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A PHAGE P22 HEAD PROTEIN

WHICH PERFORMS AN ESSENTIAL EARLY FUNCTION

by
Barbara Hoffman

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Human Genetics)
in The University of Michigan
1973

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CHAPTER ONE: INTRODUCTION

Infections with P22, a temperate phage of Salmonella typhimurium (Zinder and Lederberg, 1952; Zinder, 1953), can follow either the lytic or the lysogenic pathway. In the lytic response most phage functions are expressed, new progeny phage are produced, and the host cell is lysed. Alternatively, in the lysogenic response, the infected cells survive because vegetative phage development is repressed. After a few divisions, integration of the phage genome into the bacterial chromosome occurs by a reciprocal recombinational event (Campbell, 1962; Smith and Levine, 1967; Smith, 1968). Lysogens are stable and immune to superinfection as long as there are functional C_2 and mnt gene products present in the cells (Smith and Levine, 1964; Gough, 1968). Lysogens can be induced to produce phage by inactivating either the C_2 or mnt repressors, for example, by heating a tsC_2 or tsmnt lysogen to the non-permissive temperature, or by treatment with agents such as ultraviolet light, mitomycin C and streptonigrin.

Induction and infection pose different initial problems for the viral DNA. On induction a highly specific mechanism for excision of the phage genome from the host chromosome is required. Once excised, the phage genome is already in the cell and is probably circular. On infection the DNA must get out of the capsid and into the cytoplasm, and perhaps

undergo some conformational changes such as circularization or attachment to a membrane site. Little is known about these early events in a P22 infection, particularly about the steps between adsorption of the virion to the host cell and association of the parental phage DNA with the replication complex.

Virion proteins serve to protect the DNA while the phage is extracellular, to promote attachment to an appropriate host cell, and probably to facilitate release of the DNA from the capsid into the host cell. As will be shown in this report, the P22 gene 25 protein, a component of the virion head, plays a role after the DNA is at least partially released from the capsid, but before it associates with the replication complex. Infection is abortive in the absence of functional gene 25 protein; the host cell is not noticeably affected. The early gene 25 function is required only after infection. Induction initially proceeds normally in the absence of P25; however, newly formed progeny particles are non-infectious. Functional gene 25 protein must be incorporated into the progeny virions during maturation, on infection or induction, in order for the virions to be infectious. This is the late step in which gene 25 protein participates.

Amber and temperature sensitive mutations in gene 25 have made it possible to do an in depth study of the function(s) of this gene. Experiments are described which give information about the mechanism of action of P25 early in

infection, and various hypotheses for the early P25 function are proposed and discussed.

CHAPTER TWO: MATERIALS AND METHODS

I. BACTERIOPHAGE STRAINS

The clear mutants P22 $\underline{c_1}^7$ and P22 $\underline{c_2}^5$ were isolated by M. Levine. The mutant P22 $\underline{tsc_2}^{30}$ was obtained from M. Gough. The temperature sensitive conditional lethal mutants P22 18⁻ts18.1 and P22 25⁻ts25.1 and the amber conditional lethal mutants P22 5⁻amN114, P22 9⁻amN108 and P22 25⁻amN121 were isolated in this laboratory from wild type phage P22 mutagenized with 1-methyl-3-nitro 1-nitrosoguanidine. P22 13⁻amH101 and P22 20⁻amN20 were obtained from D. Botstein. P22 25⁻amH60 and P22 12⁻amH80 were given to us by Kolstad and Prell. P22 25⁻ts25.2 was obtained from Bezdek and was previously referred to as P22 tsX (Bezdek and Soska, 1970). P22 VirB-3 was isolated by M. Bronson in this laboratory. R. N. Rao isolated P22 siel in this laboratory. Phage L was obtained from Amati.

Wild type phage P22 and the clear mutants P22 $\underline{c_1}$ and P22 $\underline{c_2}$ have been previously described (Levine, 1957; Levine and Curtiss, 1961). Both $\underline{c_1}$ and $\underline{c_2}$ mutants cannot lysogenize the host in single infections; all infected cells lyse. $\underline{tsc_2}$ mutants behave like P22 $\underline{c_2}$ infections at 41°C, but can lysogenize the bacterium at 25°C. These lysogens induce at high temperature. Mutants in either gene 12 or 18 fail to synthesize phage DNA, but turn off host DNA synthesis and kill the host (if these mutants are also either $\underline{c_1}$ or $\underline{c_2}$; Levine and Schott, 1971; Botstein and Levine, 1968 a & b). Gene 5 mutant infections result in no head production but

normal quantities of free tail parts are produced (Botstein and Levine, 1968 a and b). Mutants in gene 9 make no tail parts, but are a source for normal phage heads (Israel, Rosen and Levine, 1972). The gene 13 mutant delays lysis and allows for continued phage production (Botstein, Chan and Waddell, 1972). Morphologically normal but noninfectious particles are produced during a gene 20 mutant infection (Botstein, Waddell and King, 1973; King, Lenk, and Botstein, 1973). VirB-3 phage can grow in a lysogen carrying a sie prophage, whereas a nonvirulent phage cannot (Bronson and Levine, 1971). Prophage bearing sie mutations do not exclude superinfecting phage but are still immune to phage P22 (Walsh and Meynell, 1967; Rao, 1968). Phage L is a temperate bacteriophage of Salmonella typhimurium which is closely related but heteroimmune to P22.

With the exception of mutants in gene 25, only one mutant allele for each gene is used in this report. These mutants will be referred to by gene number and the type of conditional lethal, ts or am, if applicable. For the temperature sensitive gene 25 mutants the allele is always given. When both am25 mutants are used, as in the complementation tests, the particular alleles will be given. However, for all other experiments only P22 25⁻amN121 is utilized and it is referred to as P22 am25.

II. BACTERIAL STRAINS

A derivative of Salmonella typhimurium LT2 cured of the PB1 phage (Zinder, 1958) was used as the sensitive wild

type strain and is designated strain 18. A galactose-negative mutant was used as the indicator on EMB galactose agar plates and is designated strain 2. Strain 192 is an LT2 amber suppressor strain (*su*$^+$) obtained from D. Berkowitz. Strains 18 and 2 are *su*$^-$. A thymidineless strain, 18*T*$^-$, derived from strain 18 is used for the preparation of ^{14}C-thymidine labeled phage (Smith, 1968). Strain DB103 (kindly supplied by D. Botstein) is strain 18 lysogenic for *sie6 int3* prophage. The integration deficient mutant, *int3*, has been described (Smith and Levine, 1967). 192(L) and 18(*C*$^+$*sie*) were isolated following infection at 37°C. Lysogens 192(*am20ts*C_2*sie*), 18(*ts*C_2), 18(*ts25.2ts*C_2), and 18(*ts25.2C*$^+$) were isolated following infection at 25°C. Lysogenic strain 18(*am12ts*C_2*sie*) was isolated following mixed infection with the clear, nonlysogenizing mutant C_2. Recipients for transduction experiments were strain LT2 carrying the *proC90* mutation (Miyake and Demerec, 1960) and strain LT2 carrying the *hisG203* mutation (obtained from P. E. Hartman). These strains were lysogenized with P22 *sie* prophage so that they are immune to superinfecting phage. Thus none of the cells are killed following infection to select for transductants.

III. MEDIA

M9CAA medium (Smith and Levine, 1964), L broth (Levine, 1957), EMB galactose agar (Levine, 1957), tryptone agar (Bronson and Levine, 1971), minimal agar (Smith, 1968), soft

agar for top layers (Levine, 1957), buffered saline (Levine, 1957), tris-buffered saline (Botstein, 1968), LCG 20 medium (Botstein, 1968) and LP/0.5 medium (Botstein, 1968) have been previously described.

IV. EXPERIMENTAL PROCEDURES

A. Purification of Phage Stocks in Cesium Chloride

Phage stocks were prepared by infecting the appropriate cells with multiplicities of 5-10 phages. After lysis, infections were stopped by shaking with a few drops of chloroform. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes in a Servall SS-34 rotor (12,000g). The particles in the supernatant were concentrated by centrifugation at 17,000 rpm for 100 minutes (34,000g). The phage pellet was resuspended in 2 ml buffered saline, which was applied to a stepwise gradient of CsCl (0.3 ml ρ = 1.7, 1.0 ml ρ = 1.6, 2.0 ml ρ = 1.4). After centrifugation at 40,000 rpm for 30 minutes at 20°C in a SW50.1 rotor, the visible phage band was collected through a hole in the bottom of the tube. CsCl was removed by dialysis overnight against 1000 volumes of buffered saline.

B. Preparation of ^{3}H-thymidine Labeled Phage

Log phase cells, grown in M9CAA medium to a concentration of 2×10^{8} cells/ml, were infected with P22 phage (moi = 10). At the time of infection, deoxyadenosine at a final concentration of 250 μg/ml and ^{3}H-thymidine (specific

activity = 22.3 Ci/mmole) at a final concentration of 10 μCi/ml were added. After lysis the phage were concentrated and purified in CsCl as described above.

C. Preparation of ^{32}P-labeled Phage

Bacteria grown overnight at 25°C with aeration in LCG20 medium, washed by centrifugation, and resuspended in tris-buffered saline was used to innoculate LP/0.5 medium to a density of about 10^7 cells/ml. Low phosphorus concentrations are used to decrease the pool of intracellular phosphorus so more $^{32}PO_4$ is incorporated into DNA. This suspension was grown at 37°C until the cell density reached 10^8 cells/ml. The culture was shifted to 25°C if temperature sensitive phage were to be labeled. Phage were added to a final multiplicity of 5 phage/cell. Simultaneously, carrier-free $^{32}PO_4$ was added to a final concentration of 10 μCi/ml. After lysis of the culture, phage were concentrated and purified as described above.

D. Preparation of ^{14}C-thymidine Labeled Phage

These labeled phage were prepared utilizing the 18T- strain. An overnight culture grown in M9CAA plus thymidine (20 μg/ml) was diluted 1:100 into fresh medium and grown to a concentration of 10^8 cells/ml. The cells were centrifuged, washed with buffered saline and resuspended in supplemented M9 containing thymidine (2 μg/ml) and ^{14}C-thymidine, 35.9 mCi/mmole (0.025μCi/ml). The cells were then infected with

phage (moi = 10). After lysis, the phage were concentrated and purified in CsCl.

E. Head Production and Purification

Su⁻ strain 18, grown to a concentration of 2 x 10^8 cells/ml at 37°C was infected at a multiplicity of 5 with P22 am9C$_1$. This infection results in a yield of about 3 x 10^{10} heads/ml and approximately 3 x 10^6 phage/ml. After lysis, heads were concentrated and banded in CsCl as previously described for phage.

F. Assay for Heads

Tail parts, at a concentration of at least 10^{10} tails/ml, were added in excess of heads so that all heads were saturated with tails (Israel, Anderson and Levine, 1967). The reaction mixture was kept at 37°C for 2 hours, then assayed for pfu.

G. Tail Production and Purification

(V. Israel, personal communication, and Israel, et al., 1972). Strain 18, grown to a concentration of 2 x 10^8 cells/ml, was infected at a multiplicity of 5 with P22 am5am13C$_1$. No head-like structures are seen in gene 5 mutant lysates; however, tail production is normal. The gene 13 mutation delays lysis and allows for continued phage production. The double mutant permits the increased accumulation of tail parts. Three hours after infection the cells were concentrated by low speed centrifugation and resuspended in buffered saline.

The cells were ruptured by sonication and the addition of chloroform, and the cell debris was removed by low-speed centrifugation. Contaminating phage were removed by centrifugation at 17,000 rpm for 100 minutes. To concentrate tails, ammonium sulfate was added to the supernatant with stirring at 0°C to 70% saturation, and the mixture was allowed to stir for about 20 minutes more. The precipitate was collected by centrifugation, resuspended in a few ml of buffered saline, dialyzed overnight at 4°C against 0.03M acetate buffer (pH 5.0). The newly formed precipitate was removed by centrifugation and the supernatant was kept at 79°C for 15 minutes to inactivate contaminating phage. The precipitate which formed was removed by centrifugation. A high concentration of tails is found in the supernatant.

H. Tail Assay

Tail titers were determined according to the methods described by Israel, Anderson and Levine (1967).

I. Measurement of the Rate of DNA Synthesis

The rate of DNA synthesis was estimated by the incorporation of ^{3}H-thymidine into acid insoluble material during a one minute pulse as described by Smith and Levine (1964).

J. Assay for Acid Soluble DNA

The assay measures the *in vivo* conversion of ^{3}H-thymidine labeled parental phage DNA to acid soluble nucleotides and oligonucleotides following infection. The *in vivo*

reaction was stopped by addition of 0.2 ml cold calf thymus DNA (2.5 mg/ml) and 0.3 ml cold 7% perchloric acid (PCA). After centrifugation at 10,000 rpm for 10 minutes in a Servall SM-24 rotor, the supernatant was immediately decanted. 0.5 ml of this supernatant was added to 10 ml of p-dioxane scintillation medium (Bray, 1960) and counted in a Packard TriCarb scintillation spectrometer.

K. Detection of Cells Carrying Phage Genomes

Cells carrying phage genomes are capable of producing phage. In these cells the intracellular phage genomes may be free or integrated as prophage. Cells to be tested were plated by the soft agar-overlay method with a background of galactose-negative cells (strain 2) on EMB-galactose agar plates. Plates were incubated for 48 to 60 hours at 25°C. Cells carrying phage gave rise to colonies with a surrounding halo of lysis due to spontaneous phage release, whereas cells not carrying phage produced no halo (Levine, 1957).

L. Thermal and Mitomycin C Induction of Lysogens

Lysogens carrying $\underline{tsC}_2$ mutant prophage are stable when grown at 25°C. When these lysogens reached a concentration of 2×10^8 cells/ml, they were pelleted by low speed centrifugation and resuspended in supplemented M9 medium prewarmed to 41°C to initiate thermal induction. Mitomycin C, used to induce $\underline{C}^+$ lysogens, was added to cell suspensions at stated concentrations (Levine, 1961). At various times after mitomycin C addition, samples were diluted 10^{-5} in fresh

medium, and 180 minutes after induction the contents of these tubes were shaken with chloroform and assayed for phage.

M. Addition of Tails

Since phage particles arising from induction of lysogens are always deficient in tails (Israel, 1967), tails were routinely added to the lysates in an amount sufficient to activate all tailless phage. Also, tail production is temperature sensitive (Israel, 1967). Therefore, tails were routinely added to all lysates from infections carried out at 41°C. Control reactions with known concentrations of heads were always run simultaneously to be sure that the tails were active.

N. Equilibrium Banding of Isotopically Labeled Phage

Equilibrium banding of phage was carried out in 5 ml of CsCl, final ρ = 1.5 gm/cm^3. Samples were centrifuged at 28,000 rpm for 38 hours at 20°C in the SW50.1 rotor. Approximately 65 fractions were collected onto strips of 3 MM Whatman chromatography paper. The strips were dried, placed in vials containing 10 ml of scintillation fluid (PPO, 5g; dimethyl POPOP, 300 mg; toluene, one liter) and counted in a TriCarb Scintillation spectrometer.

O. Preparation of Phage DNA

Phage DNA was released from phage coats by the addition of 1% sarkosyl at 65°C for 10 minutes. CsCl banded phage were used for the source of DNA.

P. Equilibrium Banding of Phage DNA

Isotopically labeled phage DNA was centrifuged to equilibrium in CsCl (final volume = 5 ml; ρ = 1.7 gm/cm^3) at 29,000 rpm for 65 hours at 25°C using the SW50.1 rotor. Approximately 95 fractions were collected onto 3 MM Whatman chromatography paper strips. After drying, the strips were placed in vials with 10 ml of scintillation fluid and assayed for radioactivity.

Q. Sedimentation of Phage DNA

To compare the sedimentation rates of various DNA molecules, 0.1 ml volume of isotopically labeled DNA was layered onto a 5 ml 5-20% sucrose gradient (0.02 M tris, 0.01 M EDTA, 0.1% sarkosyl, pH 8.0) and centrifuged for 110 minutes at 48,000 rpm at 25°C in a SW50.1 rotor. Gradients were collected in 100 fractions onto strips of Whatman 3 MM filter paper, dried and assayed for radioactivity.

R. Lysis Procedure and Sucrose Gradients for Intermediate I

The lysis procedure is that used by Botstein (1968). The lysate (0.1-0.2 ml) was layered onto a 5-20% neutral sucrose gradient (0.02 M tris, 0.01 M EDTA, 0.1% sarkosyl, pH 8.0) and centrifuged for 30 minutes at 25,000 rpm at 23°C in a SW50.1 rotor. Gradients were collected in 30-35 fractions onto strips of Whatman 3 MM filter paper. These strips were dried and soaked once in cold 10% trichloroacetic (TCA) acid for 30 minutes, twice in cold 5% TCA for 15 minutes and

and once in acetone for 5 minutes. After the strips were dried, they were assayed for radioactivity.

S. Discontinuous SDS-polyacrylamide Gel Electrophoresis of Phage Particle Proteins

Phage particles were purified in cesium chloride and dialyzed against 1000 volumes of buffered saline. Two volumes of this phage suspension was added to one volume of a mixture of SDS (6%), glycerol (30%), 2-mercaptoethanol (15%), bromphenol blue (0.006%) and tris-phosphate buffer (pH 7.1, 0.03 M). The mixture was boiled for five minutes to dissociate the virion into its component proteins. Cylindrical gels were prepared and run using the SDS-discontinuous buffer system for acrylamide gel electrophoresis as described by Maizel (1971). The gels consisted of a 10 cm resolving gel (7.5% acrylamide) and a 2.5 cm spacer gel (3% acrylamide). Samples containing 10^{11} particles in a volume no larger than 0.1 ml were carefully layered onto the top of the gels. Samples were run using fixed voltage (100 v) until the dye reached the bottom of the tube. The gels were fixed with 20% sulfosalicylic acid for 16 to 24 hours, stained with Coomassie Blue for three hours, and destained by repeated washings with 7% glacial acetic acid.

T. Transduction

Lysates from thermally induced 18($\underline{ts}C_2$) and 18($\underline{ts25.2ts}C_2$) lysogens and lysates from P22 $\underline{c}_1$ and P22 $\underline{am25c}_1$ infections of $\underline{su}^-$ strain 18 were purified in CsCl. The absolute number

of particles in each purified phage suspension was estimated from the optical density of the suspension at 260mμ. The auxotrophic recipient cells were lysogenized with P22 sie mutants to permit transduction without complications produced by killing. The recipients were grown to a concentration of 3-5 x 10^8 cells/ml in L broth, washed twice with buffered saline and infected with the appropriate phage suspension (moi = 5-10). These infected cells were diluted and plated on minimal agar plates to assay for prototrophic transductants.

CHAPTER THREE: RESULTS

I. INFECTIONS WITH P22 ts25 AT THE NONPERMISSIVE TEMPERATURE

A. Host Survival

P22 ts25.2C$_2$ infections at 25°C result in phage production and cell lysis. However, the host cell survives the infection with P22 ts25.2C$_2$ at 41°C, the nonpermissive temperature. Optical density (O.D.) and viable cells increase exponentially with time in parallel for both infected and uninfected cells at 41°C (Figure 1, A & B). Thus, P22 ts25.2C$_2$ infected cells, at the nonpermissive temperature, continue to grow and divide with the same kinetics as uninfected cells.

Survival of P22 ts25C$_2$ infected cells suggests that gene 25 may be involved in an early step. Therefore, various early phage functions were monitored. Among the parameters checked were phage adsorption, release of DNA from the phage capsid, alterations in the rate of DNA synthesis, association of parental phage DNA with the replication complex, expression of exclusion of superinfecting phage, and lysogenization of the host.

