SUPPRESSOR-SENSITIVE MUTANTS OF BACTERIOPHAGE PBS2

by

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A Thesis

Submitted to the Faculty of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

June, 1975

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DOCTOR OF PHILOSOPHY (1975)
Department of Biology

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Suppressor-sensitive mutants of Bacteriophage PBS2

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NUMBER OF PAGES: ix, 144

SCOPE AND CONTENTS:

Suppressor-sensitive mutants of Bacillus subtilis phage PBS2 were isolated from chemically mutagenized lysates. These mutants were found to comprise thirty-eight distinct complementation groups. Mutants affected in DNA synthesis and host cell lysis were identified and characterized. Two complementation groups were found to be involved in the appearance of the phage-induced DNA polymerase activity. A linear genetic map was constructed based on the results of two and three-factor genetic crosses and linkage tests.

PREFACE

The experiments described in this thesis were conducted in the Department of Biology, McMaster University, from September 1970 to November 1974. Except where others are specifically mentioned, this thesis consists of original research. To my knowledge, no similar thesis has been submitted to any other university.

I would like to thank my advisor, Dr. I. Takahashi, and other committee members, Dr. L. Prevec and Dr. H. P. Ghosh for their supervision and support throughout the course of this study. I am grateful as well to the Department of Biology for its financial assistance.

I wish to thank my fellow graduate students, Bert Rima and Muriel Herrington for their frequent advice and especially, Mrs. Darryll Bradford for her helpful technical assistance and Mr. M. Botos for always having clean glassware.

Finally, for their wonderful efforts in helping complete this thesis I express my sincere gratitude to my wife, Joan Pinkus, and to our friend, Mrs. Mary Haight.

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INTRODUCTION

PBS2 is a large, morphologically complex, transducing phage active on Bacillus subtilis. It was isolated by Takahashi (1) as a clear plaque mutant of phage PBS1. These two phages were found to be serologically related and morphologically indistinguishable. In liquid culture, at 37C, PBS2 had a latent period of 37 minutes, a rise period of 18 minutes and a burst size of 24. Eiserling (2) undertook a study of the structure of PBS1. His results showed that PBS1 is indeed very large, having an icosahedral head with a diameter of 120 nm and an estimated volume of 5×10^8 A^3, and a 200 nm long complex contractile tail, with a striated sheath having a 38 Å band period. He observed a differentiated region at the base of some uncontracted tails and a number of unique thin fibres which project outward from the base of contracted sheaths, which he termed contraction fibers. As well, long helical tail fibers (usually 3 per particle) were found to be attached to the base plate.

Joys (3) showed that PBS1 could not form plaques on or adsorb to non-motile mutants of B. subtilis which lacked flagella. However, spontaneous flagellate revertants which were motile regained susceptibility to the phage. Frankel and Joys (4) later found that PBS1 has an adsorption specificity for active flagella. Raimondo et al. (5) found that physically deflagellated cells lose the ability to adsorb PBS1, but regain it when the flagella are allowed to regenerate. Moreover, they found that

PBS1 can adsorb to non-motile protoplasts, paralyzed mutants and cells rendered non-motile with cyanide, although productive infection does not occur in these cases. Their experiments with cyanide suggested that adsorption is a two-step process. Primary adsorption is resistant to cyanide and is reversible by chloroform treatment while the second step is irreversible and sensitive to cyanide. Their electron micrographs clearly show that the helical tail fibers of PBS1 are capable of wrapping themselves around the host flagella. Salmonella typhimurium phage chi as well as two other Bacillus phages, PBP1 and SP15 were also shown to require flagella for adsorption (6,7,8).

PBS1 was at first thought to form stable lysogens in culture (9). It was later found (1) that lysogenic cultures of either PBS1 or PBS2 were unstable and reverted to the sensitive state when grown in the presence of phage antiserum. Takahashi (10) showed that phage DNA in spores carrying PBS1 was not attached physically to the host chromosome. PBS1 and PBS2 do not, therefore, exhibit classical lysogeny, as exemplified by phage lambda (11), but they can initiate a carrier state, sometimes referred to as pseudolysogeny (11,12). Bott and Strauss (13) showed that SP10, another generalized transducing phage for B. subtilis, can exist in an unstable carrier state, similar to that of PBS2. Kawakami and Landaman (14) were able to enhance the stability of SP10-carrying cells enough to study certain features of the carrier state. Under these conditions they found that the immunity of phage-carrying cells to superinfection varied with the phase of growth of the culture and that more than 90% of carrier cells do not contain mature phage particles.

They have suggested two possible models for this phenomenon. In one model, phage and host DNA replication would occur within the same cell, although at unbalanced rates, resulting in the observed segregation of sensitive cells. In the other scheme, original carrier cells, containing more than one phage genome, would begin to divide, resulting in eventual segregation of sensitive cells. In this case, no phage DNA synthesis would occur in the carrier cells. Differences in chemical composition between phage and host DNA in the case of SP10 and PBS2 would seem to dictate against the former model, however, this has not been definitely established.

The thermal denaturation temperature (Tm) of DNA and its buoyant density in a cesium chloride gradient are linearly dependent upon the guanine plus cytosine content of the DNA. This has been shown to be the case for a large number of DNA samples from various organisms, all of which contain the four normal bases in the DNA (15). Takahashi and Marmur (16) reported a Tm of 75.6C for PBS2 DNA, which would correspond to 17.5% guanine plus cytosine. Its buoyant density was found to be 1.722 g/cc, indicating 62% guanine plus cytosine. Further investigation by these authors resolved this discrepancy by revealing the presence of uracil and the absence of thymine in PBS2 DNA. The guanine plus cytosine content of the DNA was chemically established as 28%, while that of the host was 43%. Prior to this investigation, uracil had been found only in RNA. Langridge and Marmur (17) took advantage of this unique property by doing x-ray diffraction studies with PBS2 DNA fibers. Their results showed diffraction patterns consistent with DNA

in the B configuration, leading them to conclude that the 2'-hydroxyl group is responsible for the difference between double stranded RNA and DNA.

The molecular weight of PBS2 DNA was reported by Hunter et al. (18) to be 1.9×10^8. This was determined by zone centrifugation in sucrose and comparison with the DNA of Escherichia coli phage T2 which had been reported to have a molecular weight of 1.3×10^8 (19). The molecular weight of T2 DNA has since been revised downward (20), and thus that of PBS 2 should be correspondingly less. An independent determination of the molecular weight of PBS1 DNA, based on the sedimentation coefficient was made by Tyeryar et al. (8) who found it to be 1.7×10^8. B. subtilis phage SP15 DNA, by comparison, had a molecular weight of 2.5×10^8. The molecular weight of the B. subtilis chromosome has been estimated to be $3-4 \times 10^9$ (21). The chromosome of PBS1 and therefore PBS2, is extremely large for a virus and is theoretically capable of coding for a very large number of genes. Yamagishi (22), from his alkaline denaturation studies of PBS1 DNA, concluded that DNA isolated from PBS1 particles contains an average of two interruptions in each strand. His results also indicated that sealing and subsequent re-introduction of these interruptions occurs after infection. The sizes of the fragments produced by alkaline denaturation were heterogeneous indicating that the interruptions are not as specific as those of the E. coli phage T5 (23).

