptide chains of both mutant types. Aggregates of each mutant alone are inactive while hybrid aggregates are active (81, 110). As shown in Table 3, F and I, many mutant pairs with cistrons 5 and 7 of PBS 2 complement. This may be intragenic complementation.

The ts mutants of PBS 2 were mapped by two-factor crosses. The results were complicated by several facts. First, the mutants represented only a small fraction of the total number of genes. Second, interpretation of the data was difficult because 1) the recombination frequency varied extremely from experiment to experiment, 2) the additivity was poor, and 3) the maximum recombination frequency was low. The map was linear and about 50% long. It is drawn roughly to scale in Fig. 15.

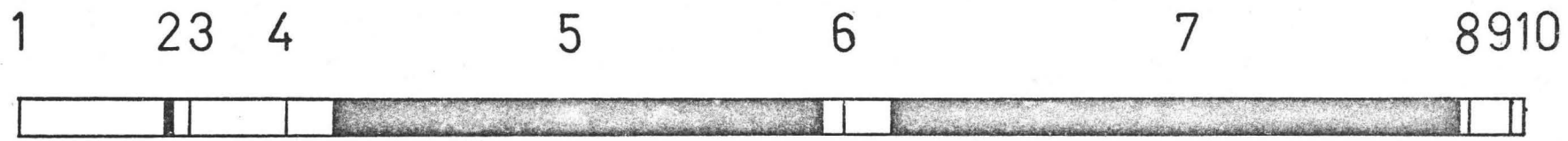
In PBS 2, two cistrons (5 and 7) are very long (20%, Fig. 15). These may be compared to some extent with the largest genes of T4 which are approximately 25% in length (21).

The adjacent mutants TAP02 and TAP13 of PBS 2 (Fig. 6 D) are loosely linked, suggesting that the linear map could be broken between these mutants to form two linear segments. However, these mutants are in the same cistron, and therefore it is unlikely that this loose linkage indicates a segmented map such as is found with T5 (38).

The following preliminary experiments carried out with one mutant from each cistron suggest that the ts mutants of PBS 2 are late

Figure 15: Genetic map of PBS 2.

The map is drawn to scale (1 cm = 2.5 % recombination). Shaded bars indicate the extent of cistrons which are identified by their mutants.


1
23
4
5
6
7
8910

mutants. All mutants were able to lyse cells at the non-permissive temperature. Nine of the ten mutants were different from the wild type phage in their heat sensitivity at 70 C. This suggests that the capsid proteins differ from those of wild type. Finally, five of the mutants appear to be tail donors in in vitro complementation tests (107).

In T4, genes coding for related functions tend to be found in clusters on the genetic map (25). Thus, if the PBS 2 mutants are late mutants, they may be a cluster of mutants. To determine whether this was a reasonable conclusion a map of the T4 ts mutants most like the PBS 2 mutants (series A and N in ref. 22) was constructed. These mutants were mapped using the data from Edgar, Denhardt, and Epstein (21), and assuming that the maximum recombination frequency was 30% (41). The map thus obtained, consisted of three linear unlinked segments and two unlinked genes. Eighteen of the twenty-six genes in this map were located in one segment, and were involved in phage maturation (25). Therefore, it seems that the limited number genes studied here represent a cluster on the PBS 2 chromosome.

Phage transformation

The first requirement in a study of phage transformation is to show that the transformation is really mediated by phage DNA. Transformation of PBS 2 was sensitive to deoxyribonuclease.

Two experiments indicated that the uptake of DNA was rapid in PBS 2 transformation. In the first experiment, the transformation mixture was diluted at different times and was assayed for wild type

infective centres. These infective centres were found less than 1 min after infection (Fig. 9). In the second experiment, transformation mixtures were treated with DNase, and then allowed to lyse. Wild type transformants were found 5-10 min after the addition of DNA (Fig. 10).