B. Adsorption of P22 ts25

Log phase cells, at a concentration of 10^8 cells/ml, were exposed to either P22 C$_2$ (moi = 14) or P22 ts25.2C$_2$ (moi = 10) at 41°C. At various times, samples were diluted into buffered saline plus a few drops of chloroform, imme-

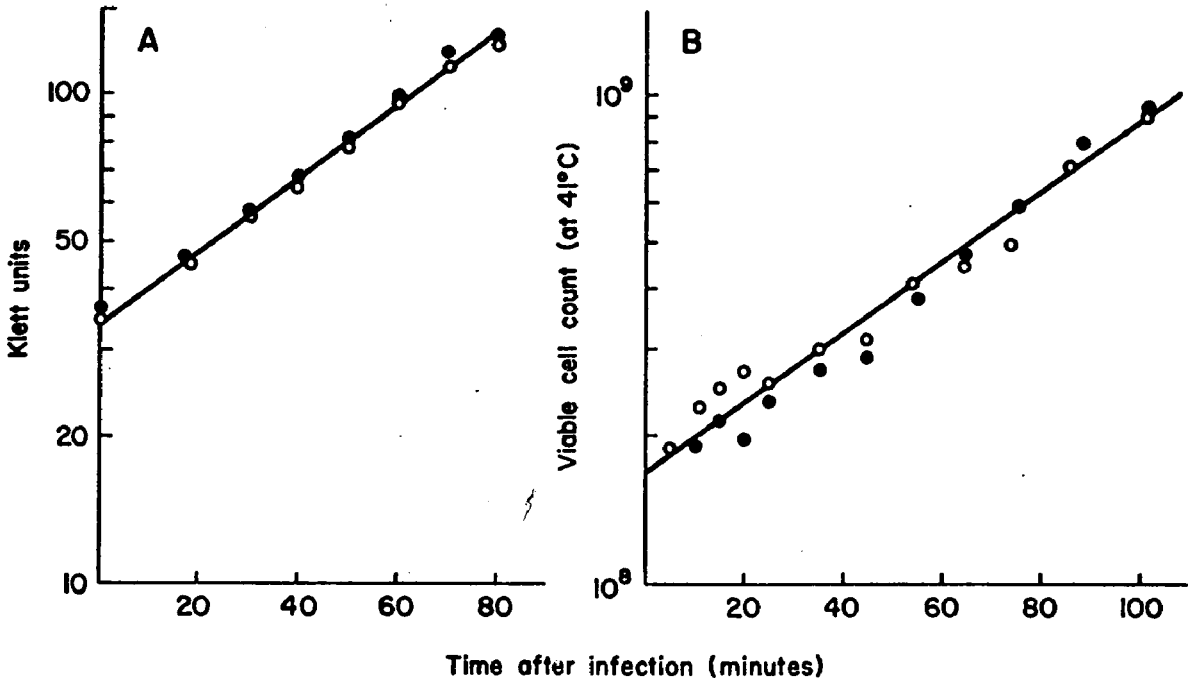


Fig. 1. Survival of P22 ts25.2C$_2$ infected cells at 41°C. Strain 18, grown to a concentration of 2 x 10^8 cells/ml, was infected with P22 ts25.2C$_2$ (moi = 5) at 41°C. (A) For both infected and uninfected cultures, the optical densities were followed using a Klett colorimeter. (B) Samples from both cultures were assayed for colony formation at 41°C. -●-●-, uninfected control cells; -o-o-, P22 ts25.2C$_2$ infected cells.

diately shaken to destroy the cells, and assayed for free phage. The adsorption velocity constants (k) were calculated (Adams, 1959). The average k value obtained for P22 C_2 is 1.67×10^{-8} and for P22 ts25.2C_2 is 2.29×10^{-8}. Thus P22 ts25C_2 adsorbs to host cells with normal kinetics under nonpermissive conditions.

C. Release of DNA from P22 ts25

A large fraction of parental phage DNA becomes acid soluble when starved cells are infected. This breakdown of DNA does not occur after infection of actively metabolizing cells. The mechanism of this phenomenon is not understood but it is presumed to be due to the accessibility of the phage DNA to host nucleases in the starved cells. These observations offer a method to determine if P22 ts25 DNA is released from the capsid. The finding of acid soluble material derived from parental phage DNA in starved cells is taken as a measure of the release of the DNA from the capsids.

Cells, grown to a concentration of 2×10^8/ml, were resuspended in M9 salts without glucose and aerated for 90 minutes at 37°C. The cells were then warmed to 41°C and infected with either ^{3}H-thymidine labeled P22 C_2 or ^{3}H-thymidine labeled P22 ts25.2C_2. At various times after infection samples were assayed for acid soluble DNA.

The amount of parental phage DNA which has become acid soluble is extensive in both infections (Table 1). By 62-63 minutes, 61% of the parental C_2 DNA and 64% of the parental ts25C_2 DNA are broken down to acid soluble products. Thus,

Table 1

The breakdown of parental phage DNA following infection of starved cells at 41°C

Phage	Time (in minutes) after infection	% input DNA made acid soluble
$\underline{C}_2$	0	3.9[a]
	7	30
	32	46
	62	60
	100	60
$\underline{ts25.2C}_2$	0	1.4[a]
	8	31
	33	50
	63	64
	101	65
$\underline{am25}$ defective particles	0	0.8[a]
	9	34
	34	57
	64	67
	102	70

[a]Phage suspensions were added to medium without cells and treated to precipitate acid insoluble DNA. The small number of counts found in the supernatant is probably due to the presence of phage which did not precipitate.

on infection of starved cells at the nonpermissive temperature P22 ts25.2C_2 parental phage DNA is rendered as acid soluble as P22 C_2 DNA. I conclude, therefore, that P22 ts25 DNA is released from the capsid after adsorption to the host cell.

D. Rate of DNA Synthesis

P22 C_2 infections show increasing rates of synthesis of phage DNA from 15 to 45 minutes after infection (Smith and Levine, 1964; Figure 2) and a concommitant drop in the rate of host DNA synthesis (Smith and Levine, 1964; Smith and Levine, 1965; Levine and Schott, 1971). To ascertain whether gene 25 product is a prerequisite for either phage DNA synthesis or turn-off of host DNA synthesis, the rates of DNA synthesis were compared in uninfected cells and in cells infected at 41°C with either P22 ts25.2C_2 or P22 C_2. The rate of DNA synthesis was estimated by measuring the incorporation of ^{3}H-thymidine into acid insoluble material during one minute pulses (Smith and Levine, 1964). P22 C_2 infections (moi = 10) at 41°C (Figure 2) give a steep and sustained rise in the rate of DNA synthesis, reaching a peak at 25 to 30 minutes after infection. At this time the rate falls rapidly and cell lysis begins. In P22 ts25.2C_2 infections, the rate of DNA synthesis increases logarithmically with time in a manner similar to that shown by uninfected cells. Thus, P22 ts25.2C_2 infections have no effect on the rate of DNA synthesis, and it is highly probable that no phage DNA is synthesized.

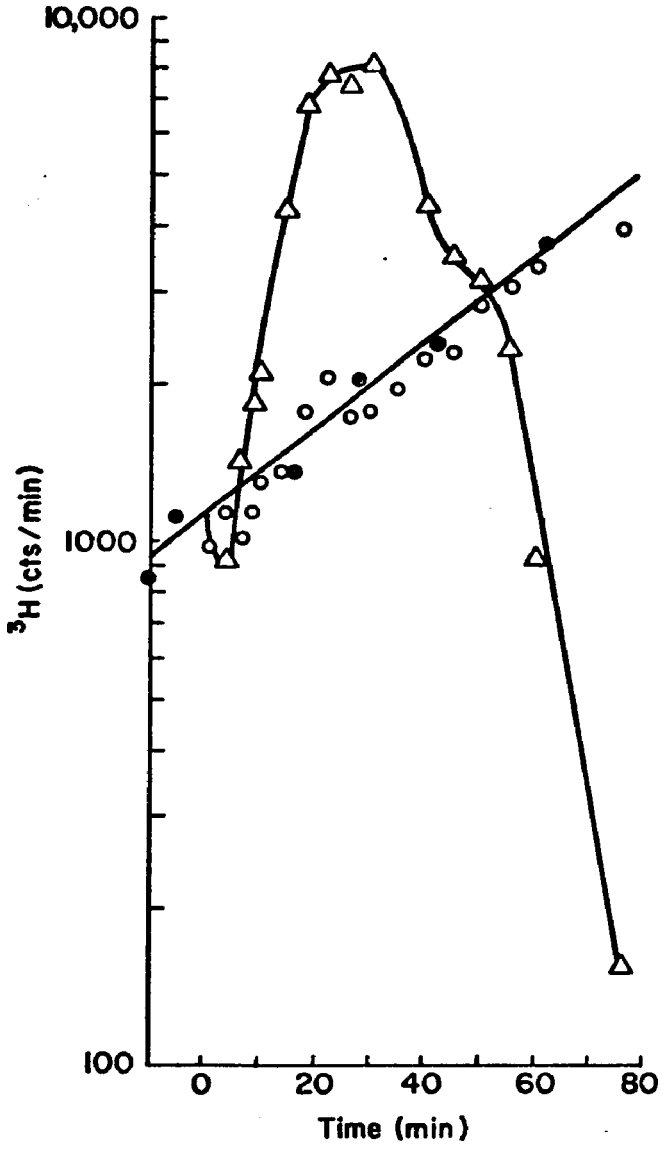


Fig. 2. Rate of incorporation of ^{3}H-thymidine into acid precipitable DNA after infection at 41°C with a multiplicity of 10 P22 $\underline{C_2}$ or 10 P22 $\underline{ts25.2C_2}$. One minute pulses of ^{3}H-thymidine were administered as described by Smith and Levine (1964). -o-o-, uninfected cells; -●-●-, P22 $\underline{ts25.2C_2}$ infected cells; -▼-▼-, P22 $\underline{C_2}$ infected cells.

E. Association of Parental Phage DNA with Intermediate I

A replication complex for phage P22 has been described (Botstein, 1968; Botstein and Levine, 1968 a & b; Levine, Chakravorty and Bronson, 1970). After gentle lysis of cells infected with ^{32}P-labeled phage, the parental phage DNA sediments in a sucrose gradient at a position indicating a sedimentation rate of at least 1000 S. This rapidly sedimenting fraction is referred to as Intermediate I and presumably represents a complex of phage DNA with host cell components. Parental DNA accumulates in Intermediate I, reaching a peak at about fifteen minutes and then is released from the complex. This complex has several properties expected of the replication machinery of phage P22. Intermediate I contains parental DNA, it is the site of synthesis of new phage DNA, and the DNA associated with it is a precursor to the DNA in progeny particles (Botstein, 1968). Association of input DNA with the replication machinery is under immunity control (Levine, Chakravorty and Bronson, 1970).

Association of parental DNA with this replication complex occurs following nonpermissive infections with mutants in genes 12 and 18, which are blocked in the synthesis of phage DNA (Botstein and Levine, 1968 a & b; Levine and Schott, 1971). Host DNA synthesis is inhibited in both mutant infections (Levine and Schott, 1971). Clearly, synthesis of phage DNA is not a prerequisite for association of parental phage DNA with the complex.

Experiments were undertaken to see if DNA from P22 ts25.2C$_2$ associates with Intermediate I under nonpermissive conditions. In a control experiment, 32% of the DNA from ^{32}P-labeled P22 am12C$_2$ infections associates with Intermediate I (Table 2, line 1). In contrast, only 1.2% of the P22 ts25.2C$_2$ parental label is found in the Intermediate I position (Table 2, line 2). It is concluded that P22 ts25.2C$_2$ parental DNA does not associate with the replication complex. In a mixed infection, using ^{32}P-labeled P22 ts25.2C$_2$ and unlabeled P22 am12C$_2$, complementation for phage production occurs (Table 2, line 3). Non-amber phage, which are mostly P22 ts25, with a small percentage of wild type recombinant phage, make up 51% of the total phage yield. Twenty-six percent of the label entering the cell as ts25 DNA is recovered in Intermediate I. Thus, in mixed infections, when P22 ts25.2C$_2$ is present in the yield, P22 ts25.2C$_2$ parental DNA is found in the replication complex.

F. Exclusion of Superinfecting Phage

Wild type P22 lysogens prevent growth of superinfecting phage P22 not only by the immunity system but also by an exclusion mechanism (Walsh and Meynell, 1967; Rao, 1968). Superinfecting P22 can neither replicate, complement, nor recombine with the prophage genome when immunity is released by induction (Rao, 1968). Within a matter of a few minutes after a primary infection with phage P22, secondary infecting phage are excluded from participating in phage production, complementation, and recombination. Exclusion of

Table 2

Association of parental phage DNA with Intermediate I

	Phage	Total radioactivity recovered (%)	Proportion recovered in intermediate I[a] (%)	Burst size[b]
1.	<u>am12C$_2$</u>	73	32	101
2.	<u>ts25.2C$_2$</u>	98	1.2	0.45
3.	<u>ts25.2C$_2$</u> x <u>am12C$_2$</u>	117	26	58

<u>Su$^+$</u> strain 192 was infected with 10 ^{32}P-labeled phage per cell at 41°C. For the mixed infection P22<u>ts25.2C$_2$</u> was ^{32}P-labeled and P22<u>am12C$_2$</u> was not. Twenty-three minutes after infection an aliquot was removed and lysis was carried out as described in materials and methods. 0.2 ml of lysate was layered on a neutral sucrose gradient and the gradients were centrifuged for 30 minutes at 25,000 rpm in a SW50.1 rotor at 23°C.

[a]Proportion recovered in intermediate I is radioactive material recovered in the first six fractions of the gradient divided by the total radioactive material recovered in the gradient expressed as a percentage.

[b]The burst size is defined as the number of plaque forming particles divided by the number of infected cells.

superinfecting phage P22 by prophage and by a primary infecting phage is due to the same exclusion mechanism. Mutants of P22 have been isolated which no longer exclude superinfecting phage; thus, exclusion is under genetic control. These mutants are designated sie for superinfection exclusion (Walsh and Meynell, 1967; Rao, 1968; Susskind, Wright and Botstein, 1971). Since P22 ts25 is defective early after infection and exclusion occurs early after a primary infection, experiments were undertaken to determine whether P22 ts25 can establish exclusion at the nonpermissive temperature.

Infections were carried out in su- strain 18 at 41°C, conditions which are nonpermissive for both mutant phages P22 ts25 and P22 am12. Sequential infections, using one of the mutant phages for the primary infection and normal P22 C_2 as the secondary infecting phage (added 15 minutes after the primary infection) will give infective centers and high yields only if P22 C_2 can actively participate in the infection. An infective center is defined as an infected bacterium which can form a plaque. If the primary infecting phage has established exclusion at the time of superinfection with P22 C_2, then both infective centers and yields are expected to be low compared to simultaneous mixed infections. As a control, infections were carried out with P22 am12C_2, an early mutant which does not synthesize phage DNA. A simultaneous mixed infection with P22 am12C_2 and P22 C_2 gives the expected number of infective centers and a normal phage

yield (Table 3, line 1). The sequential infection, with P22 am12C_2 as the primary infecting phage and P22 C_2 as the secondary infecting phage, gives one-tenth the number of infective centers and one-tenth the number of progeny phage (Table 3, line 2). That is, by 15 minutes after infection, P22 am12C_2 has established exclusion, and done so in spite of the absence of DNA synthesis. In contrast, P22 ts25.1C_2 cannot initiate superinfection exclusion. There are no differences between the simultaneous and sequential infections (Table 3, lines 3 & 4). In both cases P22 C_2 can actively participate in the infection.

G. Lysogenization of the Host Cell

Mutations in genes 12 and 18 affect functions necessary for phage DNA synthesis and also decrease the frequency of lysogeny (Levine and Schott, 1971). P22 ts25, which also does not synthesize phage DNA, was studied to ascertain whether it is capable of lysogenizing the host cell.

Normal frequencies of cell survival are observed after P22 ts25.1C^+ infection at 41°C. However, in contrast to a wild type infection, these cells do not give rise to lysogenic progeny. This is demonstrated by a study of the segregation pattern of infected cells. Infection of log phase cells was carried out at 41°C with a multiplicity of 20 phage. After a five minute adsorption period the cultures were diluted to 10^3 cells/ml in supplemented M9 medium containing anti-phage serum (K = 4). At intervals samples were plated on EMB-galactose agar using a galactose-negative mutant as

Table 3

Establishment of superinfection exclusion
by a primary infection

	Primary infection	Secondary infection	Infective centers[a]	Phage yield
1.	$\underline{am12C_2sie}^+ \times \underline{C_2}$		2.4×10^8	2.1×10^{10}
2.	$\underline{am12C_2sie}^+$	$\underline{C_2}$	2.8×10^7	2.2×10^9
3.	$\underline{ts25.1C_2sie}^+ \times \underline{C_2}$		2.1×10^8	4.7×10^{10}
4.	$\underline{ts25.1C_2sie}^+$	$\underline{C_2}$	2.7×10^8	6.0×10^{10}

Infections were carried out, using multiplicities of 10 for each phage type, in $\underline{su}^-$ strain 18 at 41°C, the nonpermissive condition for both conditional lethal mutants.

[a]Infective centers were assayed on $\underline{su}^-$ strain 18 at 41°C.

indicator bacteria and incubated at 25°C for the determination of the number of sensitive cells and the number of cells carrying phage genomes.

In the control P22 C+ infection at 41°C (Figure 3A) more than 95% of the cells survive as phage carriers. They begin dividing after a lag of about one generation, and thereafter the total number of cells increases logarithmically. The proportion of phage carriers to total cells remains high for a few divisions, but eventually cells segregate as sensitives and phage carriers. The ratio of phage carriers to total cells remains constant after about four generations. The simplest explanation for these data is that after an early period of segregation of intracellular phage genomes, prophage integration occurs and thereafter the capacity to produce phage is perpetuated in parallel with growth as expected of stable lysogenic cells (Smith and Levine, 1967).

The segregation pattern of cells infected with P22 ts25.1C+ at 41°C is different from the control infection (Figure 3B). Although a high proportion of cells survives, only about half are phage carriers. The total number of cells increases logarithmically with no lag after infection and the absolute number of phage carriers decreases. Thus, P22 ts25.1C+ does not lysogenize the host.

This result is also different from that described for int phage mutants (Smith and Levine, 1967) and mutants in genes 12 and 18 (Levine and Schott, 1971). Nonpermissive

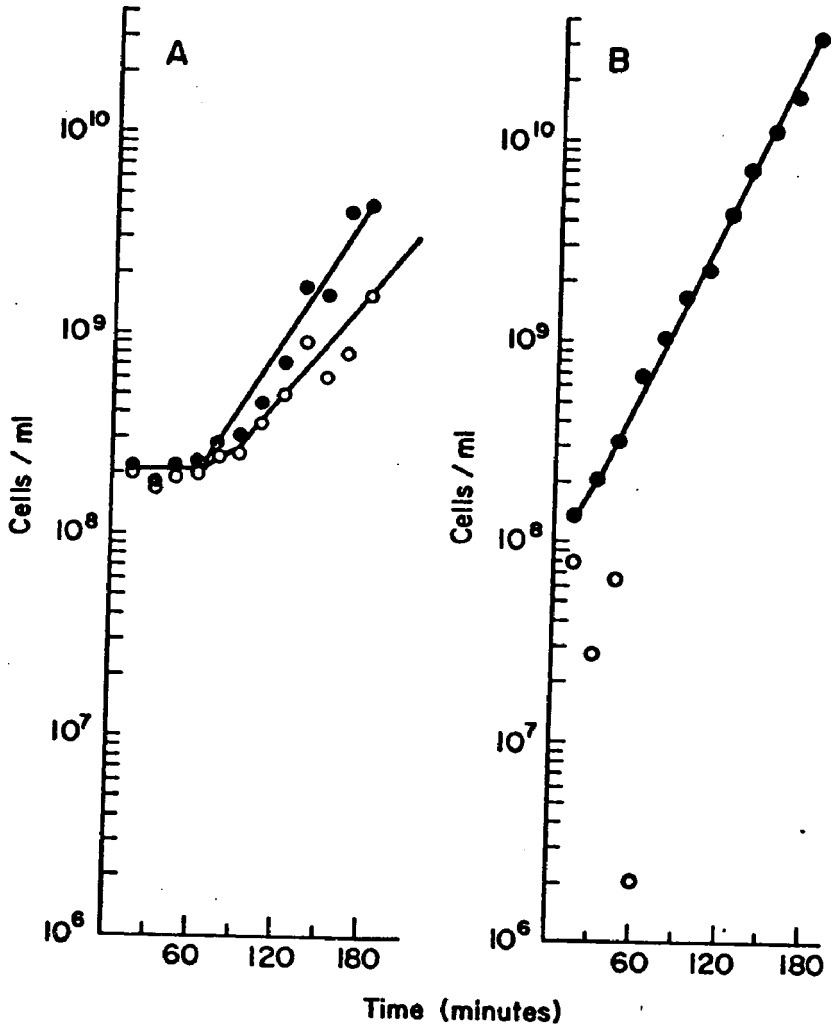


Fig. 3. Segregation of phage-carrying and sensitive cells at 41°C following infection with either (A) P22 $\underline{C}^+$ or (B) P22 $\underline{ts25.1C}^+$. -●-●-, total number of cells; -o-o-, number of phage-carrying cells.

infections with any of these three mutants show a high proportion of the cells surviving as phage carriers, but after some increase in cell titer the absolute number of phage carriers remains constant. The ratio of phage carriers to total cells continues to decrease with each division, indicating the absence of stable lysogeny. This is contrasted by P22 ts25.1C$^+$ infections where the absolute number of phage carriers decreases, as though the phage genomes are lost.

Mixed infections with P22 am12C$^+$ and P22 ts25.1C$^+$ in amber suppressor strain 192 at 41°C give some lysogens with ts25.1 prophage; therefore, P22 ts25 can be complemented for lysogeny. Ts25 mutant lysogens, formed either by complementation or by infection with P22 ts25 at 25°C, are stable at 41°C. Therefore, it can be concluded that gene 25 product is necessary for the establishment of lysogeny but not for the maintenance of the lysogenic state.

II. INDUCTION OF ts25 MUTANT LYSOGENS

At 41°C, the nonpermissive temperature, P22 ts25 adsorbs to the host cell with normal kinetics and releases its DNA from the capsid. However, no other phage functions are detected. Induction of lysogens carrying ts25 mutant prophage was carried out to determine if any phage functions are expressed under nonpermissive conditions.

A. Thermal Induction

Lysogens 18(ts25.2tsC_2) and 18(tsC_2) were grown to 10^8 cells/ml at 25°C, concentrated by centrifugation, and resuspended in prewarmed (41°C) M9CAA medium. This treatment results in induction of both lysogens, as measured by cell lysis. The lysis curves of the heat treated 18(ts25.2tsC_2) closely parallels the control (Figure 4a). There is a rise in O.D. followed by a depression indicating cell lysis. This is to be sharply contrasted with the uninterrupted growth of cells after infection with P22 ts25.2C_2 at 41°C.