Takahashi (24) reported that the frequency of joint transfer of closely linked B. subtilis markers is about the same, whether transformation

or PBS1-mediated transduction is used in the transfer. However, when more loosely linked markers were used, it was found that the frequencies of joint transfer by PBS1 transduction were much higher than those obtained by transformation. From this result, Takahashi concluded that the size of donor DNA incorporated into transducing particles may be larger than fragments of transforming DNA. Tomita and Takahashi (25) showed that extensive degradation of host DNA in PBS1-infected cells is probably absent since the co-transfer index was found to be the same for DNA from infected cells as that from uninfected cells. Similar results have been obtained with another B. subtilis transducing phage, SP10 (13) and with SP01 (26), a virulent phage for this host. Phage T4, on the other hand, is known to degrade the DNA of its host, E. coli, extensively, shortly after infection and to utilize the resulting nucleotides for its own DNA synthesis (27). Mahler et al. (28) reasoned that the differences in base composition between PBS2 and its host should rule out a mode of transduction involving integration or recombination of the phage genome with that of the host. They did not, however, observe transducing particles carrying only one DNA type characteristic of the host, in cesium chloride banding experiments. They concluded that particles of PBS2 are heterogeneous, banding at different positions in CsCl density gradients and that those capable of transduction contain fragments of host DNA either unassociated or loosely associated with the phage genome. Yamagishi and Takahashi (29) undertook the separation of transducing particles from plaque-forming particles in PBS1 lysates. This was attempted in order to characterize the DNA from transducing particles.

Using a double labelling technique, they found that all radioactivity corresponding to newly synthesized DNA from infected cells banded with plaque-forming particles at 1.443 g/cc in CsCl, while the pre-labelled host DNA banded with transducing particles at 1.433 g/cc. In their experiments, the density of transducing particles fluctuated, but was consistently 0.010 g/cc lower than that of plaque-forming particles. The results of earlier experiments by Lozeron and Szybalski (30), which indicated that the volume fraction of DNA in PBS2 particles is 0.5, taken with the difference in density of 0.0189 g/cc between phage and host DNA, led the former authors to conclude that only host DNA is contained in transducing particles. When large quantities of phage were centrifuged in a CsCl gradient, they found a band which varied in density from 1.458 to 1.483 g/cc. This band had no plaque-forming or transducing activity and electron microscopic examination revealed that it contained phage heads. During the course of their experiments, it was found that phage particles can lose their tails and shift to this band when centrifuged after storage at 5C. The DNA extracted from transducing particles was found to have a density identical to that of the host DNA. Its distribution in a sucrose gradient was broad indicating heterogeneity in size. Based on an assumed molecular weight of 1.3×10^8 for T2 DNA, they calculated the molecular weights of DNA from transducing particles to range from 4×10^6 to 1.3×10^8. The generalized transducing phage P1 of E. coli was also found to contain only host DNA in transducing particles, the molecular weight of which was similar to that of the DNA in plaque-forming particles (31). Okubo et al. (32) found that transducing particles in

lysates of B. subtilis phage SP10 contained DNA which was characteristic of the host. As in the case of PBS1 and PBS2, no physical association of phage and host DNA was detected.

Dubnau et al. (33) used PBS1 transduction, among other techniques, to construct a genetic map of B. subtilis. They found that metA3 and ura26 were the most widely separated markers to be co-transduced by PBS1. According to their map, the distance between these markers comprises about 8 percent of the host genome. Assuming an approximate molecular weight of 4×10^9 for the B. subtilis chromosome, they concluded that PBS1 transducing particles could contain DNA with a total molecular weight of at least 2.0×10^8.

Bacteriophage infection results in the utilization of some host cell components in the process of progeny phage production. This can be accomplished in a variety of ways and the number of gene functions involved depends on the particular phage and the nature of its interaction with the host. For example, RNA phages for E. coli require only three genes (34) and a viable host to assure their reproduction. Temperate phages SP02 and ø105 for B. subtilis (35,36) contain chromosomes with molecular weights of about 24×10^6 and have been estimated to code for eighteen to forty genes. Virulent B. subtilis phage ø29 has at least thirteen and perhaps as many as twenty genes (37,38), with a chromosome molecular weight of 11×10^6. The PBS2 chromosome, on the other hand, has a molecular weight of $1.6\text{-}1.9 \times 10^8$ and may therefore be expected to code for more than 200 genes, provided its DNA is not largely redundant.

Since PBS2 DNA contains uracil, one can hypothesize that major

changes in pyrimidine metabolism must occur in order for dUTP to become an available precursor for phage DNA synthesis. If the synthesis of thymine-containing and uracil-containing DNA are mutually exclusive, one might also expect some effect of the phage on thymine nucleotides in the cell. Kahan (39) described two enzyme activities in PBS2-infected cells which are not found in uninfected cells. One of these is dUMP kinase, which phosphorylates dUMP and is presumably involved in dUTP production. It might also be looked upon as being responsible for the removal of dUMP, the substrate for the enzyme dTMP synthetase (39). In the case of B. subtilis phage Øe, which contains hydroxymethyluracil in place of thymine in its DNA, thymidylate synthetase is known to be inhibited (40), however, Rima (unpublished data) and Tomita (unpublished data) have evidence that this does not occur in PBS2 infection. Kahan (39) also found a PBS2-induced dTMPase activity which may effect the removal of the substrate for dTTP synthesis. This enzyme has been further characterized by Price and Fogt (41) who showed that it also has dUMPase activity. This dUMPase activity however, has a much higher Km than the TTPase activity. Tomita and Takahashi (42) reported a novel enzyme, dCTP deaminase, induced after PBS1 infection. They did not find any dCMP deaminase activity in uninfected or infected cells. The dCTP deaminase was found to be non-competively inhibited by dTTP. The related hydroxymethyluracil-containing phages SP8, SP82, and Øe induce a dTTPase upon infection (40) and PBS1 does the same (43). The dCTP deaminase in PBS2-infected cells was further characterized by Price (44). The enzyme has an apparent molecular weight of 125,000 and exhibits

sigmoidal substrate saturation kinetics. He observed inhibition by dTTP and other thymidine nucleotides as well as by dUTP. In the case of dUTP, inhibition was observed at substrate concentrations lower than Km. Furthermore, Rima (unpublished data) has found that dCTP deaminase is not the only means of dUTP production in PBS1-infected cells. His results suggest three major contributing pathways. The other two pathways are dCDP → dUDP via a novel host enzyme, dCDP deaminase and UTP → dUTP via ribonucleotide reductase. In a variety of strains, 50% of the dUTP in phage DNA has been shown to result from UTP reduction.

Tomita and Takahashi (25,45) have conducted a study of DNase activities in B. subtilis and discovered an endonuclease which is specific for native uracil-containing DNA. A heat-stable protein inhibitor of the enzyme was found to be induced upon PBS1 infection. It was also found that spores carrying the PBS1 genome also contain the inhibitor. This would indicate that the maintenance of phage DNA in the carrier state may require this inhibitor and thus active participation of the phage in establishing this state. However, as synthesis of phage DNA and host DNA are normally incompatible in the same cell, a complex regulatory scheme involving the phage and/or the host is most likely responsible for the establishment and maintenance of the carrier state. Wovcha and Warner (47) found that E. coli DNA-polymerase I can synthesize and degrade, endonucleolytically, uracil-containing DNA. Their results showed that the nuclease activity was greater with uracil-containing DNA than with thymine-containing DNA. Destruction of the 5' → 3' nuclease portion of the DNA polymerase I resulted in the loss of both the ability to incorporate

dUTP into DNA and the uracil-specific endonucleolytic activity. In the case of B. subtilis, the uracil-specific nuclease activity is probably not associated with DNA polymerase I since no nuclease activity has been detected with this enzyme (46). The reason for the existence of these uracil-specific nuclease activities is unknown. It may be that they serve to excise dUMP from bacterial DNA should it be erroneously incorporated (47). Why this would be necessary is uncertain, since uracil should not affect subsequent replications adversely.