The number of transformants obtained decreased when the DNA was added as late as 50 min after infection. The first 15 min after infection was the period in which the rate of formation of wild type infective centres was most rapid (Fig. 9). The latent period extended for 60 min (Fig. 9). These results suggest that there may be a gradual loss in competence in the infected cells. Alternatively, the DNA may be entering the cell at later times as efficiently as at the time of infection, but may not be able to recombine with the mutant phage chromosome as efficiently as at the beginning of the infection.

There was an apparent difference in the efficiency of transformation for different markers (Table 10). This may reflect differences in the efficiency of integration of the different markers.

In this study, DNA fractionated by Hg-Cs_2SO_4 density gradients was tested for its ability to transform different ts mutants (Fig. 12 -14). The results of these experiments are summarized in Fig. 16. The mutants tested are given in the order in which they appear on the map, and for each mutant, the G + C composition (Yamagishi, unpublished results) of the DNA fraction

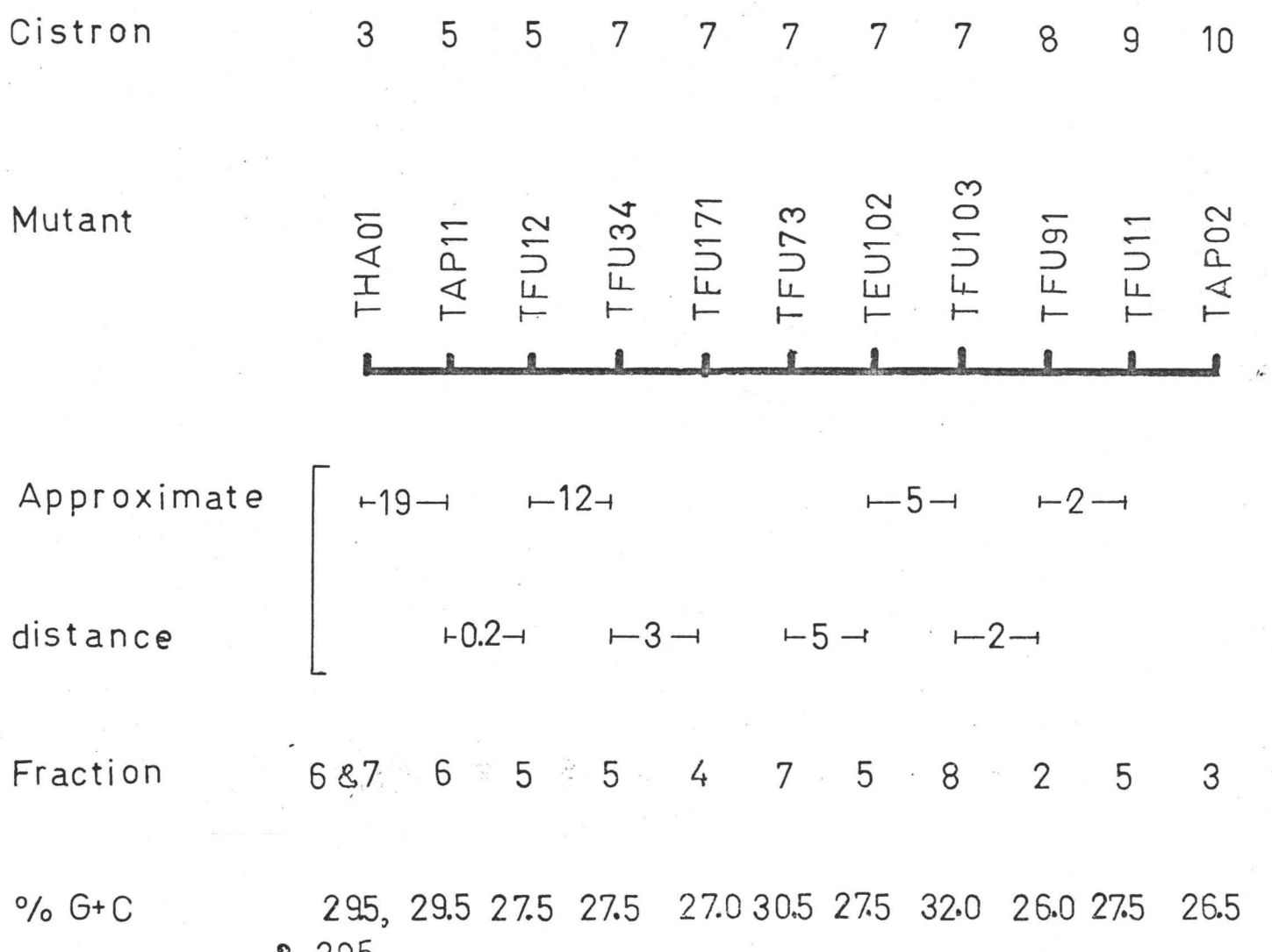
having the highest transforming activity is given.