(i). DNA Synthesis After Induction. The rates of DNA synthesis for the thermally induced lysogens were determined (Figure 4b). For both thermally induced ts25 and ts25$^+$ lysogens there is a depression in the rate between 10 and 20 minutes followed by a sustained rise, reaching a peak after forty minutes, followed by a rapid fall. Thus, the pattern of the rate of DNA synthesis after thermal induction of ts25 lysogens is similar to that of ts25$^+$ lysogens. Again, this result is different from that obtained after infection.

(ii). Infectious Particle Formation. The yield of active phage following thermal induction of ts25 lysogens is low compared to that of ts25$^+$ lysogens (Table 4). Two possible reasons for this can be put forward. (1) Induction occurs, but total particle production is markedly reduced. (2) Total particle production is normal, but most of the particles are noninfectious.

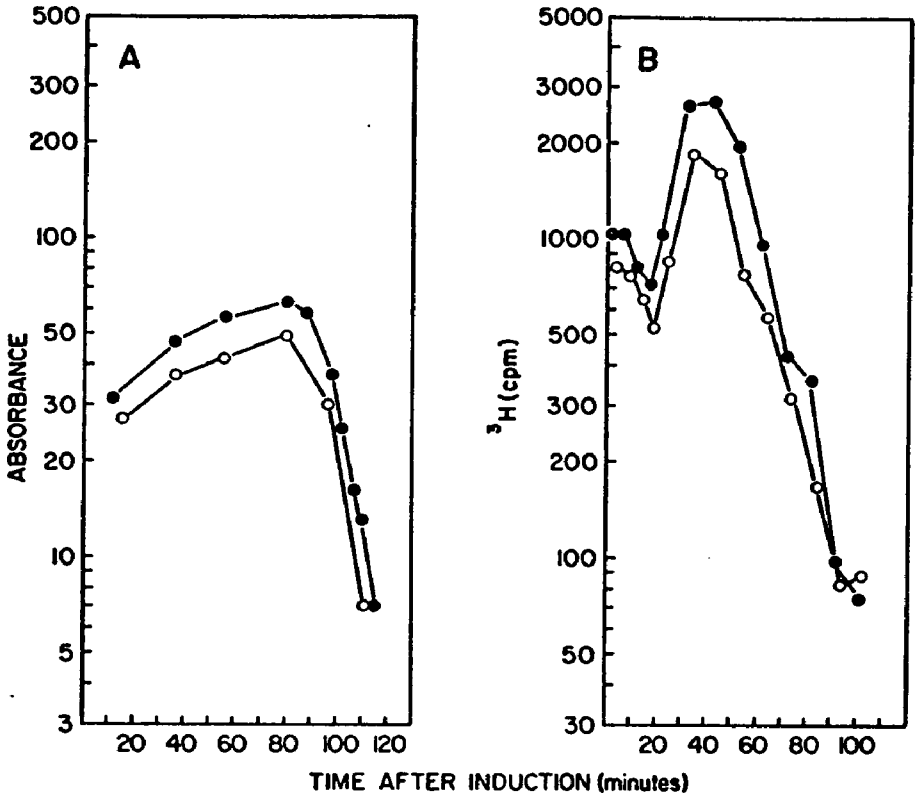


Fig. 4. Thermal induction of 18(<u>tsC$_2$</u>) and 18(<u>ts25.2tsC$_2$</u>) at 41°C as followed by optical density (A) and rate of incorporation of ^{3}H-thymidine into acid precipitable DNA (B). One minute pulses of ^{3}H-thymidine were administered as described by Smith and Levine (1964). -●-●-, 18(<u>tsC$_2$</u>); -o-o-, 18(<u>ts25.2tsC$_2$</u>).

The total yield of particles can be assayed by determining the $OD_{260\,m\mu}$ of a purified phage preparation. The phage lysates obtained from the thermal induction experiment of Figure 4 were concentrated and banded in CsCl. Both lysates gave visible bands in the same position. The $OD_{260\,m\mu}$ for the band from the mutant lysate is one-and-a-half times greater than the value obtained for the control (Table 4, column 3). Thus, total particle production is not reduced on induction of ts25 lysogens. However, for an equal concentration of particles, determined by $OD_{260\,m\mu}$, the mutant infectious yield is reduced by a factor of 30 (Table 4; active phage/$OD_{260\,m\mu}$). Therefore, the induced P22ts25 lysate must consist largely of particles which are noninfectious or defective at any temperature.

B. A Plaque Assay for Defective Particles.

A simple assay for defective particles based on a complementation test was developed. Mixed infections were carried out at 25°C in su⁻ strain 18 with P22 am12C$_2$ (moi = 5), so that all cells were infected, and with low multiplicities (less than 0.5) of ts25 defective particles, so that each cell contained no more than one defective particle. If complementation for the function missing from the defective particle occurs, the number of infective centers, using su⁻ strain 18 as the plating bacteria, should correspond to the cells which are mixedly infected. (An infective center is an infected cell which can form a plaque). These should equal the number of defective particles in the suspension.

Table 4

Evidence for defective particle formation following thermal induction of lysogenic strain 18(ts25.2tsC$_2$)

Lysogen	Active phage/ ml	OD_{260} mμ/ cm	Active phage/ OD_{260} mμ
18(tsC$_2$)	2.4×10^{11}	3.8	6.3×10^{10}
18(ts25.2tsC$_2$)	1.1×10^{10}	5.6	2.0×10^{9}

Optical density at 260 mμ wavelength and active phage/ml were measured on CsCl purified lysates of thermally induced lysogens. The ratio, active phage/OD_{260}, gives a measure of the viability of the phage suspensions.

The infective centers from single infections using only the thermally induced P22 ts25 suspension will be equal to the number of active phage in the suspension.

Ts25 defective particles can indeed be complemented (Table 5). Mixed infections show significant increases in infective centers compared to single infections: infection 3 shows a 23 fold increase compared to infection 1. Additional evidence that the defective particles are complemented for phage production is that the burst size for mixedly infected cells (infection 3) is high: 154, with both amber and temperature sensitive phage present in the yield.

The following observations provide evidence that the number of infective centers in the previously described mixed infection is a valid assay for defective particles. (1) There is a linear relationship between infective centers and defective particle multiplicity as long as each cell is infected with no more than one defective particle (Table 5, compare infection 3 and 5). Dilution of the defective particle suspension by a factor of 10 results in a ten fold decrease in infective centers. (2) In the induced ts25 lysate the number of defective particles per ml determined by the plaque assay (4.2×10^{11}) agrees with the expected number calculated by optical density (3.5×10^{11}). (3) Both the plaque assay and $OD_{260m\mu}$ give similar ratios of total particles to active phage (28 by the plaque assay and 31 by $OD_{260m\mu}$) for the lysate from thermal induction of ts25 lysogens. (4) Lysates obtained from thermal induction of $ts25^{+}$ lysogens show no difference in active phage/ml

Table 5

A plaque assay for ts25.2 defective particles[a]

	Phage	MOI[b]	Infective Centers[c]
1.	ts25.2 defective particles	0.05	3.4×10^5
2.	am12C$_2$	5	$< 10^4$
3.	ts25.2 defective particles x am12C$_2$	0.05:5	7.8×10^6
4.	ts25.2 defective particles	0.005	3.2×10^4
5.	ts25.2 defective particles x am12C$_2$	0.005:5	9.0×10^5

1.6×10^8 cells were infected at 25°C.

[a] Ts25.2 defective particles were obtained from thermal induction of strain 18(ts25.2tsC$_2$). Sufficient tails were added to saturate all heads.

[b] It was possible to estimate the moi of ts25.2 defective particles by using OD_{260} mμ/cm to determine the concentration of particles.

[c] Infective centers were plated on su$^-$ strain 18 at 25°C.

and total particles/ml using the plaque assay. This is the expected result if there are no defective particles in the phage suspension.

The term total particles will be used to refer to the number of infective centers in the plaque assay described above. Any difference between infective centers in single infection (active phage) and infective centers in mixed infection (total particles) is due to the presence of defective particles.

C. Mitomycin C Induction

Thermal induction of ts25 lysogens results in cell lysis and particle production. This response is in dramatic contrast to the apparent absence of expression of early phage functions after infection with P22 ts25 at 41°C. Since lysogens carrying a prophage with the temperature sensitive C_2 allele must be grown at 25°C, it might be argued that the response after thermal induction of ts25 lysogens is due to functional gene 25 product which has accumulated during growth of the culture at 25°C. To determine whether this is the case, ts25.2C$^+$ lysogens, carrying the wild type C_2 allele, were grown at 41°C and treated with mitomycin C at 41°C. If these lysogens induce, then accumulation of gene 25 product would not explain the thermal induction data. It would then be reasonable to conclude that gene 25 product is not needed for the expression of early genes on induction of lysogens.

As a control, 18(ts25.2C$^+$) was grown and treated with mitomycin C at 25°C. Cells exposed to mitomycin C for 15 minutes produce high yields of active phage (Table 6, line 2). The total number of particles produced, determined by the plaque assay for defective particles, is equal to the number of active phage produced, showing that there are no defective particles in this lysate. Mitomycin C induction of 18(ts25.2C$^+$), grown and induced at 41°C, results in low yields of active phage (Table 6, lines 4,5). However, the total number of particles produced is not significantly different from the number produced by induction at 25°C, the permissive temperature. The ratio of total particles to active phage in these lysates is between 25-35, which is similar to the ratios found in thermally induced lysates. The ratio is close to one for the lysate induced at 25°C. Thus, phage are produced following mitomycin C induction of 18(ts25.2C$^+$), grown and induced at 41°C; however, most of the progeny are defective. These observations are consistent with the conclusion that gene 25 product is not necessary for the expression of early genes after induction of lysogens, but it is necessary late for normal infectious phage production.

D. Revertants of ts25 Mutants

To be sure that the phenotype of the ts25 mutant is due to a single mutation, revertants at the gene 25 locus were examined. Revertants were selected by plating large numbers of P22 ts25.2ts2C$_2^{30}$ at 41°C. Phage which form a

Table 6

Mitomycin C induction of 18(ts25.2C$^+$)

	Temperature	Exposure time to mitomycin C	Lysate		
			Active phage/ml	Total particles/ml	Total particles/ active phage
1.	25°C	untreated	2.5×10^8		
2.	25°C	15 minutes	1.3×10^{10}	1.3×10^{10}	1
3.	41°C	untreated	2.6×10^6		
4.	41°C	10 minutes	1.4×10^8	4.2×10^9	30
5.	41°C	20 minutes	2.0×10^8	6.4×10^9	33

18(ts25.2C$^+$) was grown at 41°C to a concentration of 2×10^8 cells/ml in the presence of antiserum (k = 2). The culture was split; half was incubated at 25°C and the other half at 41°C. Mitomycin C, at a final concentration of 5 μg/ml was added to each culture. After stated exposure times, samples were diluted out of mitomycin C and kept in supplemented M9 medium for a total of 180 minutes. The lysates were chloroformed and sufficient tails were added to saturate all heads. The lysates were assayed for active phage and total particles.

plaque at 41°C are no longer temperature sensitive for an essential function, and therefore, are ts25$^+$. A lysogen was made from a gene 25 revertant which still retained the tsC_2 allele. Thermal induction of this lysogen produces normal yields of active phage and no defective particles. Thus, the P22 ts25 phenotype, though different after infection and induction, is due to a single mutation.

III. GENE 25 PRODUCT IS REQUIRED BOTH EARLY AND LATE AFTER INFECTION

A. Statement of the Hypothesis

A hypothesis has been developed to explain the contrasting responses with gene 25 mutants after infection and induction. Gene 25 is the structural gene for a protein. This is a component of the virion which acts very early in the infective process for the initiation of phage functions after adsorption and release of DNA. Mutant phage with a temperature sensitive gene 25 allele contain heat labile gene 25 protein. Therefore, a P22 ts25 infection at high temperature is blocked early because of inactive gene 25 protein. The early function requiring gene 25 is specific for infection; it is not necessary after induction. Therefore, induction of ts25 lysogens at high temperature proceeds normally with the expression of early genes, cell lysis, and particle production. Gene 25 product is synthesized late and is incorporated into the virion during assembly. Failure to do this during either infection

or induction results in the formation of defective particles which are not infectious in any host or at any temperature.

The hypothesis predicts that if cells are infected with P22 ts25 at 25°C, and are shifted to 41°C, after allowing sufficient time for the early step to occur, then both early and late phage genes will be expressed. Phage DNA synthesis, cell lysis and particle production will take place; however, these particles will be defective, just like the ones produced after induction of ts25 lysogens at 41°C. This type of experiment was carried out as described below.

B. Temperature Shift-Up Experiment

Sensitive cells were infected at 25°C with P22 ts25.2C$_2$ (moi = 5). After adsorption, free phage were inactivated by antiserum and diluted 10^4 fold. At intervals three samples were removed. One sample was plated for a single-step growth curve, the second was immediately shaken with chloroform and assayed for intracellular phage, and the third was shifted to 41°C and incubated for a total of 180 minutes. The latter samples were then shaken with chloroform and assayed for both active phage and total particles.

Figure 5 presents the results of the temperature-shift experiment. The latent period at 25°C is about 80 minutes and intracellular phage start to accumulate at about 60 minutes. A ten minute incubation at 25°C, followed by a shift to 41°C for the remaining time, is sufficient to give a total particle yield exceeding that obtained in the

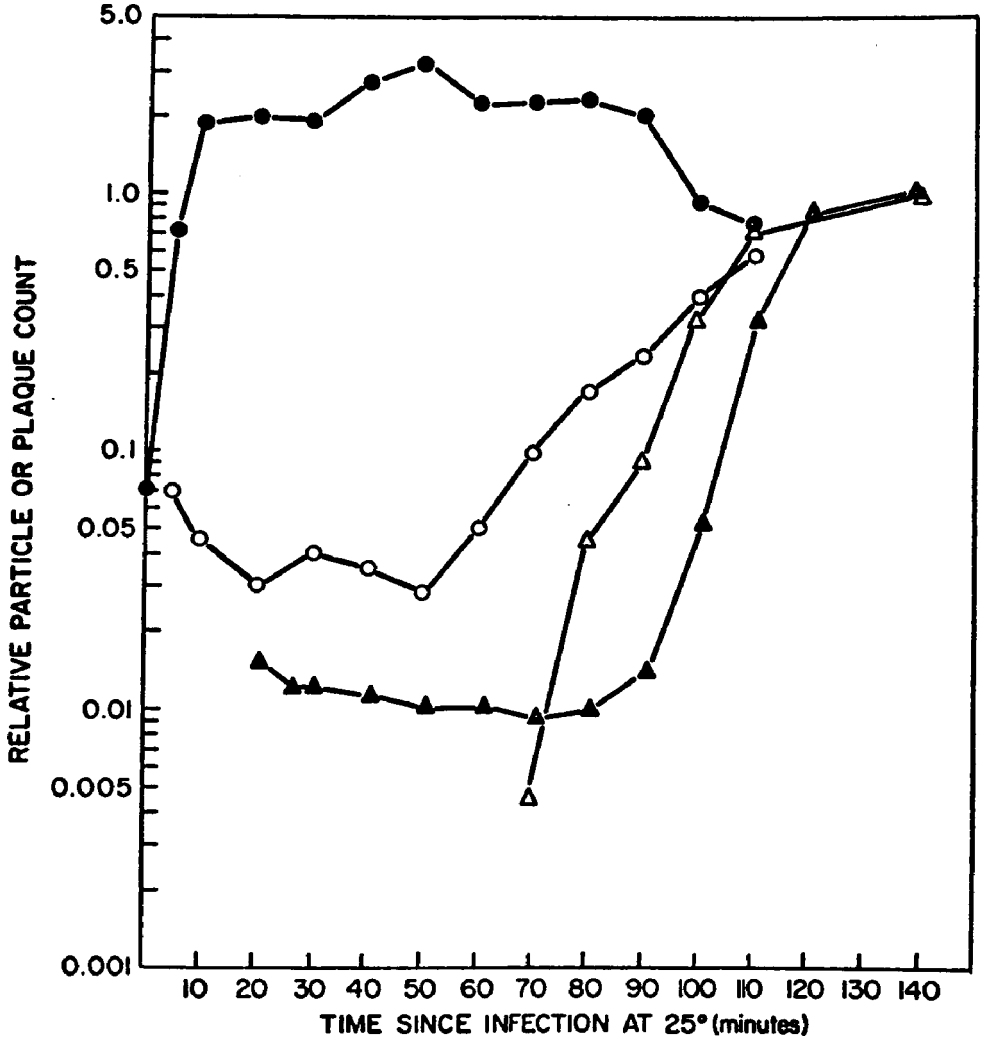


Fig. 5. Temperature-shift experiment. The infection was initiated at 25°C with P22 ts25.2C$_2$ (moi = 5). At intervals 3 samples were removed. One was plated for a single-step growth curve (-▼-▼-), the second for intracellular phage (-▼-▼-), and the third was shifted to 41°C, incubated for a total of 180 minutes, chloroformed, and assayed for total particles (-●-●-) and active phage (-o-o-). All numbers are relative to the active phage titer in the lysate from the permissive infection.

permissive infection. (The total particle yields for samples shifted to 41°C prior to 100 minutes are always slightly larger than the yield in the permissive infection.) These results demonstrate that P22 ts25 infections at 41°C are blocked very early, and the early gene 25 step occurs within the first 10 minutes after infection. However, in agreement with the prediction made from the hypothesis, defective particles, not active phage, are produced.

At the permissive temperature no defective particles are formed; total particles are always equal to active phage. However, active phage production for samples shifted up prior to 50 minutes is substantially reduced compared to the permissive infection. Incubation at 25°C for longer than 50 minutes results in an increase in active phage. This increase is correlated with the rise in intracellular phage. Near normal yields of active phage are not obtained until samples are incubated at 25°C for at least 90 minutes. These results show that there is a second block late in the nonpermissive infection which interferes with the formation of active phage.

Although an early shift-up gives a reduced number of active phage compared to a permissive infection, this phage yield is still ten times greater than that of a nonpermissive infection. This early increase in active phage is caused by leakiness. A similar fraction of the total particles from induction of ts25 lysogens at 41°C is also active phage.

In summary, temperature shift experiments show that gene 25 product is required both early and late during

infection for active phage production. If the gene 25 protein is rendered inactive early, then no phage functions are detected after release of the DNA from the capsid. If the 25 protein is inactivated between ten and fifty minutes after infection, the early step requiring gene 25 protein has occurred and phage particles are produced. However, most of these particles are defective since gene 25 protein is also required late for active phage formation. An early shift-up, permitting the first block to be bypassed, creates a situation analagous to induction of ts25 lysogens at 41°C. Samples incubated at 25°C for ten minutes prior to shifting to 41°C lyse, and release large numbers of defective particles.

IV. AMBER MUTANTS OF GENE 25

A. Expected Phenotype

According to the above hypothesis, amber mutants in gene 25 should have the phenotype of a late mutant. The gene 25 protein synthesized in the amber suppressor host and packaged in the phage particle should be functional. Thus, the early step requiring gene 25 product would occur in a P22 am25 infection of either a su^+ or a su^- host. However, in a su^- host, only fragments of the gene 25 protein would be synthesized, resulting in the production of defective particles unable to successfully infect either a su^+ or a su^- host.

B. Complementation Tests

Two amber mutants, P22 amN121 and P22 amH60, previously assigned to gene 16 fail to complement with P22 ts25.2C$_2$. Mixed infections at 41°C in a su$^-$ host do not give yields significantly different than those from single infections (Table 7, compare infections 1,2,6; 1,3,7). In contrast, P22 ts18C$_2$, an early temperature sensitive mutant which complements P22 ts25.2C$_2$, also complements P22 amN121 (Table 7, compare infections 3,4,8). In addition, P22 am20, a late amber mutant, complements P22 ts25.2C$_2$ (Table 7, compare infections 1,5,9).

The absence of complementation between am16 and ts25 mutants may be only for the early gene 25 step. Infections were carried out to look at complementation for the late step. Infections were initiated at 25°C in su$^-$ strain 18. After 20 minutes the infected cells were shifted up to 41°C and kept there long enough for phage production and cell lysis to take place. The formation of defective particles in the shift-up single infection using only P22 ts25.2C$_2$ demonstrates that the procedure permits completion of the early step (Table 8, infection 1). The shift-up mixed infection with P22 ts25.2C$_2$ and P22 amN121C$_1$ gives an active phage yield no larger than the mixed infection carried out at 41°C (Table 8, infection 2,3). Thus, P22 ts25.2C$_2$ and P22 amN121 do not complement for the late step requiring gene 25 product.

Table 7

Complementation between P22ts25.2C$_2$ and amber mutants

	Phage	Active phage in lysate
1.	ts25.2C$_2$	2.1×10^7
2.	amH60C$_1$	1.4×10^6
3.	amN121C$_1$	8.7×10^6
4.	ts18C$_2$	6.2×10^7
5.	am20C$_2$	3.0×10^6
6.	ts25.2C$_2$ x amH60C$_1$	7.2×10^6
7.	ts25.2C$_2$ x amN121C$_1$	5.4×10^7
8.	ts18C$_2$ x amN121C$_1$	2.3×10^{10}
9.	ts25.2C$_2$ x am20C$_2$	9.6×10^9

Su$^-$ strain 18, at a concentration of 2×10^8 cells/ml, was infected at 41°C with phage at a multiplicity of 5 for each genotype.