Both E. coli and B. subtilis are known to contain three distinct and somewhat analagous DNA polymerizing enzymes (48). It has recently been shown that DNA polymerase III is essential for replication in each case (49,50). A variety of other functions are also essential for replication (49). The other polymerases may also play a significant role in the process (51,52). Aposhian and Kornberg (53) observed a DNA polymerase activity induced by T2 infection of E. coli. Warner and Barnes (54) and De Waard et al. (55) showed that gene 43 of the related phage T4 is the structural gene for the phage-induced DNA polymerase. This then, was the first DNA polymerase identified which is essential for the replication of the chromosome of an organism. The enzyme has since been well characterized and is known to contain a single polypeptide chain and exonucleolytic activity as well (56). There is a considerable amount of evidence that some mutations of this enzyme result in enhanced mutagenicity and some may affect recombination frequencies as well (57,58). This evidence and the existence of conditional lethal mutations in this enzyme support the notion that it is the replicative enzyme of the phage. Oey et al. (59) identified and purified a DNA polymerase induced by phage T7 of

E. coli. They were also able to identify the structural gene for the enzyme. As in the case of T4, mutations which inactivate this enzyme are lethal for the phage. They ascertained that the T7 DNA polymerase is also a single polypeptide chain containing some exonucleolytic activity. Yehle and Ganeson (26) identified a new DNA polymerase induced by the hydroxymethyl-uracil-containing B. subtilis phage SPO1. This DNA polymerase has also further been characterized (60). Rutberg and Armentrout (61) showed that temperate B. subtilis phage SPO2 also induces a DNA polymerase activity and that this enzyme is essential for phage DNA synthesis. Since PBS2 contains uracil in its DNA, Price and Cook (62) investigated the possibility that it, too, induces a new DNA polymerase in infected cells. They did observe a new DNA polymerizing activity which, in crude extracts, incorporated dUTP and dTTP equally well. This activity was found in infected cells of a mutant host, deficient in DNA polymerase I. Induction of the activity required protein synthesis, but was resistant to rifamycin, a drug known to inhibit RNA synthesis of the host (63). It was therefore concluded that PBS2-directed mRNA synthesis is responsible for the induction of the enzyme. It was not possible for them to determine whether this DNA polymerze is essential for phage viability.

The drug 6-(p-hydroxyphenylazo)-uracil(hpUra) has been shown by Brown (64,65) to selectively inhibit semi-conservative DNA replication in B. subtilis and other gram-positive bacteria. Gass et al. (66) showed that reduced hpUra specifically and reversibly inhibits DNA polymerase III of B. subtilis. This enzyme was thus implicated to be involved in chromosome replication. Cozzarelli and Low (50) later showed that DNA

polymerase III from a B. subtilis mutant resistant to hpUra was resistant to the drug in vitro. Their results support the idea that the DNA polymerase III is essential for chromosome replication. Experiments carried out with mutants thermosensitive for DNA replication in E. coli led Gefter et al. (49) and Nusstein et al. (67) to draw the same conclusions about the DNA polymerase III of that organism.

A number of B. subtilis phages have been shown to be resistant to hpUra (64,68,69), some of which are known to induce new DNA polymerase activities upon infection. Price and Fogt (70) found that PBS2 is also resistant to this drug, indicating that the phage does not require DNA polymerase III of the host. Nalidixic acid, another drug which inhibits bacterial DNA synthesis does not affect PBS2 at concentrations which are strongly inhibitory for the host (71). Similar results have been obtained with other phage-host systems whereas certain phages are inhibited by the drug (72,73). There is, apparently, no correlation between resistance of phage to nalidixic acid and the presence of unusual bases in DNA (73).

The rifamycins are a class of antibiotics best known for their specific effect on the DNA dependent RNA polymerase of a large number of microorganisms. The history and action of these drugs has been reviewed in some detail by Wehrli and Staehelin (74,75). Rifamycin is known to specifically inhibit RNA synthesis in B. subtilis with resulting effects on protein synthesis. Phages which are known to rely on host RNA polymerase for transcription have been shown to be sensitive to rifamycin (74). The development of phage T7 for E. coli was found to become resistant to rifamycin shortly after infection (76), and this

was thought to be due to a modification of the host RNA polymerase. However, subsequent experiments showed that a new rifamycin insensitive RNA polymerase is induced by T7 (77). This enzyme was shown to be the product of gene 1 of the phage and is responsible for transcription of late phage functions. It was later discovered that certain rifamycin derivatives can inhibit the T7 enzyme (78).

Price and Frabotta (79) and Rima and Takahashi (80) showed that PBS1 and PBS2 are resistant to rifamycin, even when the drug is added several minutes prior to infection. PBS2 infection was also resistant to streptovaricin and streptolydigin, other antibiotics known to inhibit the host RNA polymerase (79). However, phage production was severely inhibited by actinomycin D and lucanthone, which affect RNA synthesis by direct interaction with the DNA template (79). Rima and Takahashi (80) observed that rifamycin reduced the rates of ^{3}H-Urd incorporation into RNA and DNA by more than 90% in infected cells. It was difficult to reconcile such a drastic apparent decrease in DNA synthesis with essentially normal yields of phage. Incorporation of ^{3}H-dCyd into DNA in rifamycin-treated cells using PBS1 and DNA-affected mutants of PBS2 revealed that phage DNA synthesis was not affected by the drug. This means however, that ^{3}H-Urd incorporation into RNA and DNA under those conditions is not an accurate measure of rates of nucleic acid synthesis. Such an effect has been predicted due to altered metabolism of nucleotide pools for bacterial systems in which the bulk of stable RNA synthesis is inhibited (81,82). Hybridization experiments showed that a portion of the pulse-labelled RNA from PBS1-infected cells hybridized specifically with phage DNA.

These results strongly suggest that PBS2 either codes for its own RNA polymerase which is resistant to rifamycin, and/or, that it somehow modifies the host enzyme rendering it insensitive to the drug. It may be that the phage injects a protein involved in RNA synthesis along with its DNA, or a secondary RNA polymerizing activity, effecting at least early phage RNA synthesis may exist in the host. Recently, Losick et al. (83) isolated a novel rifamycin insensitive RNA polymerase from PBS2 infected B. subtilis cells. The purified enzyme was found to contain four polypeptides which are presumptive subunits and distinct from those of the major host RNA polymerase. The composite molecular weight was estimated to be 262,000. Their results show that this enzyme is distinct, in a number of ways, from that of the host. The question still remains, however, as to why PBS2 is resistant to rifamycin pre-treatment.

A recent report by Rothman-Denes and Schuto (84) describes a somewhat similar phenomenon in phage N4 infection of E. coli. N4 is apparently the only known DNA-containing E. coli phage which can induce RNA synthesis despite pre-treatment with rifamycin. Experiments with N4 involving rifamycin and other drugs showed that two novel RNA synthesizing activities are involved. Induction of one requires protein synthesis after phage infection while the other does not. Although both activities in vivo are resistant to pre-treatment with rifamycin, at least some late N4 RNA synthesis is sensitive to the drug. On the other hand, both of the new activities were found to be inhibited by a rifamycin derivative known to inhibit phage T7 RNA polymerase. It is possible

that one or both of the N4-induced RNA polymerase activities represent secondary host enzymes. The only rifamycin-insensitive E. coli RNA synthesis thus far described is that involved in DNA replication (85).