The G + C composition of unfractionated DNA was 28% (96). Seven of the mutants tested were transformed by DNA having a G + C composition significantly different from the unfractionated DNA (Fig. 16). However, these mutants are interspersed with mutants transformed by DNA with the average G + C composition confirming Yamagishi's observation that large fragments (molecular weight, 6×10^7) of PBS 2 DNA have the same G + C composition as unfractionated DNA.

Five of the mutants tested were from cistron 7 (Fig. 16). The transforming activities for these mutants were present in different fractions of the gradients, indicating that this cistron must be broken into several pieces when the DNA is sheared. This supports the genetic observation that this gene is quite large.

The maximal transforming activity for TFU91 was in fraction 2, while the maximal activity for its nearest neighbours, TFU103 and TFU11, were in fractions 8 and 5 respectively (Fig. 16). Transforming activity for TFU91 was present in 5 of 8 fractions tested (Fig. 12 B). In contrast to TFU91, the mutant TFU34 was located in a relatively homogeneous region of the chromosome (Fig. 16), and the transforming activity for this mutant was present in only 3 of 8 fractions tested (Fig. 13 A). Although relatively few mutants were examined, this correlation between the broadness of the peak, and the relative homogeneity of the region of the chromosome was observed frequently. These results can be explained in the following

Figure 16: Summary of transformation by fractionated DNA. The number of the fraction with the maximum transforming activity refers to the pooled fractions numbered as in Fig. 12. The G + C are from Yamagishi (personal communication). The map and approximate genetic distances are from Fig. 6.


Cistron
3
5
5
7
7
7
7
7
8
9
10
Mutant
THA01
TAP11
TFU12
TFU34
TFU171
TFU73
TEU102
TFU103
TFU91
TFU11
TAP02
Approximate
distance
19
12
5
2
0.2
3
5
2
Fraction
6 &7
6
5
5
4
7
5
8
2
5
3
% G+C
29.5, & 30.5
29.5
27.5
27.5
27.0
30.5
27.5
32.0
26.0
27.5
26.5

way. If a mutation is located in a region of the chromosome composed of two segments of quite different nucleotide composition, then, since breakage by shear is not absolutely specific (83), fragments of DNA containing this mutation will have a wider range of nucleotide composition than fragments from a region of DNA which has a uniform nucleotide content. Therefore, fragments carrying this mutation will be distributed more widely in the gradient than the fragments carrying mutations from a homogeneous region. Therefore, the broadness of the peak of transforming activity can be used to estimate the degree of heterogeneity of the chromosome around a particular marker.

REFERENCES

1. Adams, M. H. 1959. Bacteriophages. Interscience Publisher, New York.

2. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in Escherichia coli K12. Biochem. Biophys. Res. Commun. 18: 788.

3. Baltz, R. H. 1971. Infectious DNA of bacteriophage T4. J. Mol. Biol. 62: 425.

4. Baylor, M. D., D. Hurst, S. Allen, and E. T. Bertani. 1957. The frequency and distribution of loci affecting host range in the coliphage T2H. Genetics 42: 104.