Table 8

Complementation for the late step requiring gene 25

	Phage	Temperature		Lysate	
		initial 20 min	subsequent 100 min	active phage	total[a] particles
1.	$\underline{ts25.2C_2}$	25°	41°	1.3×10^8	6.3×10^9
2.	$\underline{ts25.2C_2}$ x $\underline{amN121C_1}$	25°	41°	1.0×10^7	4.0×10^9
3.	$\underline{ts25.2C_2}$ x $\underline{amN121C_1}$	41°	41°	9.0×10^6	6.4×10^9
4.	$\underline{amN121C_1}$	41°	41°	7.0×10^5	4.3×10^9
5.	$\underline{amH60C_1}$	41°	41°	1.0×10^6	1.7×10^{10}

$\underline{Su}^-$ strain 18, at a concentration of 2×10^8 cells/ml, was infected with phage at a multiplicity of 5 for each genotype.

[a]Total particles were determined by the plaque assay for defective particles which has been described. The plaque assay was adapted to test for $\underline{am25}$ defective particles as well as $\underline{ts25}$ defective particles.

C. Production of am25 Defective Particles

By complementation tests mutants assigned to gene 16 and gene 25 appear to be in the same cistron. The amber 16 mutants should then have the phenotype described above for am25 mutants. This appears to be the case. Nonpermissive infections with either P22 amN121 or P22 amH60, result in cell lysis and defective particle production (Table 8, infections 4,5). Thus the amber mutations in gene 16 were unnecessarily assigned to a separate gene, and from now on will be referred to as gene 25 mutants.

D. Complementation Between am25 Defective Particles and ts25 Defective Particles

Am25 defective particles and ts25 defective particles should be deficient for the same function and would not be expected to complement each other. The plaque assay previously described was adapted to test for complementation between the two types of defective particles.

Su^+ strain 192, infected at 25°C with both defective particles at varying multiplicities, was diluted and plated for infective centers under conditions which distinguish between cells effectively infected with either temperature sensitive or amber phage. Table 9, infections 1 through 4, shows the number of infective centers formed by active phage present in the defective particle preparations. Mixed infections were compared with these single infections for an increase in infective centers above these background levels to indicate complementation. Complementation for ts25

Table 9

Complementation between *am25* defective particles and *ts25* defective particles

	Defective	MOI	Infective centers	
			Su$^-$, 25°[a]	*Su*$^+$, 41°[b]
1.	*ts25*	0.1	1.3×10^6	$< 10^3$
2.	*ts25*	10	1.1×10^8	$< 10^3$
3.	*am25*	0.1	$< 10^3$	3.6×10^5
4.	*am25*	10	$< 10^3$	4.0×10^7
5.	*ts25* x *am25*	0.1:10	1.0×10^6	1.6×10^7
6.	*ts25* x *am25*	10:0.1	9.0×10^7	1.3×10^5

Su$^+$ strain 192, at a concentration of 2×10^8 cells/ml, was infected at 25°C with the designated multiplicities of defective particles.

[a]These are the number of infected cells which produce temperature sensitive (or wild type recombinant) phage, and are, therefore, effectively infected with a temperature sensitive genome.

[b]These are the number of infected cells which produce amber (or wild type recombinant) phage and are, therefore, effectively infected with an amber genome.

defective particles by *am25* defective particles (Table 9, infection 5) should result in an increase in infective centers on *su*$^-$ indicator bacteria at 25°C compared to the *ts25* defective particle single infection (Table 9, infection 1). No such increase is observed. Complementation for *am25* defective particles by *ts25* defective particles (Table 9, infection 6) should result in an increase in infective centers on *su*$^+$ indicator bacteria at 41°C compared to the *am25* defective particle single infection (Table 9, infection 3). Again, no increase occurs. In conclusion, there is no complementation between *ts25* and *am25* defective particles. This result is expected if both particles have the same defect.

V. CHARACTERIZATION OF DEFECTIVE PARTICLES

Gene *25* defective particles are not infectious at any temperature or in any host. Experiments were carried out to ascertain the structural defect in these particles.

A. Density of Defective Particles

The loss of infectivity of the defective particles may be due to an alteration in either the protein or the DNA composition of the virion. If this is the case, it is possible that the density of the particle will be changed. The density of the defective particles was compared with the density of wild type phage by equilibrium centrifugation in CsCl. ^{3}H-thymidine labeled *ts25.2* defective particles obtained by thermal induction [saturated with tail parts to

eliminate density variations caused by absent or incomplete tail structures (Israel, 1967)] were mixed with ^{32}P-labeled wild type phage produced by infection and were centrifuged to equilibrium in CsCl. The ^{3}H-labeled defective particles band at a somewhat denser position in the gradient (Figure 6). Also, the densities of defective particles produced by either a shift-up infection with P22 ts25.2C_2 or a P22 amN121C_1 infection of a su$^-$ host are slightly greater than that of wild type phage (Figure 7,8). In a control experiment, the ^{3}H-thymidine labeled preparations of phage obtained from thermal induction of ts25$^+$ lysogens give a peak overlapping that of ^{32}P-labeled phage. Thus, ts25 defective particles have a slightly greater density than wild type phage.

To determine whether the density difference is due to a change in the head of the defective particles, or to an alteration in the tail structure, the buoyant densities of defective particle heads and infectious phage heads were compared. Phage lysates from induction of lysogens are deficient in tails (Israel, 1967).and routinely purified tails are added to compensate for this. However, in this case tails were not added to the induced ts25 defective particle preparation so that the density of the defective particle heads could be examined. Wild type phage heads were formed after infection, as described in Materials and Methods. When ^{3}H-thymidine labeled ts25.2 defective particle heads were mixed with ^{32}P-labeled phage heads and spun to equilibrium in CsCl, again the ^{3}H peak is in a denser position

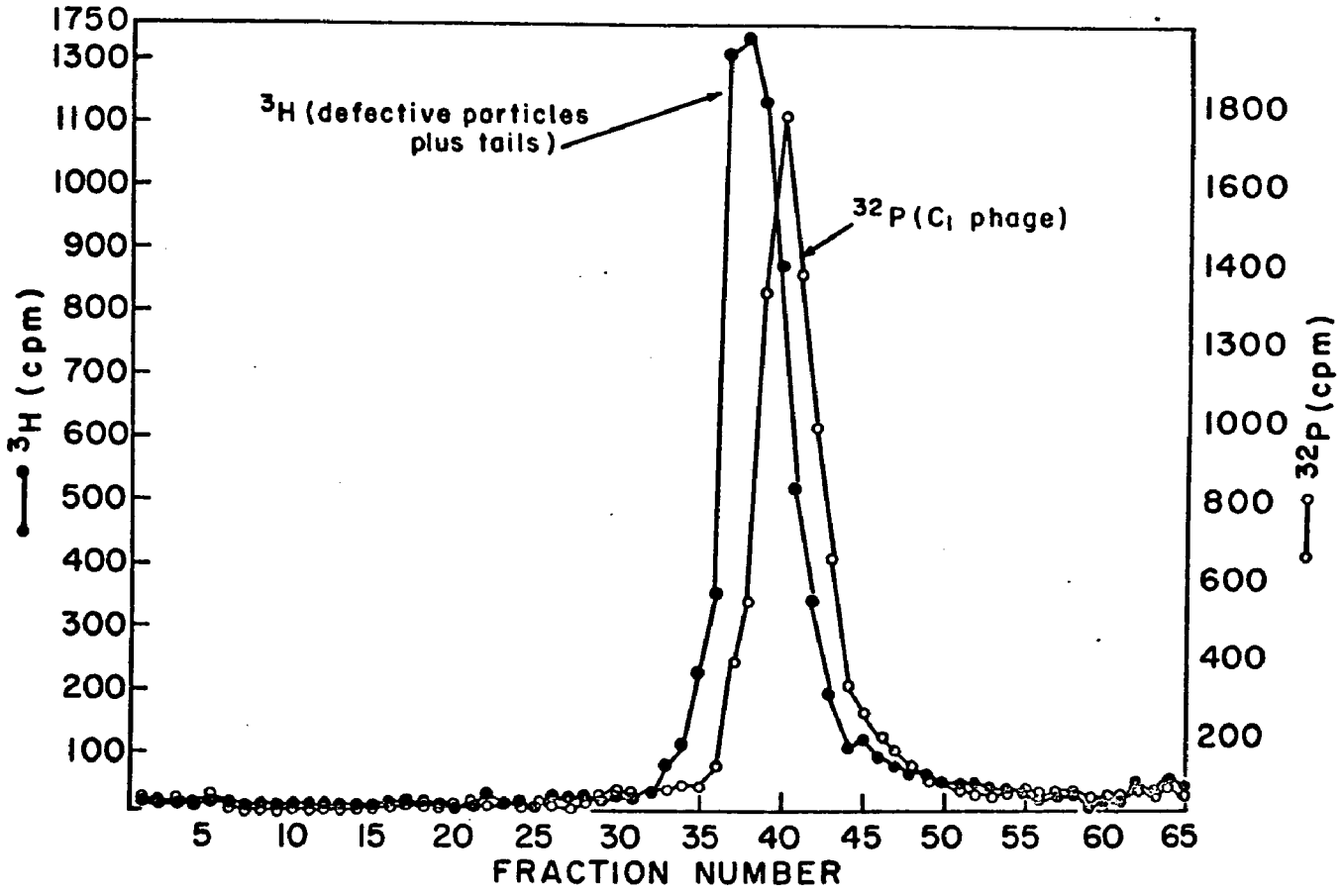


Fig. 6. Density of ^{3}H-thymidine labeled ts25 defective particles from thermal induction [saturated with tail parts to eliminate density variations caused by absent or incomplete tail structures (Israel, 1967)] and ^{32}P-labeled P22 C_1 from infection. Samples were in 5 ml of CsCl, final density of 1.5. Equilibrium banding was carried out at 28,000 rpm for 38 hours at 20°C in the SW50.1 rotor. -●-●-, ^{3}H; -o-o-, ^{32}P.

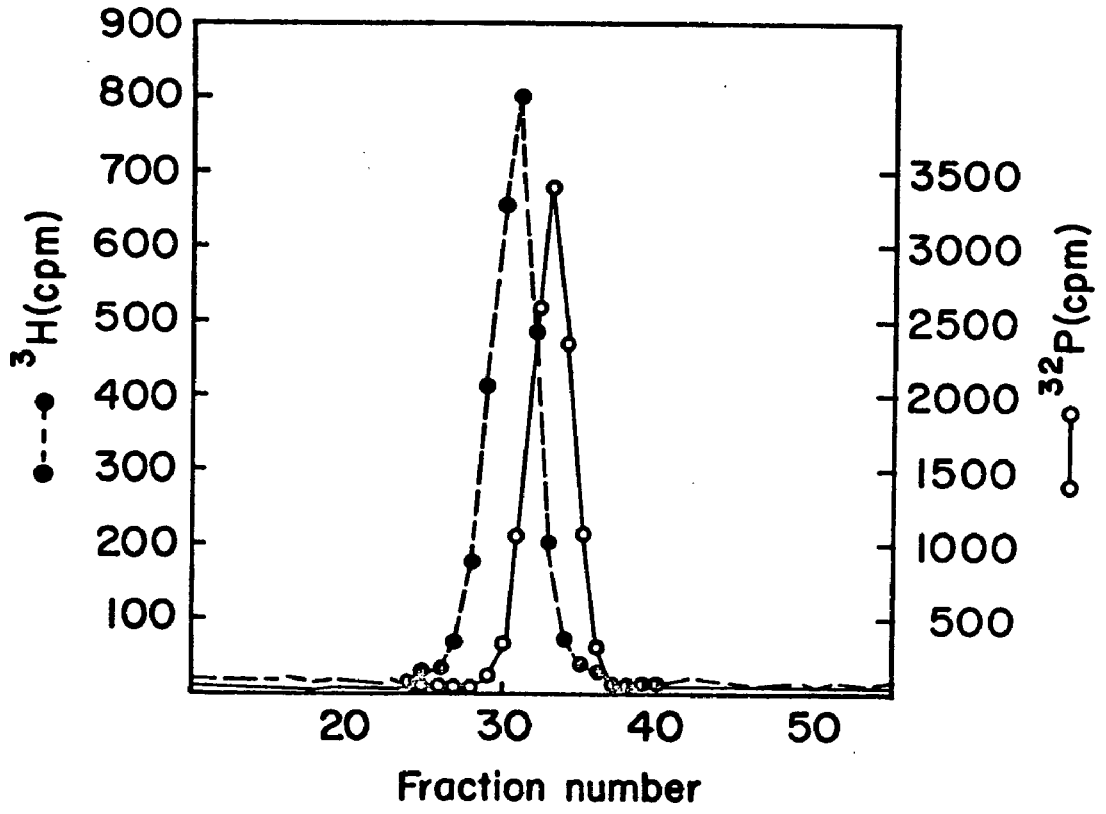


Fig. 7. Density of ^{3}H-thymidine labeled ts25 defective particles from shift-up infection and ^{32}P-labeled P22 C_1 from infection. The procedure is the same as for Fig. 6. -●-●-, ^{3}H; -o-o-, ^{32}P.

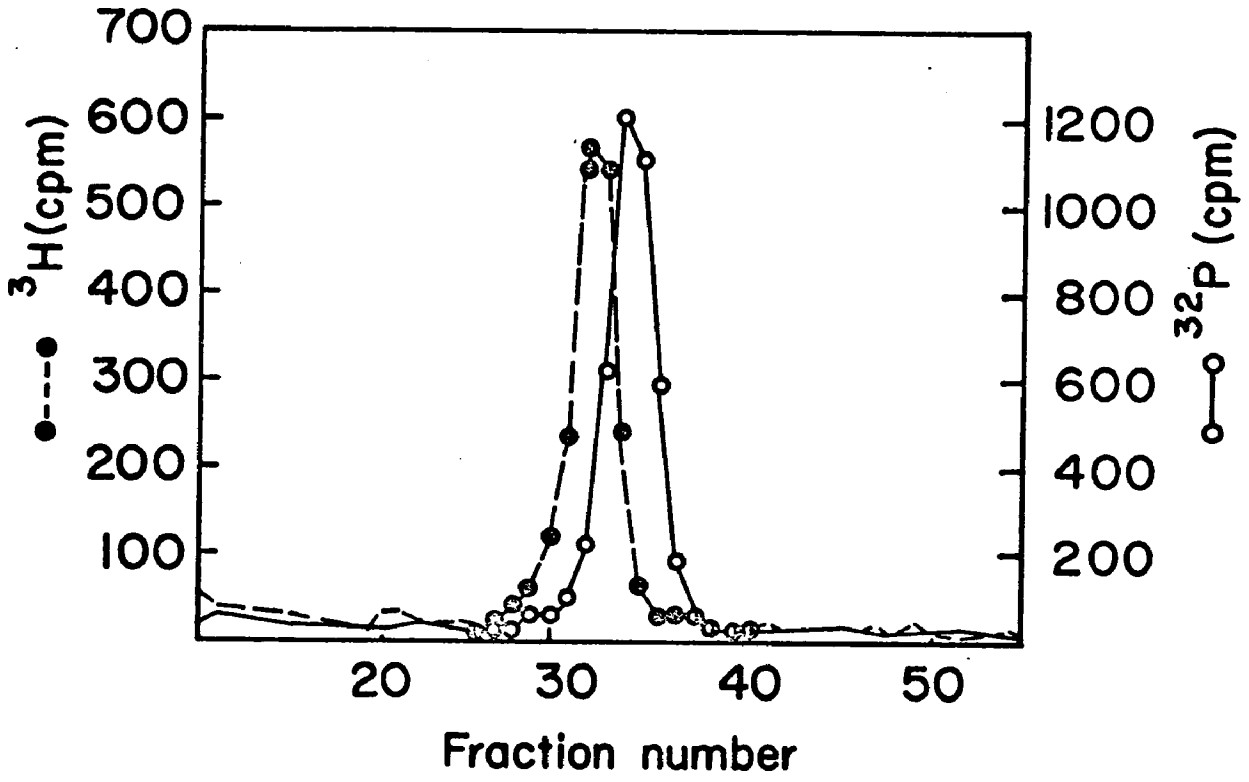


Fig. 8. Density of ^{3}H-thymidine labeled am25 defective particles and ^{32}P-labeled P22 C_1. The conditions for centrifugation are the same as Fig. 6. -●-●-, ^{3}H; -o-o-, ^{32}P.

than the ^{32}P peak (Figure 9). It can be concluded that the heads of ts25 defective particles have a slightly greater density than infectious phage heads.

B. Defective Particle DNA

If this density difference is due to altered DNA content, it could be accounted for by either an increased total amount of DNA in the defective particle head or an increased GC content. If the size of the DNA is larger than normal, then its sedimentation rate in a velocity sucrose gradient should be increased but its density in CsCl equilibrium gradients should not be altered. ^{3}H-thymidine labeled DNA from defective particles and ^{14}C-thymidine labeled DNA from wild type phage were sedimented together in a sucrose gradient to determine whether a size difference exists. The two DNA peaks coincide (Figure 10). It can be concluded therefore, that there is no detectable increase in the size of the DNA in the ts25 defective particles.

If an increase in GC content is the reason for the change in particle density, then the DNA should have a greater buoyant density in CsCl. ^{3}H-thymidine labeled defective particle DNA and ^{14}C-thymidine labeled wild type phage DNA were centrifuged to equilibrium in CsCl and the defective particle DNA peak (^{3}H) bands at essentially the same density as wild type phage DNA (^{14}C) (Figure 11). Thus, there is no evidence for a density difference between the DNAs of wild type phage and ts25 defective particles. In summary, the DNA from

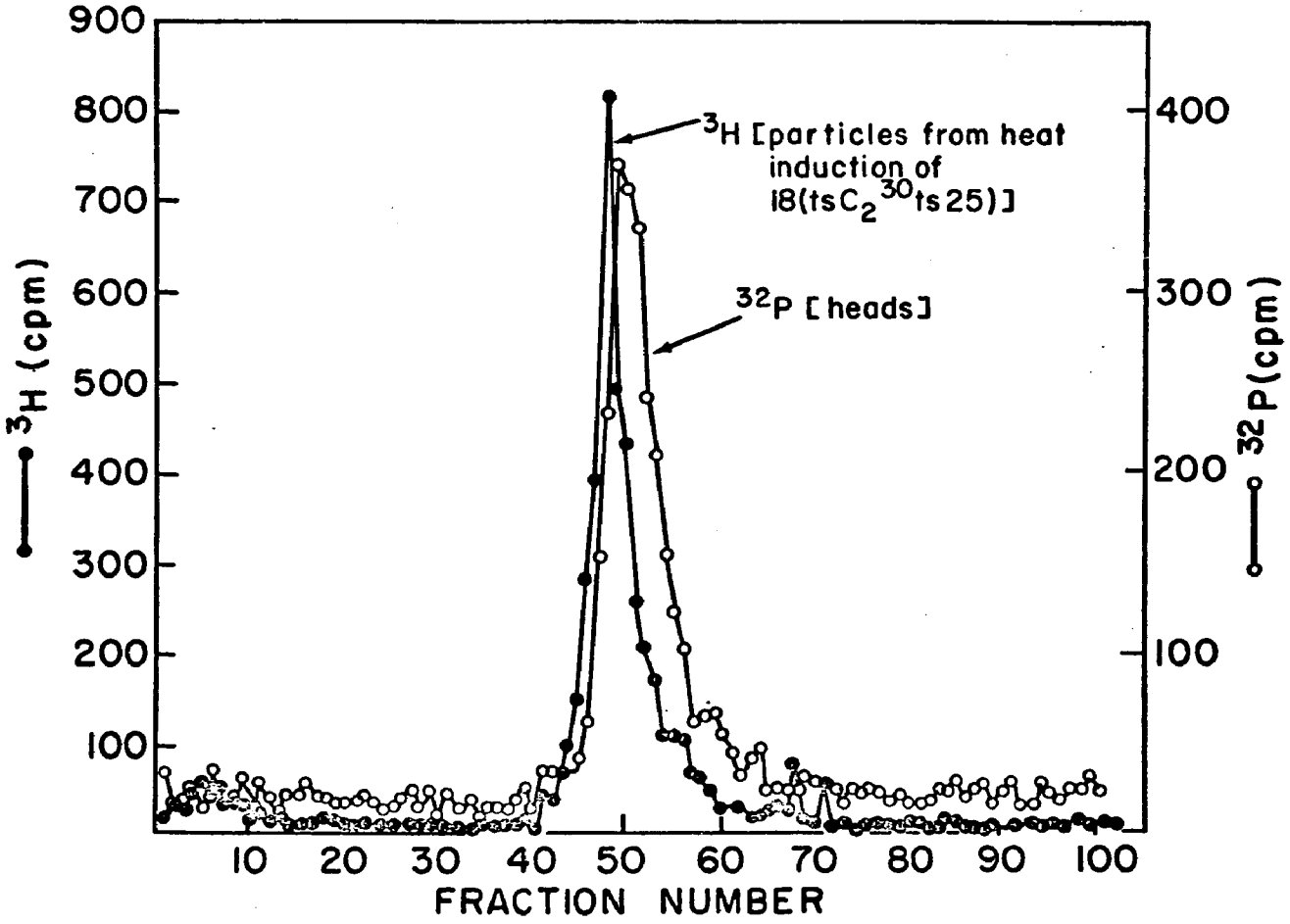


Fig. 9. Density of ^{3}H-thymidine labeled ts25 defective particle heads and ^{32}P-labeled infectious heads. Heads were prepared as described in Materials and Methods. Centrifugation conditions are the same as in Fig. 6. -●-●-, ^{3}H; -o-o-, ^{32}P.