The earliest reports of phage mutants were those of Burnet and Lush (86) and Gratia (87). Luria (88) described the isolation of host-range mutants of a phage active on E. coli. These mutants were capable of forming plaques on host strains which were resistant to wild-type phage. Hershey (89) reported plaque morphology mutants of phage T2 for E. coli. These were termed r mutants, for rapid lysis, which is actually the loss of characteristic lysis inhibition in the formation of plaques. Hershey (89) found further that these r mutants could themselves mutate with respect to host range, indicating that at least two genetic determinants were atttributable to the phage. Hershey and Rotman (90) showed that recombination between different r mutants in doubly infected cells was possible and that at least two different linkage groups leading to the r phenotype existed. The same authors (91) studied genetic recombination between plaque morphology and host range mutants in doubly infected single bacterial cells.

Collation and interpretation of the data from a number of such studies led Visconti and Delbruck (92) to formulate a theory of the mechanism of recombination in bacteriophage. Their model essentially requires consideration of the phage cross as a population of mating and replicating bacteriophage genomes. It must be noted that the simple but

elegant experiments upon which this theory was constructed were considerably facilitated by the types of phage mutants available. In crosses involving plaque morphology and host range mutants, large numbers of recombinants could be counted directly and results compared on different host strains. This was favourable for multi-factor crosses which were vital to the theory. Visconti and Delbruck (92) postulated that parental phage infect a cell, becoming vegetative phage which undergo replication. As replication proceeds, the frequency of matings becomes significant. At some point thereafter random maturation of phage into completed particles begins and proceeds at a constant rate effectively and irreversibly removing phage from the mating pool. This process continues until the time of cell lysis. Thus, in a single phage cross, a given genome may undergo a number of different mating events so that reciprocal recombinants may not be produced in equal numbers in single cells, but will be in a population of doubly infected cells.

Streisinger and Bruce (93) offered a clear and valuable operational definition of linkage of genetic markers in bacteriophage systems. Using simple criteria and three factor crosses, they were able to unequivocally establish linkage of all the then known markers of T2 and the related phage T4. This meant that all markers could be considered to exist in a single chromosome. Streisinger et al. (94) later applied the linkage test to appropriate mutants of T4, since contemporary theories regarding the physical structure of the DNA suggested a circular linkage map. Their results were consistent with a circular genetic map, however, no definite conclusion could be drawn as to the physical configuration of

the chromosome. The most common explanation of results of this sort is that the phage population contains heterogeneous chromosomes which are circular permutations of one another. Before linearity or circularity of a genetic map can be firmly established, a large amount of markers which are scattered over most of the genome must be available (95). Since the chromosome of phage P22 for S. typhimurium seemed to be similar in some respects to that of T4, Grough and Levine (95) tested the genome for circularity. Using plaque morphology mutants and conditional lethal mutants, they obtained results which were best explained by a circular map. B. subtilis phage Ø29 and vegetative E. coli phage lambda are known to have linear genetic maps and linear DNA molecules, which can circularize under certain conditions (37,96,97). B. subtilis phages SP82 and SP50 also contain linear maps and linear chromosomes (98,99,100). Phages SP02 and Ø105 are similar in that both lysogenize the B. subtilis host. Though the chromosome of both are linear, the SP02 prophage map is circularly permuted relative to mature phage, while this is not the case for Ø105 (101).

The E. coli chromosome is known to exist in circular form and recent experiments have shown both physically and genetically that the B. subtilis chromosome is also circular (102,103).

Interest centered around two possible models for recombination. A copy-choice model would explain recombination by commencement of replication along one parental template with a subsequent switch to that of a second parent. A breakage model, on the other hand, predicts fragmentation of parental chromosomes and reassociation of these into new and complete progeny genomes. Kozinski (104) using phage T4 and a

combination of radioisotope and density labelling, obtained evidence for semi-conservative replication of parental DNA. However, this parental phage DNA was fragmented and distributed among the progeny chromosomes. Shahn and Kozinski (105) in an extension of these experiments, showed that at most one DNA fragment of parental origin appeared in a given progeny genome. These results are consistent with breakage and reunion involving crossing over of semi-conservatively replicating genomes. Further experiments carried out by the same authors suggested one exchange per mating event (105).

Meselson and Weigle (106) found that the DNA of lambda is a single semi-conservatively replicating structure which is able to remain intact and unreplicated when high multiplicities of infection are used. The same authors found that discrete segments of what was originally parental DNA accounted for the density distributions among recombinant progeny and thus that recombination must have occurred by breakage and reunion. Important as well, was the finding that chromosomes need not replicate in order to recombine. Experiments such as these have not ruled out copy-choice or other modes of replication as natural processes but they have provided evidence for the occurrence of breakage and reunion of semi-conservatively replicating phage genomes.

The genetic and biochemical study of any organism depends, to a great extent, on the isolation of mutants. This in turn depends on some means of selection or identification of variants in normal populations. For this reason, mutants are commonly sought which exhibit a predictable alteration in phenotype under defined conditions. This of

course, requires knowledge of the existence of a given trait. In the case of bacteriophage genetics, the earliest mutants were those with altered plaque morphology or host range. Both of these were reasonable candidates for inheritable change in the absence of significant information on the biochemistry of phage development. Such an approach, however, can at best provide information about relatively few genetic functions. The best course to follow in the study of the organization of a particular phage would be to obtain a class of mutants which are easily identifiable, yet capable of producing defects in a large number of genes. On the assumption that the normal functioning of many genes would be essential for successful infection and thus viability of phage, one could attempt to find mutants which would be lethal under certain conditions and non-lethal under others. The theoretical assumption behind such an attempt arises as an extension of the one gene one enzyme hypothesis (107,108).

Campbell (109) applied such reasoning in his efforts to isolate mutants of phage lambda for E. coli. He succeeded in isolating mutants which were sensitive to temperature and others which were sensitive to a host suppressor. Temperature-sensitive mutants were identified by their ability to form plaques at one temperature, but not at a higher one. Suppressor-sensitive mutants were viable on host strains harbouring a suppressor allele, but not on non-suppressing strains. He demonstrated the feasibility of recombination analysis of these mutants, and devised complementation tests for the suppressor-sensitive mutants. Most significant was the fact that these mutants were found to fall in 18

complementation classes, with members of the same class mapping in the same region of the chromosome. These results showed that conditional lethal mutants can be isolated which are affected in a wide variety of phage functions. Furthermore, Campbell (109) was able to identify one complementation class as affecting the lytic enzyme induced by lambda and to establish that the ability to lysogenize is a function of the complementation class.

Edgar and Lielausis (110) reported the isolation of a large number of temperature-sensitive mutants of phage T4. Complementation tests revealed that at least 72 different genes were represented in their mutant collection. It was shown that these mutants were defective in stages of growth within the cell. Edgar et al. (111) made a comparative study of temperature-sensitive mutants of T4 and a newly discovered class of suppressor-sensitive mutants of the phage, called amber mutants. Considerable intracistronic complementation was observed between some pairs of temperature-sensitive mutants, however, none was found with amber mutants. This is not surprising since it is now known that amber mutants are nonsense or polypeptide chain-terminating mutants which can be suppressed by mutational alteration in certain transfer RNA species (112). Epstein et al. (113) first characterized temperature-sensitive and amber mutants of T4. These authors confirmed that temperature-sensitive mutations cause complete, though defective proteins to be produced whereas amber mutations result in the lack of gross alteration of the affected protein. For example, gene 23 was deduced to be involved in the formation of the head of T4. However, head components which were present in lysates of gene

23 temperature-sensitive mutants were absent from those of amber-mutants in the same gene. Thus, their work established that these mutations can affect a variety of functions and that some grouping, on the genetic map, of genes with similar function exists. Most of the mutants were found to be involved in DNA synthesis of the phage or in the complex process of morphogenesis.