5. Benbow, R. M., C. A. Hutchison III, J. D. Fabricant, and R. L. Sinsheimer. 1971. Genetic map of bacteriophage ϕX174. J. Virol. 7: 549.

6. Benzer, S. 1957. The elementary units of heredity, p. 70. In W. D. McElroy and B. Glass (eds.), The Chemical Basis of Heredity. Johns Hopkins Press, Baltimore.

7. Brody, E., L. Coleman, R. P. Mackal, B. Werninghaus, and E. A. Evans, Jr. 1964. Properties of infectious deoxyribonucleic acid from T1 and λ bacteriophage. J. Biol. Chem. 239: 285.

8. Bujard, H. 1969. Location of single-strand interruptions in the DNA of bacteriophage $T5^{+}$. Proc. Natl. Acad. Sci. U.S. 62: 1167.

9. Burnet, F. M., and D. Lush. 1936. Induced lysogenicity and mutation of bacteriophage within lysogenic bacteria. Austral. J. Exp. Biol. Med. Sci. 14: 27.

10. Campbell, A. 1961. Sensitive mutants of bacteriophage λ. Virology 14: 22.

11. Catcheside, D. G., and A. Overton. 1958. Complementation between alleles in heterocaryons. Cold Spring Harbor Symposia Quant. Biol. 23: 137.

12. Cerdá-Olmedo, E., P. C. Hanawalt, and N. Guerola. 1968. Mutagenesis of the replication point by nitrosoguanidine: Map and pattern of replication of the Escherichia coli chromosome. J. Mol. Biol. 33: 705.

13. Champe, S. P., and S. Benzer. 1962. Reversal of mutant phenotypes by 5-fluorouracil: An approach to nucleotide sequences in messenger-RNA. Proc. Natl. Acad. Sci. U.S. 48: 532.

14. Childs, J. D. 1969. Permuted partial phage within single bursts of Escherichia coli infected with a T4D mutant. Genetics 63: 239.

15. Cohen, S. S. 1961. Virus-induced acquisition of metabolic function. Fed. Proc. 20: 641.

16. Cohen, S. S., and H. D. Barner. 1962. Spermidine in the extraction of the deoxyribosyl-synthesizing system from T6 r^+-infected Escherichia coli. J. Biol. Chem. 237: PC1377.

17. Demerec, M., E. A. Adelberg, A. J. Clark, and P. E. Hartman. 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics 54: 61.

18. DeWaard, A., A. V. Paul, and I. R. Lehman. 1965. The structural gene for deoxyribonucleic acid polymerase in bacteriophages T4 and T5. Proc. Natl. Acad. Sci. 54: 1241.

19. Dunn, D. B., and J. D. Smith. 1954. Incorporation of halogenated pyrimidines into deoxyribonucleic acids of Bacterium coli and its bacteriophages. Nature 174: 305.

20. Edgar, R. S. 1958. Mapping experiments with rII and h mutants of bacteriophage T4D. Virology 6: 215.

21. Edgar, R. S., G. H. Denhardt, and R. H. Epstein. 1964. A comparative genetic study of conditional lethal mutations of bacteriophage T4D. Genetics 49: 635.

22. Edgar, R. S., and I. Lielausis. 1964. Temperature-sensitive mutants of bacteriophage T4D: Their isolation and genetic characterization. Genetics 49: 649.

23. Eisenstark, A., R. Eisenstark, and R. Van Sickle. 1965. Mutation of Salmonella typhimurium by nitrosoguanidine. Mutation Res. 2: 1.

24. Eiserling, F. A. 1967. The structure of Bacillus subtilis bacteriophage PBS 1. J. Ultrastructure Res. 17: 342.

25. Epstein, R. H., A. Bolle, C. M. Steinberg, E. Kellenberger, E. Boy De La Tour, R. Chevalley, R. S. Edgar, M. Susman, G. H. Denhardt, and A. Lielausis. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symposia Quant. Biol. 28: 375.