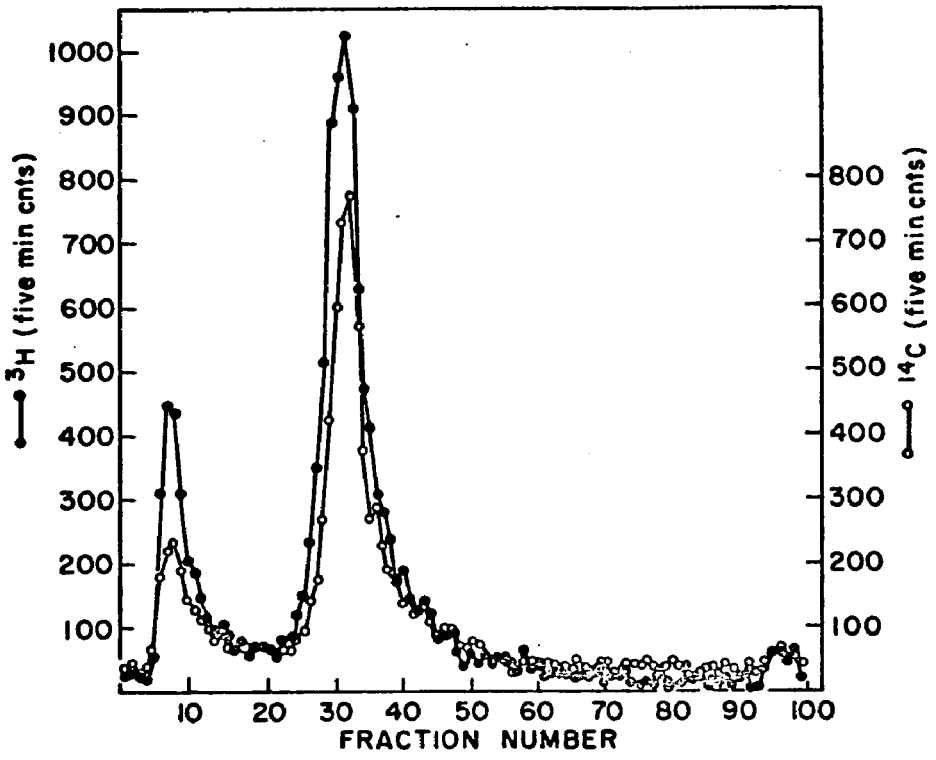


Fig. 10. Zone sedimentation of ^{3}H-thymidine labeled ts25 defective particle DNA and ^{14}C-thymidine labeled P22 C_1 DNA on a sucrose 5-20% gradient at 48,000 rpm for 110 minutes at 25°C in an SW50.1 rotor. -●-●-, ^{3}H; -o-o-, ^{14}C.

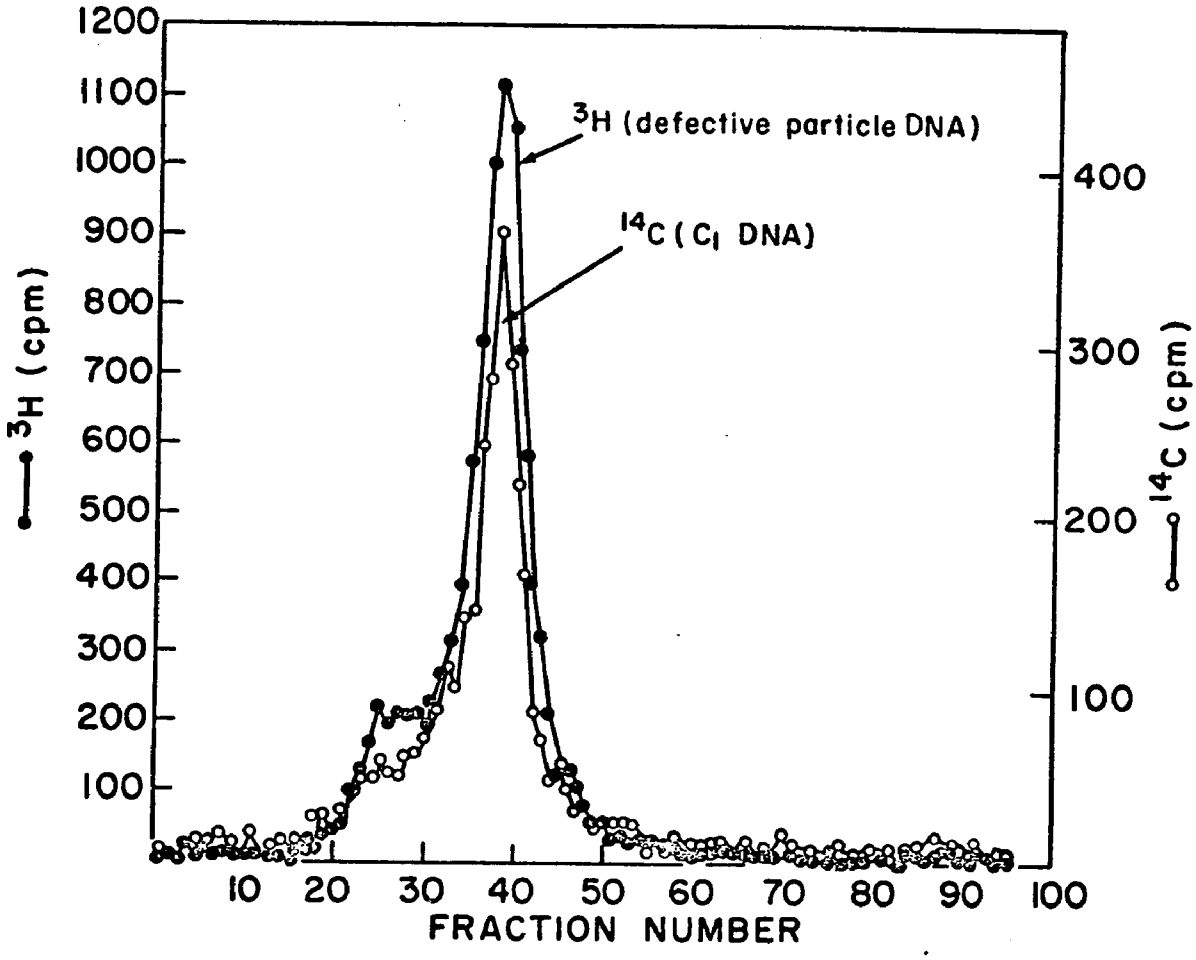


Fig. 11. Equilibrium banding of ^{3}H-thymidine labeled ts25 defective particle DNA and ^{14}C-thymidine labeled P22 c_1 DNA in CsCl (final volume is 5 ml and density is 1.7) at 29,000 rpm for 65 hours at 25°C using the SW50.1 rotor. -●-●-, ^{3}H; -o-o-, ^{14}C.

the defective particles appears to be normal in size and density. Since the tail structure does not appear to be involved, the difference in density must be due to a difference in the protein composition of the head of the defective particles.

C. Comparing the Protein Composition of Defective Particles and Phage Using Acrylamide Gel Electrophoresis

The previously described experiments support the hypothesis that gene 25 protein is part of the virion head and that the defective particles are missing this protein. Botstein et al. (1973) have shown that the mature P22 phage particle contains seven different proteins: called, in order of decreasing molecular weight, P1, P9, P16, P5 (the major protein species), P20, P26, and PX. This was demonstrated by disrupting ^{14}C-amino acid labeled phage, separating the proteins by electrophoresis in a high-resolution discontinuous SDS-polyacrylamide gel system, and analyzing the gels by autoradiography. P22 am25 was previously designated P22 am16; therefore, P25 and P16 are the same. Similar experiments were carried out with CsCl purified defective particles and phage, using a high-resolution discontinuous SDS-polyacrylamide gel system (Maizel, 1971). The phage were not labeled with radioactive amino acids, so Coomassie Blue stain was used to detect the protein bands. The banding pattern for P22 C_2 reveals five different proteins: P1, P9, P25 (P16), P5, and P20 (Plate 1). Using proteins

with known molecular weight, the bands were correlated with the virion proteins identified by Botstein et al. (1973). The most likely explanation for the failure to detect P26 and PX is that these proteins are very minor components of the virion and their concentration on the gels is too low to pick up detectable stain. Both am25 defective particles and ts25 defective particles from infection are missing P25 (Plate 1). This protein is found in P22 ts25, P22 am25, and wild type phage. Ts25 defective particles from induction are missing P25 and P9, the tail protein (Plate 2). The absence of P9 is expected since phage from induction of lysogens are deficient in tails (Israel, 1967). Tails are also missing from wild type phage made by induction.

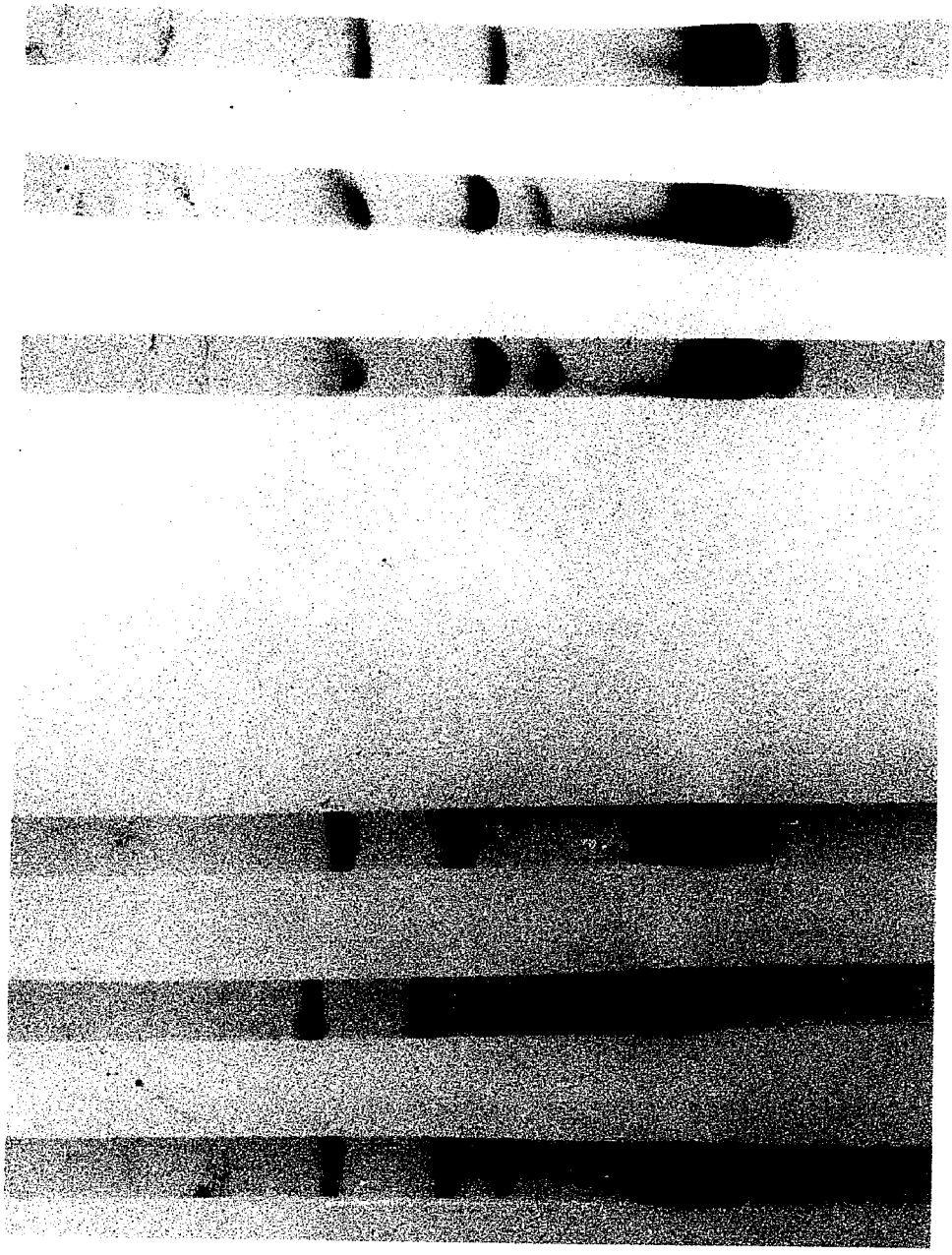
These data clearly show that am25 and ts25 defective particles, whether from infection or induction, are missing the same protein.

D. Early Functions Following Infection with Defective Particles

If defective particles are deficient for the same early function as P22 ts25 in a nonpermissive infection, then adsorption and release of the particle DNA should occur.

(i). Adsorption of ts25.2C_2 Defective Particles from Infection. To determine whether defective particles adsorb to host cells, adsorption velocity constants were calculated. Adsorption was carried out at 41°C with ts25.2C_2 defective particles (moi = 0.8) and P22 C_2 (moi = 0.6). Unadsorbed defective particle titers were determined using the plaque assay. The adsorption velocity constant obtained for

Plate 1. Protein composition of phage and defective particles from infection, resolved by SDS-acrylamide gel electrophoresis. For the first cluster of gels, samples are, from left to right: P22 $\underline{C_2}$, P22 $\underline{am25C_1}$, and $\underline{am25}$ defective particles. For the second cluster of gels, the phage used are, from left to right: P22 $\underline{C_2}$, P22 $\underline{ts25.2C_2}$, and $\underline{ts25}$ defective particles from a shift-up infection. The protein bands have been identified, from top to bottom, as: $\underline{P1}$, $\underline{P9}$, $\underline{P25}$, $\underline{P5}$, and $\underline{P20}$ (Botstein, Waddell, and King, 1973).



top

bottom

Plate 2. Protein composition of phage and defective particles from induction, resolved by SDS-acrylamide gel electrophoresis. Samples used are, from left to right: P22 $\underline{C_2}$ from infection, as a control; P22 $\underline{tsC_2}$, prepared from induction; and $\underline{ts25.2tsC_2}$ defective particles, prepared from induction. The bands can be matched with those from Plate 1.



ts25.2C_2 defective particles is 2.20×10^{-8} and for phage C_2 is 2.15×10^{-8}. These values are not significantly different from those previously found for P22 ts25.2C_2 and P22 C_2. Thus, defective particles adsorb with normal kinetics.

(ii). Release of DNA from am25 Defective Particles. The finding of acid soluble material derived from parental phage DNA when starved cells are infected is taken as a measure of the release of the DNA from the capsids. ^3H-thymidine labeled am25 defective particles (moi = 4) were used to infect starved cells at 41°C to see if the parental phage DNA is rendered acid soluble. The percentage of parental DNA from these defective particles made acid soluble by 62 minutes is not significantly different than from P22 ts25.2 or P22 C_2 at 41°C (Table 1). Thus, am25 defective particle DNA is released from the capsid. In summary, defective particles missing the gene 25 protein adsorb to host cells with normal kinetics and release their DNA from the capsid.

VI. TS25 VIRIONS CONTAIN HEAT-LABILE GENE 25 PROTEIN

A P22 ts25 infection at 41°C is similar to a defective particle infection; the phage DNA is released from the capsid following adsorption to the host cell, no phage genes are expressed, and the infected cell survives. Defective particles are missing P25, but P22 ts25 virions contain this protein (Plate 1). The abortive nature of the P22 ts25 infection at 41°C strongly suggests that P25 is thermolabile.

The following experiments demonstrate that the P22 ts25.2C_2 virion is no more sensitive to high temperatures than P22 ts25$^+$ virions. In addition, evidence will be presented suggesting that P25 is irreversibly denatured following infection with P22 ts25.2C_2 at the nonpermissive temperature.

A. P22 ts25 Virions are Not Heat Sensitive

A comparison of the heat sensitivities of P22 ts25.2C_2 and P22 C_2 was undertaken. Both phages are not affected by exposure to temperatures as high as 52°C for one hour. At 65°C, P22 ts25.2C_2 virions are at least as stable to heat as P22 C_2 virions, and may be even more resistant (Figure 12).

B. Irreversible Inactivation of P22 ts25 Infections at 41°C

Although the P22 ts25.2 virion is not differentially sensitive to high temperatures, its P25 may be thermolabile after infection at 41°C. This would explain the early block in the nonpermissive infection. If P25 is irreversibly inactivated following infection with P22 ts25.2 at 41°C then infected cells should not be killed or produce phage after a shift to 25°C.

Cells were infected with P22 ts25.2C_2 at 41°C, and at various times samples were assayed at 25°C for plaque forming ability (single step growth curve) and survivors. Only 10% of the infected cells produce plaques after 12 minutes at 41°C (Figure 13A). The number of plaques continues to fall until 35 minutes after infection and then starts to increase. This initial loss of plaque forming ability is expected if

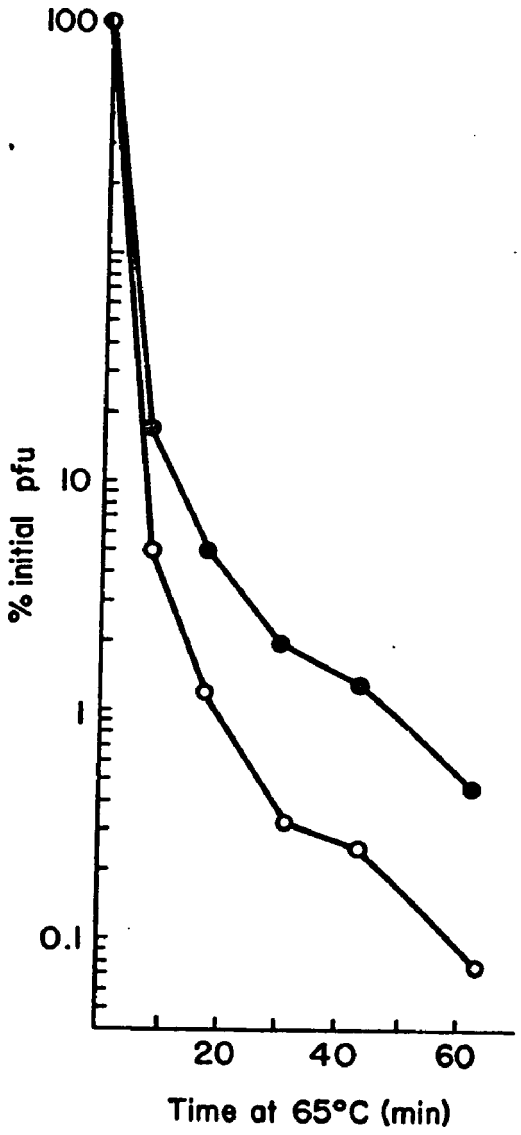


Fig. 12. Thermal inactivation of P22 ts25.2C$_2$ (-●-●-) and P22 C$_2$ (-o-o-) virions at 65°C. Phage were diluted into buffered saline at 65°C. At intervals, samples were removed and assayed for pfu.

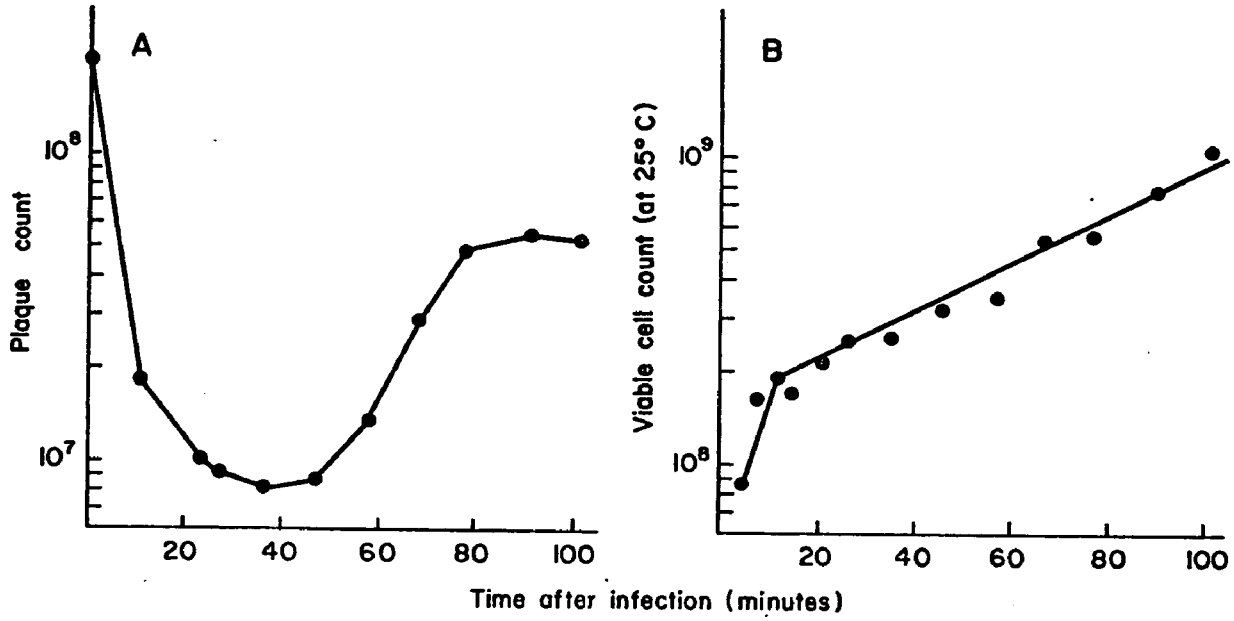


Fig. 13. Irreversible inactivation of P22 ts25.2C$_2$ following infection at 41°C. Strain 18, at a concentration of 2×10^8 cells/ml, was infected at 41°C with P22 ts25.2C$_2$ (moi = 5). Various times after infection samples were assayed for plaques and colony formers at 25°C.

irreversible inactivation of P25 occurs at 41°C. (The late increase in plaques is probably due to leakiness of the ts25 mutation.) As the number of plaques decreases, survival of the host cells increases. Five minutes after infection at 41°C, fewer than 50% of the cells form colonies when shifted to 25°C (Figure 13B). However, after 12 to 15 minutes at 41°C all the infected cells survive on shifting to the permissive temperature.