Many of the properties of amber mutants can now be accounted for by their chain-termination property. Stretton et al. (112) have reviewed the evidence which has conclusively demonstrated that amber mutations result in the generation of the nonsense triplet UAG in DNA and thus, ultimately in the premature termination of polypeptide synthesis during translation. The product is therefore a protein fragment, the relative length of which depends on the distance from the 5' end of mRNA that the nonsense codon appears. Amber suppressors are mutations affecting transfer RNA species which then acquire the ability to recognize the amber codon and insert an acceptable amino acid into the polypeptide at some frequency, which is not lethal for the cell. It had also been shown that mutations producing UAA (ochre) codons also results in premature polypeptide chain termination and suppressors for such mutants can also be obtained (112). It would be erroneous to conclude that conditional lethal mutations could be found in any gene of a complex bacteriophage or virus, however, results of experiments with phages of E. coli have shown that a considerable number of phage functions can be elucidated in this way.

Okubo and Yanagida (114) succeeded in isolating a suppressor mutant

of B. subtilis. It was isolated as a histidine independent revertant of a multiple auxotroph in which the his⁻ genotype was shown to persist by transformation experiments. They were then able to obtain mutants of phage SP01 which were not capable of growth on the parent but could grow on the suppressing strain. Georgopoulos (115) was able to isolate suppressor-mutants of B. subtilis by selecting for joint revertants of multiple auxotrophs. He also reported the isolation of suppressor-sensitive mutants of phage Øe which is similar to SP01.

Tevethia et al. (116) recently conducted a comparative study of suppressor mutants of Okubo and Yanagida and of Georgopoulos, which were designated sup-1 and sup-3, respectively. These were presumed to be nonsense suppressors, since both suppress a large number of independently isolated mutants. In support of this, these authors hoped to detect high molecular weight forms of proteins from strains carrying these suppressors. These would be presumptive read-through products caused by suppression of normal chain termination. Such grossly altered proteins have not been found in E. coli strains carrying nonsense suppressors. This may have been due to a relatively low frequency of read-through events, or to a complex post-transcriptional protein cleavage mechanism. In order to assess the affects of these suppressors in B. subtilis, the appropriate isogenic strains were constructed. By analogy to the E. coli systems, the sup-1 mutation has been tentatively classified as ochre and the sup-3 mutation as amber, based on their effects on growth and patterns of suppression. The sup-1 mutation is relatively unstable, causes a reduction in growth rate and competence for transformation, and suppresses all mutants

suppressed by sup-3 as well as some that are not. The two mutations can co-exist in the same strain and apparently effect insertion of different amino acids. Of major interest is the evidence for slowly migrating forms or new isoenzymes of L-leucine dehydrogenase and triosephosphate isomerase in strains carrying the sup-1 or sup-3 mutation (117). The new forms of triosephosphate isomerase were found to have molecular weights more than twice those of the wild-type enzyme. Although several interpretations are possible, these results are consistent with the hypothesis that these are nonsense suppressors. Ultimately, identification and characterization of altered transfer RNA species in the suppressing strains will be necessary to resolve the issue with certainty.

Temperature-sensitive mutants of a number of B. subtilis phages have been examined and in the last few years, studies with conditional lethal mutants have been extended with the use of suppressor-sensitive mutants. The work thus far has centred on the small virulent phage, ø29 (37,38,118), the hydroxymethyluracil-containing phages such as SP01, and SP82 (98,99,119,120), and two other phages which can lysogenize B. subtilis, ø105 and SP02 (35,36).

Lozeron and Szybalski (30) showed that the dUrd analogue, 5-fluorodeoxyuridine, which inhibits thymidylate synthetase and thus DNA synthesis in other systems, can be incorporated into the DNA of PBS2. Herrington and Takahashi (121) then showed that the analogue is mutagenic for the phage. Using this and other chemical mutagens, Herrington (122) isolated a number of temperature-sensitive mutants of PBS2. The low and

variable burst size of the wild-type at the restrictive temperature made meaningful complementation tests difficult. A tentative genetic map containing 10 cistrons was constructed by comparing the results of two factor genetic crosses with those of complementation tests. As well, a competence regimen for PBS2 transformation was developed which made transformation of temperature-sensitive mutants with fragmented wild-type DNA possible. Sheared PBS2 DNA was centrifuged in mercury-cesium sulphate gradients, which separates fragments of DNA of different base compositions. In this way, certain temperature-sensitive mutants were shown to be transformed by DNA from particular fractions of the gradients.

In order to extend genetic studies of PBS2, a number of suppressor-sensitive mutants have been isolated in the present study. These have been assigned to complementation groups which were then screened for the ability to perform certain phage-related functions. Mutants found to be deficient in phage-induced DNA synthesis were further characterized and subjected to genetic analysis. Mutations in two distinct cistrons were shown to be unable to induce the PBS2-related DNA polymerase during infection of non-suppressing cells. A genetic map, including a number of the complementation groups identified in this study, was presented.

MATERIALS AND METHODS

1. Symbols and abbreviations:

Symbols used to designate the genotype of bacteria were as suggested by Demerec et al. (123).

The units of length, weight, volume, and time were abbreviated as in the Journal of Bacteriology. Abbreviations of nucleic acid consistuents were according to the 1970 recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (124).

Urd	uridine
Cyd	cytidine
dUrd	deoxyuridine
dCyd	deoxycytidine
dThd	deoxythymidine
ATP	adenosine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
FdUrd	5-fluorodeoxyuridine

Other abbreviations frequently used were:

CFU	colony-forming unit
PFU	plaque-forming unit

MOI	multiplicity of infection
PCA	perchloric acid
TCA	trichloracetic acid
EDTA	ethylenediaminetetraacetic acid
PA	Penassay Broth (Difco)
AD	adsorption medium
TBB	Tryptase Blood Agar Base
NG	N-methyl-N'-nitro-N-nitrosoguanidine
CPM	counts per minute
sus	suppressor-sensitive
ts	temperature-sensitive

Some of the genotypic symbols were:

tsA	temperature-sensitive DNA mutant in B. subtilis
pyrG	mutant requiring Cyd (lacking CTP synthetase)
cdd	mutant lacking cytidine/deoxycytidine deaminase
dck	mutant lacking dCyd kinase
polA	mutant deficient in DNA polymerase I
sup^+	mutant carrying a suppressor
sup^-	non-suppressing strain
AP-2	mutant lacking alkaline phosphatase

2. Bacteriophage and bacterial strains

Bacteriophage PBS2 was obtained from a single plaque isolate of our laboratory stock. Bacteriophage SP01 and a suppressor-sensitive mutant, sus5 of SP01, were obtained from Dr. S. Okubo.

The strains of B. subtilis used in this study were listed in Table 1.

TABLE 1. List of bacterial strains

Strain	Genotype	Source
SB19E	*str*^{r}, *ery*^{r}, *sup*$^-$	Laboratory stock
pyrG1dck4	*str*^{r}, *ery*^{r}, *ts*A13, *cdd*-1, *pyrG1*, *dck4*, *sup*	B. Rima
JB0128	*his*-5, *met*-5, *AP*-2, *sup*$^-$	S. Okubo
JB0130	*his*-5, *met*-5, *AP*-2, *sup*$^+$	S. Okubo
168 *polA*	*ind*$^-$, *thy*$^-$, *polA*, *sup*$^-$	NB841 of M. Marcus
SB202	*aro*-2, *trp*-2, *his*B2, *tyr*-1, *sup*$^-$	Laboratory stock

3. Media

(a) Difco antibiotic medium 3 (PA).

(b) Minimal medium (MM) was described by Spizizen (125).