26. Fattig, W. D., and F. Lanni. 1965. Mapping of temperature-sensitive mutants in bacteriophage T5. Genetics 51: 157.

27. Fiers, W., and R. L. Sinsheimer. 1962. The structure of the DNA of bacteriophage ϕX174. J. Mol. Biol. 5: 408.

28. Foldes, J., and T. A. Trautner. 1964. Infectious DNA from a newly isolated B. subtilis phage. Z. Vererbungsl. 95: 57.

29. Fraser, D., H. R. Mahler, A. L. Shug, and C. A. Thomas, Jr. 1957. The infection of sub-cellular Escherichia coli, strain B, with a DNA preparation from T2 bacteriophage. Proc. Natl. Acad. Sci. U.S. 43: 939.

30. Freese, E. 1959. The specific mutagenic effect of base analogues on phage T4. J. Mol. Biol. 1: 87.

31. Freese, E. 1963. Molecular mechanism of mutation, p. 207. In J. H. Taylor (Ed), Molecular Genetics, Part 1. Academic Press, New York.

32. Freese, E., E. Bautz, and E. B. Freese. 1961. The chemical and mutagenic specificity of hydroxylamine. Proc. Natl. Acad. Sci. U.S. 47: 845.

33. Gough, M., and M. Levine. 1968. The circularity of the phage P 22 linkage map. Genetics 58: 161.

34. Green, D. M. 1964. Infectivity of DNA isolated from Bacillus subtilis bacteriophage, SP82. J. Mol. Biol. 10: 438.

35. Guerola, N., J. L. Ingraham, and E. Cerdá-Olmedo. 1971. Induction of closely linked multiple mutations by nitrosoguanidine. Nature N.B. 230: 122.

36. Guthrie, G. D., and R. L. Sinsheimer. 1960. Infections of protoplasts of Escherichia coli by subviral particles of bacteriophage ϕX174. J. Mol. Biol. 2: 297.

37. Hagen, E. W., V. M. Zeece, and D. L. Anderson. 1971. A genetic study of temperature-sensitive mutants of the Bacillus subtilis bacteriophage Ø29. Virology 43: 561.

38. Hendrickson, H. E., and D. J. McCorquodale. 1971. Genetic and physiological studies of bacteriophage T5. I. An expanded map of bacteriophage T5. J. Virol. 7: 612.

39. Hershey, A. D. 1946. Mutation of bacteriophage with respect to type of plaque. Genetics 31: 620.

40. Hershey, A. D., and R. Rotman. 1948. Linkage among genes controlling inhibition of lysis in a bacterial virus. Proc. Natl. Acad. Sci. U.S. 34: 89.

41. Hershey, A. D., and R. Rotman. 1949. Genetic recombination between host range and plaque-type mutants of bacteriophage in single bacterial cells. Genetics 34: 44.

42. Hogness, D. S., and J. R. Simmons. 1964. Breakage of λ dg DNA: Chemical and genetic characterization of each isolated half-molecule. J. Mol. Biol. 9: 411.

43. Horowitz, J., and E. Chargaff. 1959. Massive incorporation of 5-fluorouracil into a bacterial ribonucleic acid. Nature 184: 1213.

44. Hunter, B. I., H. Yamagishi, and I. Takahashi. 1967. Molecular weight of bacteriophage PBS 1 deoxyribonucleic acid. J. Virol. 1: 841.

45. IUPAC-IUB Commission on Biological Nomenclature (CBN). 1970. Abbreviations and symbols for nucleic acids, polynucleotides, and their constituents. Biochem. 9: 4022.

46. Kahan, E. 1966. A genetic study of temperature-sensitive mutants of the subtilis phage SP82. Virology 30: 650.

47. Kahan, F. M. 1963. Novel enzymes formed by Bacillus subtilis infected with bacteriophage. Fed. Proc. 22: 406.

48. Kaiser, A. D. 1962. The production of phage chromosome fragments and their capacity for genetic transfer. J. Mol. Biol. 4: 275.