The segregation pattern of P22 ts25.1C$^+$ infected cells at 41°C (Figure 3B) shows that, although the host cells survive, few survivors are detected as phage carriers at 25°C. The number of cells carrying phage decreases the longer the infection is kept at 41°C. This observation is additional evidence for the irreversible inactivation of P22 ts25 infections at the nonpermissive temperature.

C. Rescue of a P22 ts25.2C$_2$ Infection by Superinfecting with P22 am20C$_2$

It is tempting to conclude that P22 ts25 infections at 41°C are inactivated because the P25 component of the virion is irreversibly heat labile and that the infection aborts at the step controlled by P25. Alternatively, in the absence of functional P25, the phage genome may be rendered irreversibly inactive. Since complementation for the early step requiring P25 can occur in a simultaneous mixed infection, superinfection by a P25$^+$ phage may also give complementation and, therefore, rescue a primary P22 ts25 infection. Complementation would not be expected if the phage DNA is

damaged. However, if the only cause for irreversible inactivation of the P22 ts25 infection is heat denaturation of P25, then the infection might be rescuable.

The following infections were carried out to determine whether superinfecting P22 am20C$_2$ can complement P22 ts25.2C$_2$. Su$^-$ strain 18 was infected at 41°C with P22 ts25.2C$_2$ (moi = 0.1). Ten minutes later an aliquot was diluted and assayed for infective centers using su$^-$ strain 18 plating bacteria at 25°C. At this time a second aliquot was superinfected with P22 am20C$_2$ (moi = 10). In addition, an aliquot of uninfected cells was infected with P22 am20C$_2$. After adsorption, these last two samples were assayed for infective centers.

Only 12% of the infected cells form plaques after infection with P22 ts25.2C$_2$ (Table 10, infection 1). No infective centers are detected for the P22 am20C$_2$ infection (Table 10, infection 2). In the sequential infection, all mixedly infected cells produce plaques (Table 10, infection 3). Superinfecting P22 am20C$_2$ can complement and, therefore, rescue a P22 ts25.2C$_2$ primary infection at 41°C. Two conclusions can be drawn from this result. (1) The phage genome is not irreversibly inactivated in infections carried out in the absence of functional P25. (2) P22 ts25.2C$_2$ infections at 41°C are abortive, probably because P25 is irreversibly denatured.

VII. THE NATURE OF THE EARLY STEP WHICH REQUIRED GENE 25 PROTEIN

Evidence has been presented that gene 25 protein is part of the virion head and that it plays a role early in

Table 10

Rescue of a P22 $\underline{ts25.2C_2}$ infection

by superinfecting P22 $\underline{am20C_2}$

	Infection	Superinfection	Infective Centers $\underline{Su^-}$, 25°C
1.	$\underline{ts25.2C_2}$		2.4×10^6
2.	$\underline{am20C_2}$		$<10^5$
3.	$\underline{ts25.2C_2}$	$\underline{am20C_2}$	2.1×10^7

$\underline{Su^-}$ strain 18, at a concentration of about 2×10^8 cells/ml, was infected at 41°C with P22 $\underline{ts25.2C_2}$ (moi = 0.1), so that 1.9×10^7 cells/ml were infected with this phage.

infection, after release of the DNA. The following experiments were carried out to obtain information about this early reaction.

A. The Early Step is Independent of Active Cell Metabolism

Three lines of experimental evidence support the idea that the early gene 25 reaction is independent of active cellular metabolic processes. The design of all three experiments is similar. P22 ts25.2C$_2$ infections are initiated at 25°C, the permissive temperature. However, other conditions are imposed which prevent normal phage production. The infection is initiated (1) in cells treated with chloramphenicol (CM), and, therefore, in the absence of new protein synthesis; (2) in starved cells, and, therefore, in the absence of energy requiring processes; and (3) in lysogens, in which transcription and translation of most phage genes are repressed. After allowing sufficient time for the early step requiring P25 to occur, the infected cells are shifted to 41°C, the nonpermissive temperature for the early reaction. Simultaneously the imposed restrictions on phage are lifted. The purpose of these experiments is to determine whether the early step can occur under these restrictive conditions.

(i). P25 Action in the Presence of Chloramphenicol. CM, at a final concentration of 100 μg/ml, was added to strain 18 cells at 25°C to inhibit protein synthesis. This concentration of CM is sufficient to prevent phage production when present for the entire incubation period (Table 11,

infection 1). However, no amino acid incorporation studies were done to ensure that protein synthesis was completely turned off under these conditions. Five minutes after the addition of CM, P22 ts25.2C$_2$ (moi = 10) was added to the cell suspension. After 10 minutes free phage were inactivated with antiserum. The infected cells were diluted out of antiserum and into growth medium free of CM at either 25°C or 41°C. Parallel infections were carried out in cells not treated with CM. After 180 minutes, chloroform was added to all infections. The lysates were assayed for active phage, and in some cases also for total particles.

The short treatment with CM has no significant effect on the phage yield of a permissive infection (Table 11, compare infections 2 and 3). Infections initiated in CM and then shifted to 41°C give yields of active phage and total particles similar to a parallel infection carried out without CM (Table 11, compare infections 4 and 5). Since CM is present during the period the infection is at the permissive temperature, the early gene 25 reaction must occur in the absence of new protein synthesis.

(ii). P25 Action in Starved Cells. The early step requiring P25 is independent of new protein synthesis. The following infections were carried out to see if the early step is independent of energy requiring processes. The basic design of the experiment is the same as for the previous experiment, except that starved cells are infected with P22 ts25.2C$_2$ at 25°C.

Table 11

P25 action in the presence of chloramphenicol

	Conditions of Infection				Progeny	
	0-20 min		20-180 min		Active phage/ml	Total particles/ml
	Temp. (°C)	CM	Temp. (°C)	CM		
1.	25	+	25	+	1.8×10^6	
2.	25	+	25	-	5.4×10^9	
3.	25	-	25	-	1.9×10^{10}	
4.	25	+	41	-	5.9×10^8	8.3×10^9
5.	25	-	41	-	4.2×10^8	9.8×10^9

Strain 18 cells were grown in M9CAA to a concentration of 10^9/ml, washed twice with M9 salts, resuspended in M9 salts without glucose, and aerated for 70 minutes. Samples of these starved cells were infected at 25°C with either P22 $\underline{C_2}$ or P22 $\underline{ts25.2C_2}$ (moi = 10). Eighty minutes later each sample was diluted into M9CAA at either 25°C or 41°C, and the infections were permitted to go to completion. After lysis, the phage particles were concentrated and purified by banding in a CsCl gradient. The $OD_{260m\mu}$ (a measure of total particles) and pfu/ml for each phage preparation were determined. The number of active phage in a fixed concentration of particles, active phage/$OD_{260m\mu}$, is the same for the P22 $\underline{C_2}$ infections at both temperatures (Table 12, lines 1 and 2). For P22 $\underline{ts25.2C_2}$, active phage/$OD_{260m\mu}$ is 34 times lower in the 41°C infection than in the 25°C infection (Table 12, lines 3 and 4). However, lysis and defective particle production do occur in the 41°C infection. Thus, the early function requiring $\underline{P25}$ is able to take place at 25°C in starved cells.

(iii). $\underline{\text{P25 Action on Superinfection of Lysogens}}$. Since the early $\underline{P25}$ reaction requires neither new protein synthesis nor energy, it probably can occur in a lysogenic cell in which most phage transcription and translation are turned off. However, it is possible that the $\underline{C_2}$ repressor can block this reaction by binding to the phage DNA. In other words, the reaction may be under immunity control. The following

Table 12

P25 action in starved cells

Phage	Final temperature of incubation	CsCl banded phage suspensions		
		$OD_{260m\mu}$	Active phage/ml	Active phage/ $OD_{260m\mu}$
1. $\underline{C_2}$	25°C	4.1	1.7×10^{12}	4.1×10^{11}
2. $\underline{C_2}$	41°C	3.6	1.7×10^{12}	4.6×10^{11}
3. $\underline{ts25.2C_2}$	25°C	1.4	5.2×10^{11}	3.7×10^{11}
4. $\underline{ts25.2C_2}$	41°C	9.3	1.0×10^{11}	1.1×10^{10}

Starved cells were infected at 25°C with either P22 $\underline{C_2}$ or P22 $\underline{ts25.2C_2}$ (moi = 10). Eighty minutes later each sample was diluted into supplemented M9 at either 25°C or 41°C, and the infection was permitted to go to completion.

infections were carried out to ascertain whether the early P25 reaction can occur in a lysogen.

18(am12tsC_2sie) was grown to 10^8 cells/ml and then superinfected with P22 ts25.2C_2 at 25°C. Ten minutes later, the superinfected cells were pelleted and resuspended in M9CAA at 41°C. This temperature thermally induces the lysogen and is nonpermissive for P22 ts25.2C_2. In the absence of superinfection the phage yield is low (9.0×10^5/ml) since strain 18 is nonpermissive for amber phage. For the superinfected lysogen, total phage production is high (6.0×10^9/ml), with amber and temperature sensitive phage produced. An induced prophage cannot complement superinfecting phage for the early step requiring P25 (Hoffman, unpublished data). Therefore, P22 ts25.2C_2 had to carry out the early P25 reaction at 25°C, the permissive temperature, in the lysogenic host. This early reaction is, therefore, independent of most phage transcription and translation and is not under immunity control by C_2 repressor.

B. Phage with Inactive Genomes as the Complementing Agent

P25 is a structural component of the virion which can apparently act in the absence of active metabolic processes. A phage particle containing an inactive genome should complement for this early step, even though it is unable to complement for other early functions which require transcription of the DNA. One means of inactivating the virion genome is by UV irradiation.

A suspension of P22 am12C$_2$ was treated with a dose of UV irradiation (15 watt G. E. germicidal lamp at a distance of 50 cm for three minutes), resulting in a three log reduction in the active phage titer. Although the plaque forming ability of these UV-irradiated phage is greatly reduced, it is necessary to show that its DNA cannot be transcribed. A mixed infection at 41°C with UV-irradiated phage and P22 ts18C$_2$ does not result in complementation (Table 13, infections 1, 2, and 3). This suggests that gene 18 is not transcribed from the UV-irradiated DNA.

In a single infection at 41°C with P22 ts25.2C$_2$, most of the infected cells cannot form plaques at 25°C nor do they lyse (Table 13, infection 4). However, in the mixed infection with P22 ts25.2C$_2$ and UV treated phage, all infected cells form plaques and lysis is observed (Table 13, infection 5). Thus, P22 ts25.2C$_2$ is complemented by coinfecting UV-irradiated phage for the early step requiring P25.

The active phage titer from the mixed infection is low (Table 13, infection 5). This is expected since newly synthesized wild type gene 25 protein is necessary for active phage formation, and the UV-irradiated ts25$^+$ phage DNA most likely cannot be transcribed. These data are interpreted to mean that complementation for the late gene 25 step requires a transcribable phage genome, but complementation for the early step does not.

Table 13

Complementation with UV-irradiated phage

for the early step requiring P25

	Infection	Infective centers[a]	Lysis	Progeny Active phage/ml
1.	UV $\underline{am12C_2}$	9.0×10^5	-	$<10^5$
2.	$\underline{ts18C_2}$	1.3×10^8	-	7.6×10^7
3.	UV $\underline{am12C_2}$ x $\underline{ts18C_2}$	1.3×10^8	-	1.8×10^8
4.	$\underline{ts25.2C_2}$	1.5×10^7	-	4.6×10^7
5.	UV $\underline{am12C_2}$ x $\underline{ts25.2C_2}$	1.2×10^8	+	2.1×10^8

$\underline{Su^+}$ strain 192 was infected at 41°C with the appropriate phage at a multiplicity of 5 for each genotype

[a] The infected bacteria were plated at 25°C on $\underline{su^+}$ indicator bacteria for plaque formation.

C. P25 Dosage Experiments

A number of lines of evidence have been presented showing that phage, carrying conditional lethal alleles of gene 25, and defective particles, missing P25, can be complemented for the early step requiring gene 25 protein. In all the examples of complementation described above, the multiplicity of the P25$^+$ phage was greater than or equal to the multiplicity of the P25$^-$ phage (P25$^-$ phage are particles either with thermolabile P25 in a 41°C infection or missing P25). Mixed infections were carried out to determine whether complementation for the early step is affected by the amount of P25 available relative to the number of phage used in the infection. Since the am25 defective particles contain no P25, the availability of this protein relative to the number of infecting genomes can be manipulated by varying moi of P25$^+$ phage while holding the moi of am25 defective particles constant.

(i). Criteria for Determining Complementation. Mixed infections with am25 defective particles and P22 ts18C$_2$ were carried out in su$^+$ strain 192 at 25°C, conditions which are permissive for both temperature sensitive and amber mutant phage. Am25 defective particles are not productive in any host at any temperature unless P25 function is provided. Therefore, infective centers will appear on su$^+$ indicator bacteria at 41°C and ts$^+$ phage will be produced only if complementation for P25 function occurs.

Two measurements of complementation were used. (1) One

measurement is the fraction of mixedly infected cells which form infective centers on *su*$^+$ indicator bacteria at 41°C. This fraction, number of infective centers at 41°C on *su*$^+$ indicator bacteria divided by number of mixedly infected cells, is referred to as P_c. P_c values near one indicate that *am25* defective particles are complemented for the early P25 reaction in most mixedly infected cells. P_c values significantly less than one are indicative of the failure of P22 *ts18C*$_2$ to complement the *am25* defective particles. (2) The percentage of *ts*$^+$ phage in the total yield reflects the marker contribution of the genomes carried in the *am25* defective particles, and thus, is another measure of complementation for the P25 function.

(ii). *Complementation is Sensitive to the Input Ratio of Defective Particles and Phage*. As previously demonstrated, mixed infection of a *su*$^+$ host at 25°C, with high multiplicities of P22 *ts18C*$_2$ and low multiplicities of *am25* defective particles results in an infective center titer equal to the defective particle titer. By definition, P_c for these infections is equal to one since the number of mixedly infected cells (the denominator) is determined by the number of infective centers (the numerator) (Table 14, infection 1 and 2). Infections with low multiplicities of both P22 *ts18C*$_2$ and *am25* defective particles, such that mixedly infected cells contain only one copy of each, give P_c values close to one and high percentages of *ts*$^+$ progeny (Table 14, infections 3 and 4).

Table 14

Effect of multiplicity on complementation for the early step

	MOI		P_c [a]	% $\underline{ts}^+$ phage in yield[b]
	P22 $\underline{ts18C_2}$	$\underline{am25}$ defective particles		
1.	5	0.1	1[c]	20
2.	5	0.5	1[c]	22
3.	0.5	0.1	1.3	42
4.	0.5	0.5	0.7	27
5.	0.5	3	0.1	5
6.	0.5	7	0.06	3
7.	0.5	13	0.05	2
8.	0.5	5	0.04	4
9.	5	5	0.5	23
10.	10	5	0.7	32
11.	0.5	10	0.05	2
12.	5	10	0.5	10
13.	10	10	0.6	22
14.	5	20	0.2	5
15.	10	20	0.5	10

[a] $P_c = \frac{\text{number of infective centers at } 41^\circ C \text{ on } su^+ \text{ indicator}}{\text{number of mixedly infected cells}}$

[b] The number of plaques on $\underline{su}^+$ indicator bacteria at $41^\circ C$, expressed as a percentage of the total yield from mixedly infected cells.

[c] P_c equals one by definition.

Thus, one phage can complement one defective particle for the early step.

Cells infected with one copy of P22 ts18C$_2$ and increasing multiplicities of am25 defective particles give decreasing P_c values (Table 14, infections 5-7), and decreases in the percentages of ts$^+$ phage in the yield. In other words, the efficiency of complementation for the early P25 step in cells infected with one P22 ts18C$_2$ phage and more than one am25 defective particle decreases as the number of am25 defective particles increases.

The principle that the amount of P25 affects the degree of complementation is illustrated in another way by the fact that for high multiplicities of am25 defective particles, raising the multiplicity of P22 ts18C$_2$ increases the level of complementation, measured by both P_c and the percentage of ts$^+$ progeny (Table 14, infections 8-15).

Figure 14 is a plot of the multiplicity ratio, am25 defective particles/P22 ts18C$_2$, versus P_c. For a multiplicity ratio less than one, fewer defective particles than phage, P_c is approximately one. P_c drops precipitously when the multiplicity ratio exceeds one. The presence of active phage in the am25 defective particle suspension can account for the observation that P_c never falls below 0.04.

In all these mixed infections, P22 ts18C$_2$ progeny production is normal. This is shown by the fact that (1) the number of infective centers at 25°C, plated on either su$^+$ or su$^-$ indicator bacteria, is always equal to the number

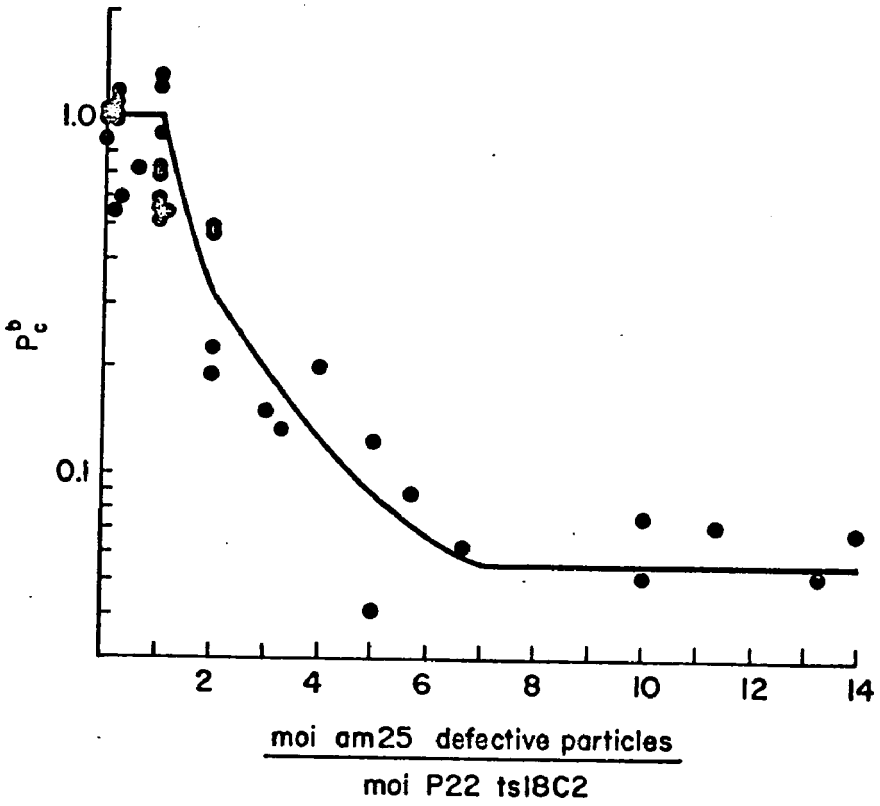


Fig. 14. The effect of the multiplicity ratio (am25 defective particles/P22 ts18C_2)[a] on complementation for the early P25 function.

[a]These multiplicities are for infected cells.. For example, if a multiplicity of 0.1 is used, then the multiplicity for the infected cell is one.

[b]P_c = the number of infective centers at 41°C using su$^+$ indicator bacteria / the number of mixedly infected cells

of P22 ts18C$_2$ infected cells and (2) the number of temperature sensitive progeny in the yield is always high (Hoffman, unpublished data).

(iii). Summary and Conclusions. Complementation for the early step requiring P25 depends on the ratio of defective particles to P25$^+$ phage. One phage complements one defective particle efficiently, but complements two or more defective particles poorly. Thus, the presence of two defective particles hinders complementation of either one by the P25$^+$ phage. In general, if there are more defective particles than phage, complementation is inhibited. As the number of am25 defective particles relative to phage increases, the extent to which P25$^+$ phage can complement the defective particles drops. In all instances, the replication of P22 ts18C$_2$ is not inhibited by any number of coinfecting am25 defective particles.

D. Complementation for the Early Step Requiring P25 in Sequential Infections

It has been reported in an earlier section (section VI, C) that a primary P22 ts25.2 infection at 41°C can be complemented for the early function by superinfecting phage added at 10 minutes post infection. Superinfecting as late as 20 minutes post infection still permits rescue of the ts25 genome (Table 15, compare infections 1-4). The reciprocal sequential infection, P22 am12tsC$_2$sie as the primary phage and P22 ts25.2tsC$_2$sie as the secondary

Table 15
Complementation in sequential infections

	Primary infection	Secondary infection	Progeny in yield
1.	<u>am12tsC_2sie</u>		4.5×10^7
2.	<u>ts25.2tsC_2sie</u>		3.0×10^7
3.	<u>am12tsC_2sie</u> x <u>ts25.2tsC_2sie</u>		3.5×10^{10}
4.	<u>ts25.2tsC_2sie</u>	<u>am12tsC_2sie</u>	1.3×10^{10}
5.	<u>am12tsC_2sie</u>	<u>ts25.2tsC_2sie</u>	1.3×10^{10}

<u>Su$^-$</u> strain 18, at a concentration of 2.5×10^8 cells/ml, was infected at 41°C with the appropriate phage (moi = 5). Superinfections were carried out 20 minutes after the primary infection.

one, was also examined for complementation. In this case, P25 is provided early, and one can ask how long it is available to the superinfecting genome. Complementation for phage production, and therefore for the early P25 function, occurs in this sequential infection. The active phage titer following superinfection at 20 minutes is almost as high as that in a simultaneous mixed infection (Table 15, infections 3 and 5). Similar results were obtained with am25 defective particles as the secondary infecting phage (Hoffman, unpublished data).