$(NH_4)_2SO_4$	2.0 g
K_2HPO_4	14.0 g
KH_2PO_4	6.0 g
Sodium citrate	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
Distilled water	1.0 l

The pH was adjusted to 7.0 and after autoclaving, 50 ml of 10% glucose were added per litre of medium. Minimal agar (MA) contained 15g of Difco agar per litre of medium. Amino acids were added at 25-50 ug/ml when required.

(c) Difco Tryptase Blood Agar Base (TBB)

(d) Adsorption medium (AD) (29) was used for all phage dilution unless otherwise stated.

NaCl	4.0 g
K_2SO_4	5.0 g
KH_2PO_4	1.5 g
$Na_2HPO_4 \cdot 7H_2O$	5.68 g
$MgSO_4 \cdot 7H_2O$	0.12 g
$CaCl_2$ solution (1.6 g/100 ml)	1.0 ml
$FeCl_3$ solution (0.5 g/100 ml)	2.0 ml
Difco Yeast Extract	1.0 g
Distilled water	1.0 l

(e) Sporulation medium (SM) was as described by Schaeffer (126).

Difco Nutrient Broth	8.0 g
$MgSO_4 \cdot 7H_2O$	0.25 g
KCl	1.0 g
$MnCl_2$ solution (1.9%)	0.1 ml
Difco agar	15.0 g
Distilled water	1.0 l

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

$FeSO_4$ solution (0.27%)	0.1 ml
$Ca(NO_3)_2 \cdot 4H_2O$ solution (2.3%)	10.0 ml

(f) Vogel and Bonner supplemented medium (VBS) (127).

$MgSO_4 \cdot 7H_2O$	0.2 g
Citric acid ($C_6H_8O_7 \cdot H_2O$)	2.0 g
KH_2PO_4	14.0 g
$NaNH_4HPO_4 \cdot 4H_2O$	10.0 g
Difco Casamino Acids	1.0 g
tryptophan	10.0 mg
$FeCl_3 \cdot 6H_2O$	10.0 mg
Distilled water	1.0 l

The pH was adjusted to 7.0 and after autoclaving 50 ml of 10% glucose was added per litre of medium.

(g) Basic Growth Medium (BGM) (122).

minimal medium	100 ml

After autoclaving, the following were added:

glucose (10%)	5.0 ml
tryptophan (0.5%)	1.0 ml
Difco Yeast Extract (10%)	1.0 ml
Difco casamino acids (1%)	2.0 ml
tyrosine (0.5%)	1.0 ml
phenylalanine (1.0%)	0.5 ml

Just before use, 0.1 ml of arginine (100 mg/ml) per 10 ml medium was added.

(h) Competence medium (CM) (122).

minimal medium	100 ml

The following were added after autoclaving:

glucose (10%)	5.0 ml
tryptophan (0.5%)	0.1 ml
Difco Yeast Extract (10%)	1.0 ml
Difco Casamino Acids (1.0%)	1.0 ml
tyrosine (0.5%)	0.5 ml
histidine (1.0%)	0.25 ml
shikimic acid (0.5%)	0.5 ml

Just before use, the following were added:

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

4. Culture conditions

Strain JOH130 was maintained on MA since it was more stable on it than on TBB agar. Strain SB19E pyrG1, dck4, was maintained on TBB supplemented with 10 ug/ml of Cyd. Other strains were usually maintained on TBB agar plates. Unless otherwise stated liquid cultures were routinely prepared by inoculating PA medium with less than 10^7 cells/ml from overnight cultures on TBB agar. Cultures were normally grown at 37C and aerated by shaking. Cultures reached stationary phase within 4-5 h. When necessary, growth was followed by measuring turbidity with a Klett-Summerson colorimeter equipped with a #59 (green) filter.

5. Phage techniques

PBS2 and SP01 were assayed for plaque-forming units by the method described by Takahashi (1), except AD medium was used for dilutions. However, in order to obtain good lawns of JB0130 and clear PBS2 plaques of reasonable size (about 1 mm), assays of suppressor-sensitive mutants on this host were performed using 0.7% agar instead of 1.0% for the TBB overlay. The final concentration of agar in the top layer was therefore 0.35%, which was still sufficiently solid for routine plaque assay and lysate preparation. PBS2 could not be assayed with 0.7% agar on other host strains since the resulting plaques were too large and diffuse.

High titre lysates were routinely obtained from confluently lysed plates. The plating technique was as above but 10^5-10^6 PFU per plate were used to obtain confluent lysis. After overnight incubation at 37C, 5 ml of PA were added to each plate and left at room temperature

for 3-5 h with occasional agitation. The phage suspension was centrifuged at 6000 x g for 15 min to remove cell debris and stored at 4C. All centrifugation steps reported here were at 4C. Lysates prepared this way had titres of 1-7 x 10^{10} PFU/ml as compared to 1-5 x 10^{9} PFU/ml in lysates obtained in broth culture.

6. Preparation of N-methyl-N'-nitro-N-nitrosoguanidine (NG)-treated lysates

Four hour cultures of JB0130 (2.3 x 10^{8} cells/ml) in PA were infected with PBS2 at an MOI of about 1. Immediately after infection, NG was added to the culture at 20 ug/ml. The preparation of NG solutions and determination of NG concentrations were done according to Takahashi and Barnard (128). The treated cultures were shaken for 1.5 h at 37C and incubated overnight at the same temperature. Lysates were clarified by centrifugation at 6000 x g for 15 min and assayed on JB0130. Titres obtained varied from 2 x 10^{7} to 2 x 10^{8} PFU/ml.

7. Preparation of 5-fluorodeoxyuridine-treated lysates

Four hour cultures of JOH130 were diluted 10 fold into VBS medium and shaken for 1 h at 37C (to approximately 10^{8} cells/ml). Cultures were then centrifuged at 6000 x g for 10-15 min, resuspended in $\frac{1}{2}$ volume of VBS medium and infected with PBS2 at an MOI of about 1. Infected cultures were then shaken for 20 min at which time FdUrd was added to a final concentration of 5 ug/ml. The cultures were shaken for an additional 1 h and incubated overnight without shaking at 37C. Lysates were clarified by centrifugation for 15 min at 6000 x g and assayed on

JB0130. Titres obtained were from 1.5×10^7 to 2.5×10^8 PFU/ml.

8. Isolation and purification of suppressor-sensitive (sus) mutants

A mixed indicator technique which was devised for SP01 and SP01 sus5 could not be applied to PBS2 due to the variation in plaque size and clarity of the PBS2 plaques on JOB130. Mutants therefore had to be isolated by the nondiscriminate picking of plaques. Mutagenized lysates were plated on JB0130 to give 100-300 plaques per plate. These were then picked with sterile toothpicks onto pairs of plates, one seeded with JB0130 and the other with JB0128. These were then incubated overnight at 30C and scored for lysis the following day. Isolates which produced lysis on JB0130 and not on JB0128 were selected for purification. These putative mutants were subjected to at least two successive single plaque isolations on JB0130. Lysates were prepared from those which continued to express conditional lethality. Mutant titres were generally $1-4 \times 10^{10}$ PFU/ml when obtained from confluently lysed plates. The frequency of revertants determined by plating on JB0128 ranged from 10^{-5} to 10^{-9}.

9. Mutant nomenclature

Forty-nine suppressor-sensitive mutants were isolated from twelve NG-treated lysates of PBS2. These were given the prefix SNG and numbered from 1-49 in order of isolation. Seventy mutants obtained from eleven FdUrd-treated lysates were given the prefix SFU and numbered 1-70 in order of their isolation. The frequency of mutants was 1.1% from NG-treated lysates and 0.6% from FdUrd-treated lysates.