49. Kaiser, A. D., and D. S. Hogness. 1960. The transformation of Escherichia coli with deoxyribonucleic acid isolated from bacteriophage λ dg. J. Mol. Biol. 2: 392.

50. Kejzlarová, J., P. Donini, T. Eremenko-Volpe, and F. Graziosi. 1970. Genetic map of bacteriophage ∝. J. Virol. 6: 49.

51. Kozloff, L. M., M. Lute, and K. Henderson. 1957. Viral invasion I Rupture of thiol ester bonds in the bacteriophage tail. J. Biol. Chem. 228: 511.

52. Kramer, G., H. G. Wittmann, and H. Schuster. 1964. Die Erzeugung von Mutanten des Tabakmosaikvirus durch den Einbau von Fluorouracil in die Virusnukleinsäure. Z. Naturforsch. 19b: 46.

53. Levine, M. 1969. Phage morphogenesis. Ann. Rev. Genetics. 3: 323.

54. Lindahl, G. 1969. Genetic map of bacteriophage P2. Virology 39: 839.

55. Litman, R. M., and A. B. Pardee. 1956. Production of bacteriophage mutants by a disturbance of deoxyribose-nucleic acid metabolism. Nature 178: 529.

56. Lozeron, H. A., and W. Szybalski. 1967. Incorporation of 5-fluorodeoxyuridine into the DNA of Bacillus subtilis phage PBS 2 and its radiobiological consequences. J. Mol. Biol. 30: 277.

57. McFall, E., and G. S. Stent. 1958. Three star mutants of coliphage T2. J. Gen. Microbiol. 18: 346.

58. Mahler, I. 1968. Procedures for Bacillus subtilis transformation, p. 846. In L. Grossman, and K. Moldave (Eds), Methods of Enzymology, XII B. Academic Press Inc., New York.

59. Mandel, M., and A. Higa. 1970. Calcium-dependant bacteriophage DNA infection. J. Mol. Biol. 53: 159.

60. Mandell, J. D., and J. Greenberg. 1960. A new chemical mutagen for bacteria, 1-methyl-3-nitro-1-nitrosoguanidine. Biochem. Biophys. Res. Commun. 3: 575.

61. Mandell, J. D., and A. D. Hershey. 1960. A fractionating column for analysis of nucleic acids. Analytical Biochem. 1: 66.

62. Marsh, R. C., A. M. Breschkin, and G. Mosig. 1971. Origin and direction of bacteriophage T4 DNA replication. II A gradient of marker frequencies in partially replicated T4 DNA as assayed by transformation. J. Mol. Biol. 60: 213.

63. Mizobuchi, K., G. C. Anderson, and D. J. McCorquodale. 1971. Abortive infection by bacteriophage BF23 due to the colicin Ib factor. I. Genetic studies of nonrestricted and amber mutants of bacteriophage BF23. Genetics 68: 323.

64. Mosig, G. 1963. Genetic recombination in bacteriophage T4 during replication of DNA fragments. Cold Spring Harbor Symposia Quant. Biol. 28: 35.

65. Mosig, G. 1968. A map of distances along the DNA molecule of phage T4. Genetics 59: 137.

66. Munro, J. L., and J. S. Wiberg. 1968. Evidence that gene 56 of bacteriophage T4 is a structural gene for deoxycytidine triphosphate. Virology 36: 442.

67. Murphy, J. S. 1953. Recombinations of mutant phages of Bacillus megatherium 899A. J. Exp. Med. 98: 657.

68. Nandi, U. S., J. C. Wang, and N. Davidson. 1965. Separation of deoxyribonucleic acids by Hg (II) binding and Cs_2SO_4 density-gradient centrifugation. Biochemistry 4: 1687.

69. Nester, E. W., M. Schafer, and J. Lederberg. 1963. Gene linkage in DNA transfer: a cluster of genes concerned with aromatic biosynthesis in Bacillus subtilis. Genetics 48: 529.