In summary, complementation for the early gene 25 function can occur in sequential infections, in which either the primary or secondary phage is missing functional P25. The former case permits the conclusion that no irreversible damage is imposed on the genome of the P25⁻ phage during the first 20 minutes of a nonpermissive infection. From the latter case it can be concluded that the trans effect of P25 persists for at least 20 minutes.

E. Specificity of the Early P25 Function

In the absence of P25 activity, infecting phage DNA does not initiate the synthetic processes leading to phage production. P22 generalized transducing particles contain primarily bacterial DNA and little or no phage DNA (Ebel-Tsipis, Botstein and Fox, 1972). The mechanisms of injection of this DNA into the bacterial cell is presumed to be the same as for phage DNA. The question can be

asked if P25 activity is needed for successful transduction, or is it specific for phage DNA? As another aspect of specificity, complementation tests between am25 defective particles and phage L, a related but heteroimmune phage (Bezdek and Amati, 1967, 1968, 1970), were carried out to see if phage L contains a protein which can supply the missing gene 25 function. Phage L and P22 are closely related such that recombination occurs between the two phages.

(i). Transduction Requires Functional P25. A comparison of the transducing activity of $P25^+$ and $P25^-$ phages for bacterial proC and hisG markers was carried out. No transductants were detected for either the proC or hisG marker with am25 defective particles as donor, although normal frequencies were obtained for P22 C_1 (Table 16, lines 1 and 2). In phage suspensions from thermal induction of 18(ts25.2tsC_2), only one out of 30 particles has P25. The frequencies of transduction for both the proC and hisG markers are about 15 fold lower using this suspension as donor than the one from thermal induction of 18(tsC_2), in which P25 is present in all particles (Table 16, lines 3 and 4).

It can be concluded that the presence of P25 in the virion is necessary for transduction. Therefore, the early P25 function is not unique to infecting phage DNA, but is also essential for infecting bacterial DNA.

Table 16

Frequency of transduction of bacterial markers

	Phage	Method of preparation	Transductants per 10^6 particles[a] hisG201	proC90
1.	$\underline{C_1}$	infection	0.25	0.12
2.	$\underline{am25C_1}$ defective particles	infection	$<10^{-3}$	$<2 \times 10^{-4}$
3.	$\underline{tsC_2}$	induction	1.3	7.0
4.	$\underline{ts25.2tsC_2}$ defective particles	induction	0.08	0.4

CsCl purified phage suspensions, prepared by infection or induction, were assayed for transducing particles using auxotrophic recipients lysogenic for P22 siel. Only complete transductants were scored.

[a]Absolute phage particle concentrations were determined by $OD_{260m\mu}$.

(ii). Complementation for the Early Step with Phage L. Mixed infections with phage L (moi = 10) and am25 defective particles (moi = 0.3) fail to show any infective centers at 41°C on 192(L) indicator bacteria (Table 17, infections 1,2 and 4). P22 amber mutants form plaques on 192(L) indicator, but phage L does not. In contrast, a control infection with P22 $ts18C_2$ (moi = 10) and am25 defective particles (moi = 0.3) gives more than a three log increase in infective centers (Table 17, infections 1,3 and 5).

The failure of phage L to complement for the early P25 reaction can be explained by the following possibilities: (1) Phage L has no protein analogous to P25. (2) The P25 reaction is phage specific. The phage L protein analogous to P25 cannot substitute for P25. (3) The P25 analog in phage L cannot act in trans. At the moment, no distinction can be made between these possibilities. However, analysis of the protein components of the L virion by high resolution SDS-acrylamide gel electrophoresis reveals a banding pattern similar to the one from P22 virions (Hoffman, unpublished data). L virions have a minor protein component with a molecular weight close to P25, suggesting that there is a protein analogous to P25.

F. Am25 Defective Particles Cannot Titrate Repressor

Superinfection of a lysogen with sufficiently high numbers of phage overcomes lysogenic immunity. The injected

Table 17

Complementation between phage L

and *am25* defective particles

Infection	MOI	Infective Centers[a]
1. *am25* defective particles	0.3	1.0×10^4
2. L	10	$<10^5$
3. *ts18C*$_2$	10	$<10^5$
4. *am25* defective particles x L	0.3:10	$<10^5$
5. *am25* defective particles x *ts18C*$_2$	0.3:10	2.2×10^7

Su$^+$ strain 192 was infected at 25°C.

[a]Infective centers were plated on 192(L) at 41°C. Neither P22 *ts18C*$_2$ nor phage L forms plaques under these plating conditions.

DNA titrates out repressor molecules, repression of phage gene expression is released, and both the prophage and superinfecting genomes are replicated (Weismeyer, 1965; Rao, 1968). Similar experiments were carried out with am25 defective particles to see if the released DNA can titrate repressor. The results should be informative as to the intracellular localization of the released defective particle DNA.

Strain 18(C^+sie) was grown at 37°C to a final concentration of 2 x 10^8 cells/ml, and superinfected at multiplicities of 50 with either P22 am12C_2, as a control, or am25 defective particles. Antiserum was added (K=2) and five minutes later the superinfected lysogens were diluted and assayed for infective centers, on su^- indicator bacteria, and surviving bacteria.

Superinfection of a lysogen with P22 am12C_2 induces the majority of the cells, since only 10% of the infected lysogens survive as colony formers (Table 18, line 2). Although 90% of the infected lysogens are killed, not all of these form infective centers. Only infected cells in which both repressor is titrated out and at least one prophage genome is packaged into a mature phage particle will form an infective center on su^- indicator bacteria. That only 37% of the cells do so is not unreasonable, since each superinfected cell contains 50 am genomes for every prophage.

Am25 defective particles do not titrate out repressor.

Table 18

Induction of lysogens by superinfection

Superinfection	% survivors[a]	% infective centers[b]
1. ---	100	1.2
2. *am12C2*	10	37
3. *am25* defective particles	112	1.4

18(*C⁺sie*) was superinfected with the appropriate phage (moi = 50). After allowing sufficient time for adsorption and treatment with antiserum, samples were diluted and assayed for survivors and infective centers on *su⁻* indicator bacteria.

[a] % survivors = $\frac{\text{\# colonies from sample}}{\text{\# colonies from uninfected lysogen}} \times 100$

[b] % infective centers = $\frac{\text{\# infective centers from sample}}{\text{\# colonies from uninfected lysogen}} \times 100$

Following superinfection with am25 defective particles, no infective centers above background level appear and no killing is detected (Table 18, lines 1 and 3). The significance of this result with regard to the localization of the DNA from am25 defective particles will be discussed below.

VIII. THE EFFECT OF MULTIPLICITY ON THE LATE STEP REQUIRING GENE 25 PRODUCT

P25 is a component protein of the virion and therefore, it is required in stoicheometric amounts for the maturation of active particles. Gene dosage experiments in T4 infections have shown that, for stoicheometric functions, the burst size decreases rapidly as the am^+ to am input ratio in a su^- host is decreased (Snustad, 1968). Similar infections were done with am25 mutants to demonstrate this effect.

Mixed infections were carried out in su^- strain 18 at 25°C, varying the multiplicities of P22 $ts18C_2$ and P22 $am25C_1$. These conditions are permissive for the temperature sensitive phage and nonpermissive for P22 $am25C_1$. Free phage were inactivated by antiserum and diluted 10^4 fold. After 180 minutes, samples were chloroformed and assayed for active phage. Cells mixedly infected with P22 $ts18C_2$, at a multiplicity of one, and increasing multiplicities of P22 $am25C_1$ show a decrease in the yield of active phage

Table 19

Effect of multiplicity on active phage yield

Infection	MOI	Active phage yield
1. ts18C$_2$	1	2.3×10^{10}
2. "	5	4.1×10^{10}
3. "	10	3.9×10^{10}
4. am25C$_1$	1	2.0×10^{5}
5. "	5	1.4×10^{6}
6. "	10	7.4×10^{6}
7. ts18C$_2$ x am25C$_1$	1:1	1.8×10^{10}
8. " x "	1:5	4.2×10^{9}
9. " x "	1:10	1.7×10^{9}
10. " x "	5:1	3.5×10^{10}
11. " x "	5:5	1.1×10^{10}
12. " x "	5:10	1.0×10^{10}
13. " x "	10:1	4.0×10^{10}
14. " x "	10:5	1.5×10^{10}
15. " x "	10:10	8.4×10^{9}

Su$^-$ strain 18, at a concentration of 2×10^8 cells/ml, was infected at 25°C with the appropriate phage.

(Table 19, infections 1 and 7-9). Raising the multiplicity of P22 ts18C_2 gives smaller reductions in the yields (Table 19, infections 2-3, 10-15). In conclusion, decreasing the ratio of am25$^+$ to am25 phage in a su$^-$ host results in a decrease in the phage yield.

IX. P22 ts25.2 AND P22 am25 ARE NOT NORMAL PHAGES, EVEN IN PERMISSIVE INFECTIONS

Three lines of evidence will be presented which suggest that both P22 ts25.2 and P22 am25 differ from wild type phage even under permissive conditions.

A. Complementation for the Early Function Requiring P25 with either P22 ts25.2 or P22 am25 as the P25$^+$ Phage

Complementation tests for the early gene 25 step, with the gene 25 mutant phages, was carried out to see if they complement as well as gene 25$^+$ phage. The procedure and calculations to detect and quantitate complementation have been described in section VII, C and in Table 14. The appropriate plating conditions were employed when amber phage was used as the P25$^+$ phage.

When P22 ts25.2C_2 is the P25$^+$ phage (moi = 5), only about 50% of the mixedly infected cells complement am25 defective particles (Table 20, infections 1 and 2). Increasing the multiplicity of am25 defective particles, while maintaining only one P22 ts25.2C_2 per cell, results

in a decrease in complementation (Table 20, infections 3-7). Complementation is consistently poorer with P22 ts25.2C$_2$, rather than P22 ts18C$_2$, as the P25$^+$ phage (compare Table 14 and Table 20).

Infections were also carried out with either P22 am12C$_2$ or P22 am25C$_1$ as the P25$^+$ phage and ts25 defective particles as the P25$^-$ phage. P_c values are lower if P22 am25C$_1$, rather than P22 am12C$_2$, is the P25$^+$ phage. Therefore, P22 am25C$_1$ complements less well for the early function than phage carrying the gene 25$^+$ allele.

Two possible explanations for the weak complementation are: (1) The P25 component of these mutant virions is less active, even at 25°C, than wild type P25. (2) The mutant virions contain reduced amounts of P25, which renders them less able to complement.

B. P22 ts25.2 and P22 am25 Have Slightly Greater Densities than Wild Type Phage

A phage particle containing less protein than wild type phage should be denser. This is the case for both ts25 and am25 defective particles, which are missing P25. If P22 ts25 and P22 am25 contain fewer P25 molecules than wild type phage, their density should also be slightly increased.

The density of the mutant phages were compared with the density of wild type phage by equilibrium centrifugation in CsCl. ^{3}H-thymidine labeled P22 ts25.2C$_2$ was

Table 20

Complementation for the early step with P22 ts25.2C$_2$ as the P25$^+$ phage

Infection	MOI	P_c[a]	% ts$^+$ phage in yield[b]
1. ts25.2C$_2$ x am25 defective particles	5:0.1	0.4	2
2. " x " " "	5:0.5	0.6	3
3. " x " " "	0.6:0.1	0.3	8
4. " x " " "	0.6:0.5	0.2	6
5. " x " " "	0.6:3	0.06	2
6. " x " " "	0.6:7	0.03	1
7. " x " " "	0.6:13	0.03	1

The infections were carried out in su$^+$ strain 192 at 25°C. The procedure and calculations have been described in section VII, C and Table 14.

[a]Samples plated for infective centers were kept at 25°C for 30 minutes, which is sufficient time for the early step to occur. Then plates were incubated at 41°C.

[b]The infected cells were kept at 25°C for 180 minutes, treated with chloroform and assayed for phage.

Table 21

Complementation for the early step with P22 $\underline{am25C_1}$ as the $P\underline{25}^+$ phage

	Infection	MOI	P_c
1.	$\underline{am12C_2}$ x $\underline{ts25}$ defective particles	0.3:0.05	1.3
2.	" x " " "	5:0.05	1.0
3.	" x " " "	20:0.05	1.0
4.	$\underline{am25C_1}$ x $\underline{ts25}$ defective particles	2:0.05	0.4
5.	" x " " "	10:0.05	0.5
6.	" x " " "	20:0.05	0.3

Infections were carried out in $\underline{su}^+$ strain 192 at 25°C. The procedure and calculations have been described in section VII, C and Table 14. The appropriate plating conditions ($\underline{su}^-$ indicator bacteria at 25°C) were employed to determine if $\underline{ts25}$ defective particles are complemented for the early step.

mixed with ^{32}P-labeled P22 $\underline{c_2}$ and centrifuged to equilibrium in CsCl. The ^{3}H label bands in a slightly denser position in the gradient (Figure 15). The density of the mutant phage was also compared to that of ts25 defective particles. A mixture of the three types of particles, ^{3}H-thymidine labeled ts25 defective particles, ^{32}P-labeled P22 $\underline{ts25.2c_2}$ and ^{14}C-thymidine labeled P22 $\underline{c}^+$, was centrifuged to equilibrium. Three peaks are observed, and the ^{32}P label is found in the intermediate position (Figure 16). Thus, P22 $\underline{ts25.2c_2}$ has a slightly greater density than P22 $\underline{c}^+$, but it is not as dense as ts25 defective particles. This intermediate density supports the prediction that this mutant phage contains a reduced quantity of P25. The density of ^{3}H-thymidine labeled P22 $\underline{am25c_1}$ is also slightly greater than the density of ^{32}P-labeled wild type phage (Figure 17).

C. The Effect of Multiplicity on the Late Step

If the mutant phage contain fewer P25 molecules than wild type, it is probably because fewer molecules are available for assembly of virions. If this is the case, then mixed infections, similar to those described in section VIII, with P22 $\underline{ts25.2c_2}$ as the P25$^+$ phage, should show more dramatic decreases in the phage yields than when P22 $\underline{ts18c_2}$ is the P25$^+$ phage. The results agree with this expectation (compare Table 19, infections 10,11 and 12 with Table 22, infections 6,7 and 8; Table 19, infections 13, 14

and 15 with Table 22, infections 9, 10 and 11).

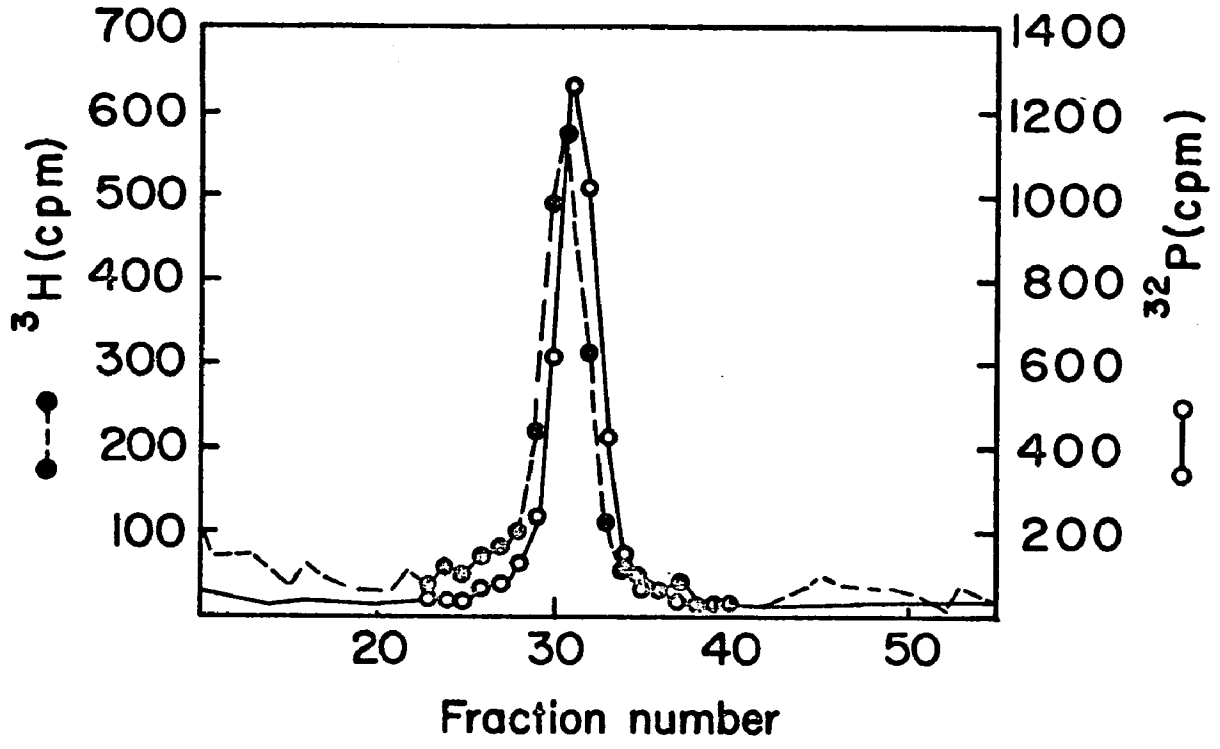


Fig. 15. Density of ^{3}H-thymidine labeled P22 ts25.2C$_2$ and ^{32}P-labeled P22 C$_2$. The conditions for centrifugation are the same as for Fig. 6. -●-●-, ^{3}H; -o-o-, ^{32}P.

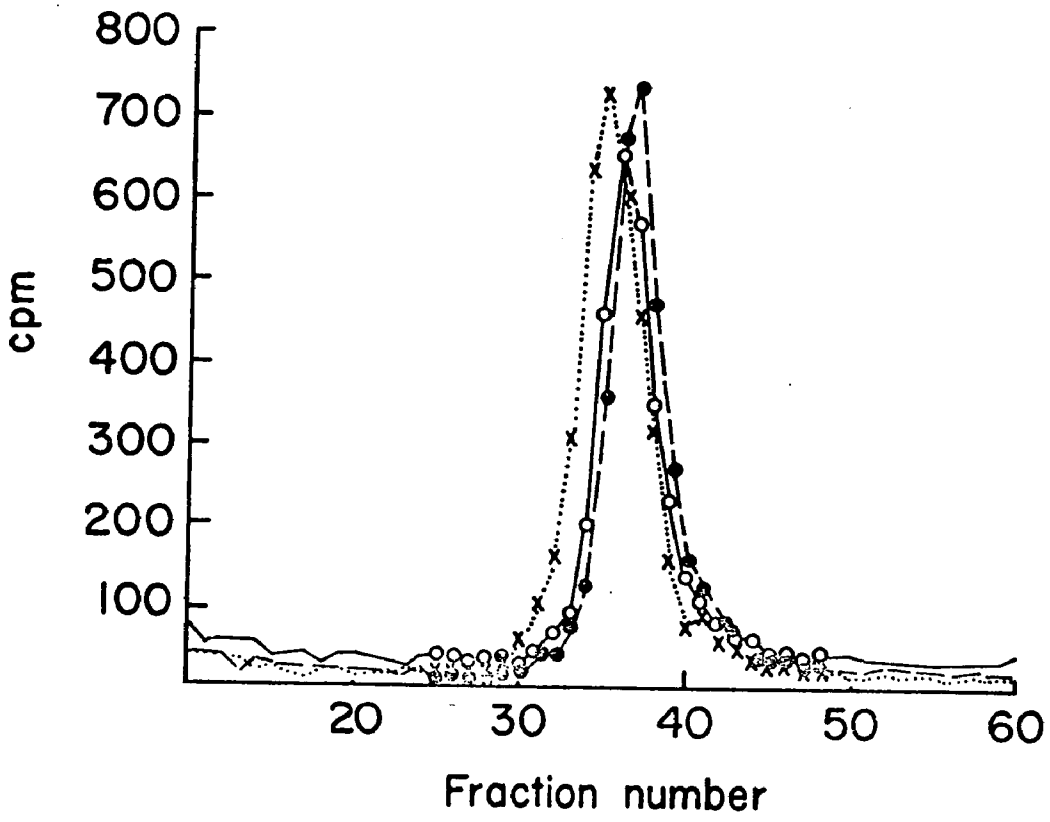


Fig. 16. Density of ^{3}H-thymidine labeled ts25 defective particles, ^{32}P-labeled P22 ts25.2C$_2$, and ^{14}C-thymidine labeled P22 C^{+}. The conditions for centrifugation are the same as in Fig. 6. ..x..x.., ^{3}H; -o-o-, ^{32}P; -●-●-, ^{14}C.