10. Complementation tests

A spot test for complementation was devised to facilitate classification of these mutants. Mutant lysates were diluted to 1-4 x 10^6 PFU/ml and drops of these were mixed pairwise on plates seeded with cells of JB0128. Plates were incubated overnight at 30C and scored for lysis the following day. In all experiments, singly infected controls were included. Complementing pairs sometimes produced only slight lysis or a number of single plaques. In most cases, however, results were quite straightforward. When difficulties did arise, pairs were retested several times, often from different lysates, before being classified.

Quantitative complementation tests were carried out in PA medium. Four hour cultures of JB0128 were diluted 10-fold in fresh PA and grown 1 h to 10^8 cells/ml. These were distributed to appropriate tubes and infected with pairs of mutants, each at an MOI of 10. After 15 min, unadsorbed phage were inactivated by treatment with antiserum for 5 min. The antiserum used was obtained from I. Takahashi and had a K value of 400 against PBS2 when assayed according to Adams (129). Cultures were then diluted 1,000-fold in fresh PA at 37C. At 90 min, total phage yields were estimated with JB0130 as host. Single infections with wild-type PBS2 and mutants were included as controls in each experiment. Relative phage yields as a measure of complementation were calculated as:

$$\frac{\text{(yield from mixed infection) - (sum of individual yields)}}{\text{wild-type yield}} \times 100$$

Mutants said to complement one another gave relative yields of 20% or more and individual mutants and noncomplementing pairs gave relative

yields of 1% or less. In some experiments, the sum of individual yields of noncomplementing mutants exceeded the total yield from mixed infection.

11. Genetic crosses

Genetic crosses between suppressor-sensitive mutants or between suppressor-sensitive and temperature-sensitive mutants were performed in the suppressor-carrying host, JB0130. Four hour cultures of JB0130 were diluted 10-fold in fresh PA and grown to 1×10^8 cells/ml (75 Klett units). Pairs of mutants were diluted together in PA so that each had a titre of 1×10^9 PFU/ml in the final dilution tube. At the desiredtime, infection was begun by diluting the cultures $\frac{1}{2}$ in PA containing the mutants to be tested. At 15 min after infection, unadsorbed phage were inactivated with antiserum. Five minutes after the addition of antiserum, infected cells were diluted 1,000-fold in prewarmed PA. Ninety min after infection (lysis was usually apparent by this time), the progeny were either assayed immediately or stored at 4C in the presence of a few drops of chloroform. Crosses between suppressor-sensitive mutants were assayed on JB0128 for wild-type recombinants and on JB0130 for total progeny. Recombination frequency was calculated as:

$$\frac{\text{wild-type recombinants} \times 2}{\text{total progeny}} \times 100$$

These crosses, unless otherwise stated were conducted at 37C. Crosses between suppressor-sensitive and temperature-sensitive mutants were usually done at 30C. Recombination frequency was determined by plating the progeny of the cross on JB0130 and testing the resulting plaques for the ability to grow on JB0128 at 45C. Those which produced lysis were

wild-type recombinants and recombination frequency was calculated as:

$$\frac{\text{plaques which produced lysis at 45C X 2}}{\text{total plaques tested}} \times 100$$

for each cross, at least 200 plaques were tested in this way. When necessary, the relative abundance of parental types was determined, and the appropriate correction applied to the calculation.

12. Isolation of double mutants

Double mutants of the sus-sus type were isolated from the progeny of the appropriate genetic cross. Progeny were plated on JB0130 and plaques were transferred with sterile toothpicks onto each of 3 plates. One was seeded with cells of JB0130, the second with cells of JB0128 and about 1×10^6 of one of the parent mutants, and the third with cells of JB0128 and about 1×10^6 PFU of the parent mutant. The plates were incubated overnight at 30C. Putative double mutants were those which produced lysis on the plate containing JB0130 but on neither of the other two plates. The latter observation was considered an indication that such isolates could not complement either of the parents crossed. These were retested and if the double mutant phenotype persisted, high titre lysates were prepared after at least one single-plaque isolation. In some cases, genotypes were tested with the regular spot test procedure.

Double mutants of the sus-ts type were also isolated from the progeny of the appropriate two-factor crosses. Plaques formed on JB0130 from the progeny were picked onto 3 plates, two of which were seeded with cells of JB0130 and the third with JB0128. One of the JB0130-seeded plates and the JB0128-seeded plate were incubated overnight at 30C. The other plate seeded with JB0130 was

incubated overnight at 45C. Putative double mutants were those which produced lysis only on JB0130 at 30C. These isolates were retested and double mutants were purified through at least one single-plaque isolation. This technique had inherent difficulties since some of the sus mutants alone produced little or no lysis on JB0130 at 45C when picked from single plaques. When necessary, spot complementation tests were used to certify mutant genotypes.

13. Phage transformation with PBS2

Transformation was carried out as described by Herrington and Takahashi (130). Cultures of SB202 were grown to the end of log phase in BGM and diluted to 10-fold in CM. After an incubation of 90 min at 37C mutant phage and DNA from wild-type PBS2 were added simultaneously. At 90 min after infection, cultures were assayed for wild-type transformants on JB0128.

14. Preparation of crude extracts

Crude extracts of PBS2-infected and uninfected cells for enzyme assays were prepared as follows: 4 h cultures were diluted 10-fold in PA and grown to about 1 x 10^8 cells/ml. These were then infected and at the appropriate time after infection cells were harvested by centrifugation at 6,000 x g for 15 min. When not used immediately, pellets were stored at -15C. To obtain crude extracts, cells were resuspended in the appropriate buffer and passed twice through a French pressure cell (American Instrument Co.) at 15,000 pounds per square

inch. Undisrupted cells and debris were removed by centrifugation at 15,000 x g for 30 min and supernatants were used as crude extracts. The concentration of protein in crude extracts was estimated by the method of Lowry et al. (131) using bovine serum albumin as a standard.

In early experiments with PBS2-induced DNA polymerase activity, crude extracts were prepared essentially as described by Price and Cook (62). Cells were grown and infected as above. At the appropriate times, cells were rapidly cooled and centrifuged at 6000 x g for 15 min. Pellets were washed once and either stored at -15C or resuspended for extraction in 1/100 volume of 10 mM tris-chloride buffer (pH 7.5) containing 1.0 mM EDTA. The cells were treated with lysozyme (200 ug/ml) for 30 min at 37C. Lysates were centrifuged at 15,000 x g for 30 min to remove cell debris and dithiothreitol was added to the supernatant at 0.1 mM. When not used immediately, the extracts were stored at -15C with little loss in DNA polymerase activity for at least one month. Prior to use, extracts were dialysed overnight against a buffer containing 50 mM tris-chloride (pH 7.5), 10 mM NaCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol.

15. Enzyme assays

(a) dCTP deaminase

This activity was assayed spectrophotometrically according to the method of Neuhard and Thomassen (132). Cells of JB0128 were grown and infected as above and harvested 30 min after infection. Cells were resuspended in 25 mM tris-chloride (pH 7.5) and French press extracts were prepared as described above. The reaction mixtures contained: 25 mM tris-chloride (pH 7.5); 365 µM dCTP; 250 µM $MgCl_2$ and aliquots

of crude extract in a final volume of 0.5 ml . The mixtures were incubated at 37C and at various time intervals the reaction was terminated by transferring 0.1 ml samples into 0.9 ml of 5% PCA in ice water. After 30 min on ice, precipitates were removed by centrifugation. The deamination of dCTP was followed by measuring the absorbance of the samples at 290 nm in a Beckman spectrophotometer, Model DU, assuming E^{1M}_{1cm} dCTP - E^{1M}_{1cm} dUTP = 10.1×10^3. Therefore, $\Delta A290 = 10.1 \times 10^3 \times \Delta$ [dCTP]. Reactions were linear for the first 15-20 min. No activity was detectable in crude extracts of uninfected cells or boiled extracts (100C for 15 min) of PBS2-infected cells.