70. Nishihara, M., and W. R. Romig. 1964. Temperature-sensitive mutants of Bacillus subtilis bacteriophage SP3. I. Isolation and characterization. J. Bacteriol. 88: 1226.

71. Okubo, S., B. Strauss, and M. Stodolsky. 1964. The possible role of recombination in the infection of competent Bacillus subtilis by bacteriophage deoxyribonucleic acid. Virology 24: 552.

72. Parma, D. H. 1969. The structure of genomes of individual petit particles of the bacteriophage T4D mutant E920/96/41. Genetics 63: 247.

73. Price, A. R., and S. J. Cook. 1972. New deoxyribonucleic acid polymerase induced by Bacillus subtilis bacteriophage PBS 2. J. Virol. 9: 602.

74. Radding, C. M., and A. D. Kaiser. 1963. Gene transfer by broken molecules of λ DNA: Activity of the left half molecule. J. Mol. Biol. 7: 225.

75. Raimondo, L. M., N. P. Lundh, and R. J. Martinez. 1968. Primary adsorption site of phage PBS 1: The flagellum of Bacillus subtilis. J. Virol. 2: 256.

76. Reilly, B., and J. Spizizen. 1965. Bacteriophage deoxyribonucleate infection of competent Bacillus subtilis. J. Bacteriol. 89: 782.

77. Romig, W. R. 1962. Infection of Bacillus subtilis with phenol-extracted bacteriophages. Virology 16: 452.

78. Rubenstein, I., C. A. Thomas, Jr., and A. D. Hershey. 1961. The molecular weight of T2 bacteriophage DNA and its first and second breakage products. Proc. Natl. Acad. Sci. U.S. 47: 1113.

79. Rutberg, L. 1969. Mapping of a temperate bacteriophage active on Bacillus subtilis. J. Virol. 3: 38.

80. Schaeffer, P. 1961. Etude genetique, biochemique, et cytologique de la sporulation de Bacillus subtilis. D. Sc. Thesis, Faculty of Science, University of Paris, Paris.

81. Schlesinger, M. J. and C. Levinthal. 1963. Hybrid protein formation of Escherichia coli alkaline phosphatase leading to in vitro complementation. J. Mol. Biol. 7: 1.

82. Sekiguchi, M. 1958. Inactivation of urea-disrupted phage by proteinases. Virology 6: 777.

83. Skalka, A. 1971. A method for the breakage of DNA and resolution of the fragments, p. 341. In Methods in Enzymology, XXI D. Academic Press Inc., New York.

84. Skalka, A., E. Burgi, and A. D. Hershey. 1968. Segmental distribution of nucleotides in the DNA of bacteriophage Lambda. J. Mol. Biol. 34: 1.

85. Spizizen, J. 1957. Infection of protoplasts by disrupted T2 virus. Proc. Natl. Acad. Sci. U.S. 43: 694.

86. Spizizen, J. 1958. Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate. Proc. Natl. Acad. Sci. U.S. 44: 1072.

87. Stent, G. S. 1963. Molecular Biology of Bacterial Viruses. W. H. Freeman and Company, San Francisco and London.

88. Streisinger, G., and V. Bruce. 1960. Linkage of genetic markers in T2 and T4. Genetics 45: 1289.

89. Streisinger, G., R. H. Edgar, and G. H. Denhardt. 1964. Chromosome structure in phage T4. Proc. Natl. Acad. Sci. U.S. 51: 775.