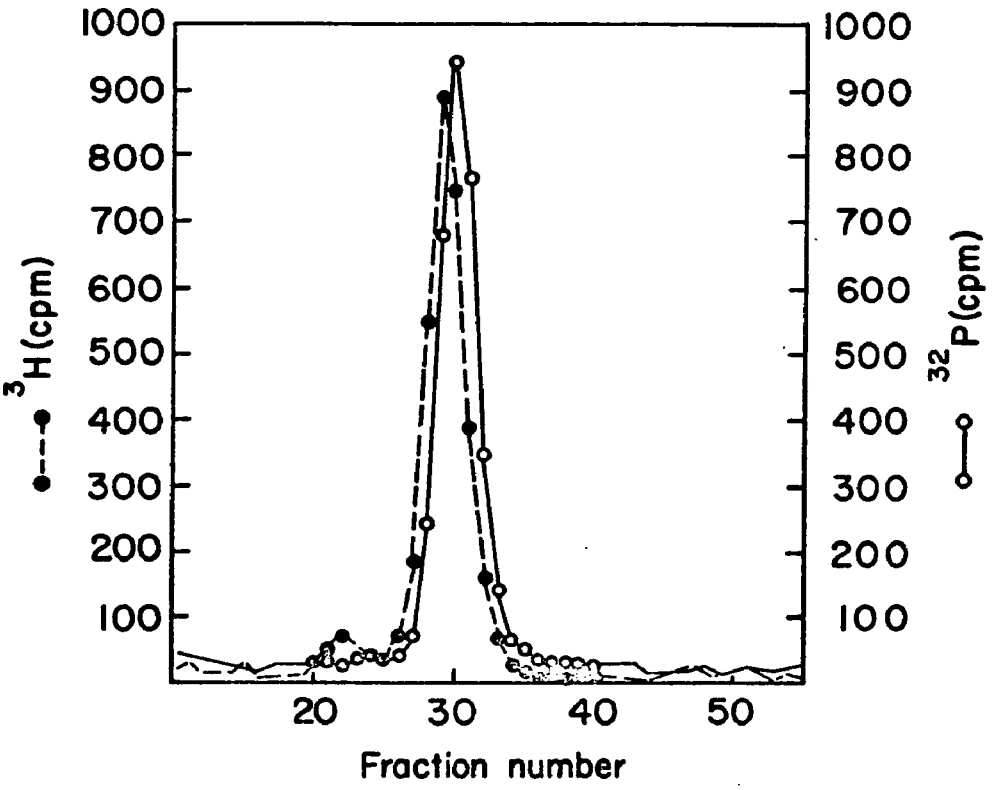


Fig. 17. Density of ^{3}H-thymidine labeled P22 $\underline{am25C_1}$ and ^{32}P-labeled P22 $\underline{C_2}$. The procedure for centrifugation is the same as in Fig. 6. -●-●-, ^{3}H; -o-o-, ^{32}P.

Table 22
Effect of multiplicity
on active phage yield

Infection	MOI	Active phage yield
1. ts25.2C$_2$	5	2.3×10^{10}
2. "	10	1.5×10^{10}
3. am25C$_1$	1	2.0×10^{5}
4. "	5	1.4×10^{6}
5. "	10	7.4×10^{6}
6. ts25.2C$_2$ x am25C$_1$	5:1	1.3×10^{10}
7. " x "	5:5	2.1×10^{9}
8. " x "	5:10	4.6×10^{8}
9. " x "	10:1	1.3×10^{10}
10. " x "	10:5	2.2×10^{9}
11. " x "	10:10	1.3×10^{9}

Su$^-$ strain 18, at a concentration of 2×10^8 cells/ml, was infected at 25°C with the appropriate phage.

CHAPTER FOUR: DISCUSSION

I. P25 IS REQUIRED BOTH EARLY AND LATE IN INFECTION

The gene 25 protein is a component of the P22 virion head, which plays a role after the DNA is at least partially released from the capsid, but before it associates with the replication complex. A nonpermissive infection with P22 am25 results in the production of particles which are noninfectious, but are morphologically indistinguishable from wild type virions (Botstein, Waddell and King, 1973). These defective particles are missing P25, as determined by SDS polyacrylamide gel electrophoresis (Botstein, et al., 1973; Hoffman, this report). P25 specified by a ts25 mutant allele appears to be thermolabile and is inactive on infection at 41°C. No functional P25 is available on infection with P22 ts25 at high temperature or with am25 defective particles under any conditions.

The following features of infection in the absence of functional P25 are well established. Adsorption proceeds with normal kinetics and phage DNA is released from the capsid. The host cells are not detectably affected; they continue to grow and divide with the same kinetics as uninfected cells. No change in the rate of DNA synthesis in the infected culture is observed, suggesting that no phage DNA is synthesized. The parental phage DNA does not associate with the replication complex and superinfection

exclusion is not induced. Two alterations in enzymatic activity are detected early in P22 infections: a loss of host deoxyribonuclease activity specific for denatured DNA (Israel, Woodworth-Gutai and Levine, 1972) and an increase in a phage specified deoxyribonuclease activity specific for native DNA (Woodworth-Gutai, Israel and Levine, 1972). Infections in the absence of P25 activity fail to show either of these effects. These infections do not result in the transcription of any phage specific RNA, as determined by RNA-DNA hybridization (E.N. Jackson, personal communication). Finally, the host is not lysogenized. In conclusion, in the absence of functional P25, there is an early block in the infective process which prevents the expression of any phage functions after release of phage DNA from the capsid.

Since P25 is a virion component, it is synthesized as a late protein to be incorporated into the particle during assembly. Therefore, the gene 25 product is required not only early, for an effective infection, but also late, for the production of infectious progeny. Temperature-shift experiments with P22 ts25 clearly demonstrate that P25 is necessary both early and late for active phage production. If P25 is rendered inactive early, no phage synthetic functions are detected. If P25 is inactivated between ten and fifty minutes after infection, the early step has occurred and phage particles are produced. However, most of these particles are defective since, like the am25 defective

particles, these are also missing P25.

II. THE NATURE OF THE EARLY GENE 25 FUNCTION

This section will summarize all of the data about the early gene 25 function and discuss the implications of these findings.

1. In the absence of functional P25, induction of lysogens proceeds with the expression of early and late genes, resulting in defective particle production and cell lysis. This response is in dramatic contrast to infections with P25⁻ phage. That the excised prophage genome bypasses the requirement for P25 demonstrates that P25 is not an integral part of the phage replication machinery.

2. Generalized transduction requires functional P25. This shows that the early gene 25 step in the infective process is not specific for phage DNA. Ebel-Tsipis, Fox and Botstein (1972) have shown that transducing DNA is integrated into recipient bacterial DNA in fully conserved, double stranded form. Therefore, the initial events following transduction do not require replication of the transducing DNA. The fact that transduction requires P25, but not replication of the transducing DNA, may be taken as further evidence that the early P25 function is not directly involved with replication of P22 DNA.

3. The early P25 reaction is independent of active cell metabolism, since it can occur in the presence of

chloramphenicol and in starved cells. The occurrence of the early step on superinfection of lysogens shows that it is not under immunity control. This suggests that the early function depends solely on the activity of P25 contained in the infecting phage.

4. P25$^-$ particles can be complemented for the early P25 event. Not only viable phage but also phage with inactivated DNA (UV-irradiated) can be the complementing agent. Thus, only the active protein need be provided by the complementing phage.

5. Varying the multiplicities of P25$^+$ and P25$^-$ phage in mixed infections reveals restrictions on the conditions for complementation. When the number of P25$^+$ phage is equal to or greater than P25$^-$ phage efficient complementation results. However, in cells infected with fewer P25$^+$ than P25$^-$ phage, complementation is inhibited. In all instances, the production of P25$^+$ phage is not affected by the coinfecting P25$^-$ particles. P25$^+$ phage production is normal whether or not complementation for the early gene 25 function has taken place.

These data suggest that P25 acts initially in *cis*. Yet the capacity of a P25$^+$ virion to complement a P25$^-$ particle shows that the protein can also act in *trans*. If the action of P25 causes a generalized change in the host cell, such as an alteration in large areas of the host membrane or inactivation (or modification) of host cell enzymes, then a single P25$^+$ particle should initiate these

changes. Any number of other particles, whether P25$^+$ or P25$^-$, should then be able to effectively infect the cell. This is clearly not the case. Thus, all models of P25 action which suppose a generalized change in the infected cell can be ruled out. P25 action must be localized, either affecting only a small region of the cell or acting directly on a phage or phage genome. If the latter is the case, it could be further argued that P25 must be released from the capsid to accomplish complementation. One explanation for the finding that an excess of defective particles prevents complementation for any of them is that there is dilution of P25 among the many DNA molecules or phage particles.

6. Complementation for the early P25 reaction occurs when the primary infecting phage is P25$^+$ and the secondary infecting phage is P25$^-$. That is, P25 from the primary infection, or the consequence of its action, is still available to superinfecting P25$^-$ phage very late in the latent period.

7. P25$^-$ primary infections can be complemented, and the 25$^-$ genome rescued by superinfecting P25$^+$ phage. Thus, in the absence of P25, the infection is blocked at some point in the normal sequence of events, and the phage genome is not irreversibly damaged. Without P25, the released DNA is not susceptible to host induced damage, thereby eliminating a protecting role for P25.

8. Superinfecting DNA from P25$^-$ phage does not titrate

repressor. This may be because the released phage DNA either is not in the compartment where the repressor molecules are, or it is in a conformation which prevents binding to repressor. These possibilities will be discussed in the following section.

III. MODELS FOR THE EARLY P25 FUNCTION

Bezdek and Soska (1970) claimed that gene 25 product has a basic regulatory function similar to the N function of phage λ (Dove, 1968). The temperature sensitive mutant which they describe, P22 tsX, is identical with P22 ts25.2. All their observations agree with those presented in this report, except that they did not detect lysis following UV induction of LT2(tsXC$^+$) at 39°C. This observation, plus the absence of active phage production following induction, led them to conclude that gene 25 product is necessary for the expression of any phage genes on induction as well as on infection.

In this paper we report that thermal induction of 18(ts25.2tsC$_2$) at 41°C results in cell lysis and defective particle production and that mitomycin C induction of 18(ts25.2C$^+$) at the nonpermissive temperature gives a high yield of defective particles. In agreement with these data, lysis was observed following UV induction of 18(ts25.1C$^+$) at 39°C (Hoffman, unpublished data). These observations show that early and late genes are expressed

in the absence of functional P25 following induction.

These data, plus the requirement for P25 in transduction, allow the conclusion that gene 25 product is not involved in an early regulatory function and has no resemblance to the N function of phage λ. A P25 role as an enzyme or an enzyme cofactor necessary for phage production is also ruled out by these findings.

Since parental DNA does not appear in Intermediate I in the absence of functional P25, Levine, Chakravorty, and Bronson (1970) suggested that P25 may be active in binding parental DNA to the phage replication machinery. Because P25 is not necessary for phage DNA synthesis on induction, P25 cannot be an integral part of the phage replication machinery. However, P25 may be involved in binding infecting DNA to the replication complex. The following observations show that the P25 function occurs prior to association with the complex, and is, therefore, not directly involved in binding parental DNA to Intermediate I. (1) DNA from P25$^-$ phage does not titrate repressor ; that is, the P25 function must occur before phage DNA can bind repressor. In addition, repressor prevents association of parental DNA with Intermediate I (Levine et al., 1970). Therefore, P25 function occurs prior to either binding repressor to phage DNA or association of parental DNA with Intermediate I. (2) Since transducing DNA is integrated into the bacterial chromosome without being first replicated (Ebel-Tsipis, Fox, and Botstein, 1972),

it is not likely to be associated with the replication machinery. Yet P25 function is required for transduction.

Little is known about the early events in a P22 infection, particularly about the steps between adsorption of the virion to the host cell and association of the parental phage DNA with Intermediate I. The P25 function occurs after at least partial release of the DNA from the capsid and prior to association with the replication machinery, and it is specific for infecting DNA. The role of P25 may be one of the following: (1) completing the release of DNA from the capsid, (2) bringing the released DNA to the appropriate compartment in the cell, (3) removal of virion proteins which are bound to the injected DNA, and (4) conversion of injected DNA to a conformation which renders it active.

1. Phage DNA is susceptible to host degradative enzymes in P25$^-$ phage infections of starved cells. Thus, the DNA is at least partially released from the capsid into the cell. There is no direct evidence that the DNA is completely released and disengaged from the capsid. This process may require P25. The *trans* activity of P25 implies that it is able to act after it is released from the capsid.

There is precedence for partial release of phage DNA from the particle. Transfer of the phage T5 DNA molecule from the infecting particle to an *Escherichia coli* cell is divided into two steps, transfer of an initial portion

(the first-step-transfer or FST section) amounting to about 8 percent of the whole molecule, and transfer of the remainder (Lanni, 1960; McCorquodale and Lanni, 1964; Lanni, McCorquodale and Wilson, 1964). The second transfer is under control of FST-DNA genes (Lanni, 1965; Lanni, 1969). This is a case in which the release of most of the phage DNA from the particle requires a phage specified function dependent on newly synthesized proteins in the host.

2. P25 may be involved in bringing the released DNA to the appropriate compartment within the cell. In order for the DNA to be effectively infective it must either attach to or pass through host membrane to reach the proper cellular site.

There is no direct evidence that the released phage DNA gets beyond the cytoplasmic membrane in the absence of P25. The DNA may be in the periplasmic space, between the cell wall and membrane, and the action of P25 is required to get it beyond this barrier. It is difficult to visualize how complementation can occur, particularly in sequential infections, if the 25⁻ DNA is blocked at the cell membrane.

3. There may be virion proteins which bind to the infecting DNA. Unless they are removed transcription and replication are prevented. P25 may act to clear the DNA of these proteins and render it infectious.

The bound proteins may be residual capsid proteins

which serve no specific function after release of the DNA. Alternatively, the DNA-associated protein may be one which plays a specific role after DNA release. P20 is another P22 virion protein which has no direct role in particle assembly, but is necessary for infectivity. P20⁻ defective particles, which are missing P20, are produced in an am20 nonpermissive infection (Botstein, Waddell, and King, 1973; King, Lenk, and Botstein, 1973). If P25 is required to remove P20, then the interesting question would concern the role played by P20 early in infection.

4. P25 may produce an essential structural alteration in the injected DNA. The structural change may be in the primary structure, such as nicking a strand, which might make sites available to particular enzymes. The tumor virus SV40 contains a minor capsid protein; one role proposed for this protein is an endonuclease activity necessary for the conversion of supercoiled form I viral DNA to nicked open circular form II DNA before transcription can be initiated (Robb and Martin, 1972). Another possibility is that the secondary or tertiary structure of the P22 DNA is changed by the action of P25. The T4 gene-32 protein is required in stoichiometric amounts for replication of T4 DNA in vivo (Sinha and Snustad, 1971). Huberman and Kornberg (1971) suggest that 32-protein stimulates T4 DNA polymerase by removing inhibitory secondary structures from the template DNA strand.

Transfection is the term used for infections initiated

directly with naked phage DNA. The frequency of transfection varies, depending on the phage DNA and the method employed to make cells competent. The highest frequency of transfection reported for P22 is one infective center per 3×10^7 molecules of P22 phage DNA when lysozyme-EDTA spheroplasts of E. coli are transfected in the presence of 25 μg of protamine sulfate/ml (Lawhorne, Kleber, Mitchell, and Benzinger, 1973). For penicillin spheroplasts of S. typhimurium, the frequency approached 10^{-8} (Benzinger, and Kleber, 1971). The frequency of transfection in E. coli for phage λ is often as high as 10^{-3} (Benzinger, Kleber, and Huskey, 1971), but for T7 it is at best 3×10^{-6} (Lawhorne et al., 1973). The infectivity of P22 DNA is low compared to that of most other phages. Perhaps this is because the infecting DNA requires the function normally supplied by P25.

Infection with helper phage is one of several procedures employed to make cells competent for transfection. If the frequency of transfection of P22 DNA is low because of the absence of P25 function, the $P25^+$ helper phage should increase the infectivity of the DNA. As a control, $P25^-$ helper phage should not increase the transfection frequency. A positive result with this type of experiment would favor models 2 and 4 and tend to eliminate models 1 and 3.

There are other phages with capsid proteins that perform essential early functions after adsorption and are

infected into the cell with the nucleic acid. M13, a filamentous, male-specific, single-stranded DNA virus, contains a minor protein constituent, P3, which enters the cell with the phage DNA (Jazwinski, Marco, and Kornberg, 1973). The authors suggest that P3 may perform an essential function in the synthesis of double stranded replicative form DNA by linking the phage DNA to a cellular replicative system in or on the inner cell membrane. In MS2, an RNA phage which adsorbs to F-pili on E. coli, a minor capsid protein is injected into the host in association with the viral RNA (Kozak and Nathans, 1971). The infecting RNA-protein complex may play a role in either the penetration process or subsequent expression of phage genomes. The A protein in R17, another RNA male-specific phage, is transferred into the cell along with equimolar amounts of phage RNA (Krahn, O'Callaghan, and Paranchych, 1972). This protein is responsible for the initial attachment of phage to F-pili (Roberts and Steitz, 1967). Krahn et al. suggest that this protein may also play a rôle in promoting penetration of phage RNA into the cell and/or in the replication process.

IV. THE ROLE OF P25 IN MORPHOGENESIS

P25 is an essential component of the P22 virion. Temperature sensitive mutants in gene 25 contain apparently heat labile P25. There is evidence suggesting that these

mutant virions also contain fewer P25 molecules than wild type phage. It seems possible that at 41°C P25 is synthesized but is not incorporated into the virion because it is denatured. This results in the formation of defective particles missing P25. Perhaps, even at low temperature the configuration of P25 limits the number of these molecules packaged into the P22 ts25 particle. Am25 mutant virions may also contain fewer P25 molecules than wild type virions. Nonpermissive infections with P22 am25 result in defective particle production because only P25 fragments are synthesized.

King, Lenk, and Botstein (1973) have shown that P25 is present in the 240S prohead particles, which are early intermediates in the head morphogenesis pathway. The absence of P25 has no effect on the subsequent assembly process because phage DNA is packaged into particles which are morphologically indistinguishable from wild type virions when examined by electron microscopy (Botstein, Waddell, and King, 1973). These noninfectious particles cannot be activated *in vitro* (Botstein *et al.*, 1973; Hoffman, unpublished data); however, they can be complemented in mixed infections. P20 defective particles were briefly described above. These particles cannot be activated *in vitro* (Botstein *et al.* 1973) and are poorly complemented in mixed infections (Hoffman, unpublished data).

The internal proteins of T-even coliphages are

packaged in the virion and can be separated from phage ghosts by osmotic shock. They are transferred into the infected cells along with the phage DNA (Backrach, Levin, and Friedman, 1970; Stone, Kenneth, and Cummings, 1972). Amber mutants of T4 have been isolated which fail to synthesize internal proteins (Showe and Black, 1973). Internal proteins are dispensible for growth since a T4 mutant constructed from an amber point mutation in each of the three internal protein genes grows on a su⁻ host. Phage particles, which lack all three internal protein species have normal infectivity. Thus, the internal proteins packaged in the T4 virion do not have any role early in the next infection. T4 assembly is not affected by the absence of internal proteins I or II. However, the absence of internal protein III does have an effect; the rate of head assembly is only one-half that of wild type, and large numbers of polyheads are produced (Showe and Black, 1973). Thus internal protein III has a dispensible assembly function.

There are two groups of amber mutants in T7 which produce particles similar to P25⁻ and P20⁻ defective particles. Nonpermissive infections with mutants in either gene 7 or gene 13 result in the formation of DNA-containing particles which lack only the protein specified by either gene 7 or 13 (Studier, 1972). They appear normal in electron micrographs. However, these particles are not infectious and cannot be activated in vitro.

Complementation for these noninfectious particles in mixed infections has not been tried (Studier, personal communication). Transfection frequencies for T7 are low, perhaps because P7 and/or P13 are required for the infecting DNA. For phages P22 and T7 there are at least two head proteins which are not essential for normal head assembly, but are required in the ensuing infection.

V. SUMMARY AND CONCLUSIONS

P25 is a capsid protein, and as such, is incorporated into the phage head as a step in morphogenesis. The role in assembly is not essential since particles are formed without this protein, but these particles are defective. P25 is essential when the particle infects a cell in the following cycle of infection. In the absence of functional P25 the infection does not appear to proceed beyond release of phage DNA from the capsid, since no known genes are expressed, no DNA is transcribed, and the host cell survives the infection, continuing to grow and divide normally.

The P25 function is required only during infection for the expression of phage functions. Induction in the absence of P25 proceeds with the expression of early and late genes and particle formation. The P25 function is necessary for transduction as well as for infection. Its activity is independent of active cellular metabolism and it is not

under immunity control. P25 can act in *trans*, but appears to act preferentially on the phage or phage DNA with which it is packaged. The data from complementation studies are compatible with P25 release from the capsid with the phage DNA. In the absence of P25 the infection is blocked, but the phage genome is not degraded.

The various roles which have been ruled out for P25 are: (1) an early regulatory function, (2) an enzymatic activity necessary for phage production, (3) protection of phage DNA from host degradation enzymes, (4) binding parental DNA to the replication complex, and (5) any generalized alteration of the host cell.

The following functions for P25 have not been excluded by the data. P25 is responsible for: (1) complete release of the DNA and disengagement from the capsid, (2) bringing the released DNA to some necessary cell site or compartment such as the cytoplasm, (3) removal of other virion proteins from the injected DNA, and (4) alteration of the structure of the injected DNA.

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