90. Takahashi, I. 1961. Genetic transduction in Bacillus subtilis. Biochem. Biophys. Res. Commun. 5: 171.

91. Takahashi, I. 1963. Transducing phages for Bacillus subtilis. J. Gen. Microbiol. 31: 211.

92. Takahashi, I. 1964. Incorporation of bacteriophage genome by spores of Bacillus subtilis. J. Bacteriol. 87: 1499.

93. Takahashi, I. 1965. Transduction of sporogenesis in Bacillus subtilis. J. Bacteriol. 89: 294.

94. Takahashi, I. 1965. Localization of spore markers on the chromosome of Bacillus subtilis. J. Bacteriol. 89: 1065.

95. Takahashi, I., and R. Barnard. 1967. Effect of N-methyl-N'-nitro-N-nitrosoguanidine on the F factor of Escherichia coli. Mutation Res. 4: 111.

96. Takahashi, I., and J. Marmur. 1963. Replacement of thymidylic acid by deoxyuridylic acid in the deoxyribonucleic acid of a transducing phage for Bacillus subtilis. Nature 197: 794.

97. Tan, K. B., J. F. Sambrook, and A. J. D. Bellett. 1969. Semliki forest virus temperature-sensitive mutants--Isolation and characterization. Virology 38: 427.

98. Tessman, I. 1959. Mutagenesis in phage ϕX174 and T4 and properties of the genetic material. Virology 9: 375.

99. Thomas, C. A. Jr., and L. A. MacHattie. 1964. Circular T2 DNA molecules. Proc. Natl. Acad. Sci. U.S. 52: 1297.

100. Thomas, C. A., Jr., and I. Rubenstein. 1964. The arrangements of nucleotide sequences in T2 and T5 bacteriophage DNA molecules. Biophys. J. 4: 93.

101. Tomita, F. 1968. Biochemical studies on bacteriophage PBS 1. Ph.D. Thesis, Faculty of Graduate Studies, McMaster University, Hamilton, Canada.

102. Tomita, F. and I. Takahashi. 1969. A novel enzyme, dCTP deaminase, found in Bacillus subtilis infected with phage PBS 1. Biochim. Biophys. Acta. 179: 18.

103. Van de Pol, J. H., G. Veldhuisen, and J. A. Cohen. 1961. Phage transformation: A new criterium for the biological activity of bacteriophage DNA. Biochim. Biophys. Acta 48: 417.

104. Warner, H. R., and J. E. Barnes. 1966. Deoxyribonucleic acid synthesis in Escherichia coli infected with some deoxyribonucleic acid polymerase-less mutants of bacteriophage T4. Virology 28: 100.

105. Watson, J. D. 1965. Molecular Biology of the Gene. W. A. Benjamin, Inc., New York.

106. Watson, J. D., and F. H. C. Crick. 1953. Genetical implications of the structure of deoxyribonucleic acid. Nature 171: 964.

107. Weigle, J. 1966. Assembly of phage lambda in vitro. Proc. Natl. Acad. Sci. U.S. 55: 1462.

108. Winkler, U., U. Kopp-Scholz, and Ch. Haux. 1970. Nonsense mutants of Serratia phage Kappa. I. Their isolation and use for proving circular linkage of the phage genes. Molec. Gen. Genetics 106: 239.

109. Wittman-Liebold, B., and H. G. Wittman. 1965. Lokalisierung von Aminosäureaustauschen bei Nitritmutanten des Tabakmosaikvirus Z. Vererbungsl. 97: 305.

110. Woodward, D. O. 1960. A gene concept based on genetic and chemical studies in Neurospora. Quart. Rev. Biol. 35: 313.

111. Yamagishi, H. 1968. Single-strand interruptions in PBS 1 bacteriophage DNA molecule. J. Mol. Biol. 35: 623.

112. Yamagishi, H. 1970. Nucleotide distribution in the DNA of Escherichia coli. J. Mol. Biol. 49: 603.

113. Yamagishi, H., and I. Takahashi. 1971. Heterogeneity in nucleotide composition of Bacillus subtilis DNA. J. Mol. Biol. 57: 369.

114. Zampieri, A., J. Greenberg, and G. Warren. 1968. Inactivating and mutagenic effects of 1-methyl-3-nitro-1-nitrosoguanidine on intracellular bacteriophage. J. Virol. 2: 901.