(b) Deoxyribonuclease

The uracil-specific DNase in B. subtilis cells was assayed by the radioactive method of Tomita and Takahashi (25). Infected cells of JB0128 were harvested 30 min after infection by cooling on ice and centrifuging at 6,000 x g for 15 min. Cells were resuspended in 1/20 volume of 25 mM tris-chloride (pH 7.5) and crude extracts prepared with a French press. Reaction mixtures contained 1.25 ug of ^{3}H-Urd-labelled PBS1 DNA (9216 CPM/µg); 19 µg of unlabelled PBS2 DNA; 0.02 ml of B-mercaptoethanol (1.4 M); 0.02 ml of $CaCl_2$ (67.5 mM); 0.1 ml of tris-chloride buffer (1 M, pH 8.5); distilled water and 0.05 ml of crude extracts corresponding to 0.13 - 0.18 mg protein in a final volume of 0.27 ml. Reactions were carried out at 37C for 30 min and terminated by the addition of 0.2 ml PCA (1.8 M), 0.04 ml of bovine serum albumin (5 mg/ml) and 0.5 ml calf thymus DNA (0.5 mg/ml). These were kept on ice for 30 min and centrifuged for 10-15 min at 15,000 x g. Acid soluble

counts were determined by mixing 0.4 ml of supernatants with 10 ml of Aquascint II (International Chemical and Nuclear Corp.) and counting in a Beckman LS230 scintillation counter.

(c) DNA polymerase

Early assays (shown in Table 11) of DNA polymerase activity were done according to Price and Cook (62). Cells of JB0128 were grown and infected as described above. At the appropriate times after infection, the cultures were cooled and centrifuged for 15 min at 6,000 x g. Crude extracts were then prepared with lysozyme as described above. Reaction mixtures contained: 0.02 ml of $MgCl_2$ (80 mM); 0.02 ml each of dATP (0.4 mM), dGTP (0.4 mM), dCTP (0.4 mM); 0.02 ml of dUTP (2 mM); 100 µg of calf thymus DNA (heated for 15 min at 100C); 1 µCi of ^{3}H-dCTP (specific activity, 22.6 Ci/mMole); 64 mM tris-chloride buffer (pH 7.5); and aliquots of crude extract in a final volume of 0.25 ml.

Reactions were terminated at appropriate times by pipetting 0.05 ml of the incubation mixture onto Whatman #3 filter paper discs which were placed immediately in cold 10% TCA. The filter paper discs were washed several times with cold 5% TCA, dried under an infra-red lamp and placed in vials containing Spectrafluor (Amersham/Searle Corp.). Samples were then counted in a Beckman scintillation counter, Model LS230.

In later experiments, cells were grown and infected as described above, centrifuged at appropriate times and resuspended in 1/20 volume of 25 mM tris-chloride (pH 7.5). Crude extracts were prepared using a French press as previously described. Reaction mixtures were as described in Fig. 10. The mixtures were incubated at 37C and at appropriate

times 0.05 ml samples were added to 5 ml of cold 10% TCA. After at least 30 min at 0C, precipitates were washed onto membrane filters (Schleicher and Schuell, Type B6 Bac-T-Flex, pore size: 0.45 um) and washed 3 times with 7 ml of cold 5% TCA. Filters were dried under infra-red lamp and placed in scintillation vials containing Spectrofluor and counted in a Beckman scintillation counter Model LS230.

16. Incorporation of radioactive nucleosides into nucleic acids

Appropriate amounts of labelled cultures were pipetted into 5 ml of cold 20% TCA. These were allowed to precipitate for at least 30 min and washed onto membrane filter discs. They were washed 3 times with cold 5% TCA, dried under an infra-red lamp and counted in a Beckman scintillation counter, Model LS230 or LS250. Radipactivity incorporated into DNA was determined by pipetting labelled cultures into NaOH (final concentration 0.3 N) and incubated for 16-18 h at 37C (133). To samples which were neutralized with 1 N HCl, TCA was added to 7% and kept on ice for at least 30 min. Radioactivities were then determined as above. Radioactivity incorporated into RNA was estimated from the difference between counts in the total nucleic acid fraction and those in the DNA fraction.

17. Materials

Common chemicals were obtained from Fisher Scientific and were all Reagent grade. N-methyl-N'-nitro-N-nitrosoguanidine and spermine were obtained from Aldrich Chemical Co. Nucleosides and nucleotides

were from Sigma Chemical Co. Enzymes were from Worthington Biochemical Corp. Amino acids were obtained from Calbiochem. Rifamycin was a gift from Dr. K. B. Freeman and 5-fluorodeoxyuridine was a gift from Dr. W. Szybalski and Dr. R. J. Horsley.

The following labelled compounds were obtained from Schwarz Mannheim BioResearch Corp.: 5-^{3}H-Cyd (19 Ci/mMole), 5-^{3}H-dCyd (25 Ci/mMole), 5-^{3}H-CTP (22.6 Ci/mMole), methyl-^{3}H-dThd (6.7 Ci/mMole), 5-^{3}H-dUrd (18 Ci/mMole) and 5-^{3}H-dATP (16 Ci/mMole).

RESULTS

ISOLATION AND CHARACTERIZATION OF MUTANTS

Growth experiments

Since PA broth was the liquid medium employed throughout this study, the growth of strains JB0130 and JB0128 were investigated in this medium. Ten-fold dilutions of 4 h cultures of these strains in fresh PA medium resulted in resumption of exponential growth following a very short lag. Under these conditions, the generation times determined from turbidity and CFU, of JB0130 and JB0128 were found to be 29 and 19 min respectively. The frequency of spores in strain JB0130 was 0.05% and that in strain JB0128 was 3.6%. The frequency of spores was determined by plating cells which were grown in SM for 24 h and heated at 85C for 10 min.

The growth of PBS2 has previously been characterized by Takahashi (1) using the prototrophic strain SB19E as host. One-step growth experiments of PBS2 with cultures of JB0130 and JB0128 were carried out as follows: 4 h cultures were diluted ten-fold in fresh PA medium and grown to 10^8 cells/ml. Cells were infected with PBS2 at an MOI of 0.1. After 15 min, serial dilutions were made in PA and incubated at 37C. Tubes were assayed for plaque-forming units at appropriate intervals and burst sizes were calculated by dividing the final yield of phage by the number of originally infected cells. Under these conditions, the latent

period of PBS2 in strain JB0130 was approximately 50 min and that in strains JB0128 was about 40 min. The rise period was about 20 min in both cases and burst sizes for both varied from 10 to 40.

The effects of phage infection in cultures at various stages of growth were determined as follows: A 4 h culture of strain JB0128 was diluted ten-fold into fresh PA and shaken at 37C. At various times thereafter, 1 ml samples were transferred to separate flasks, assayed for colony-forming units and infected with PBS2 at an MOI of 10. At the 20th min after infection, cultures were diluted and plated on TBB agar to determine colony-forming units. The following day, survivors were tested for the ability to produce phage by transferring cells with sterile toothpicks onto plates seeded with JB0128 cells. These were then scored for lysis after incubation overnight at 30C. As shown in Table 2 the fraction of cells surviving infection increased with the age of the culture. The fraction of phage-carrying cells, on the other hand, decreased with the age of the culture.

Complementation

In order to begin characterizing a group of mutants it is important to determine the number of independent functions or genes that are represented in the mutant collection. This can usually be done with bacteriophage mutants by the cis-trans complementation test which was originally devised by Benzer (134). In this test, two different phage mutants co-infect the host cells under conditions which would normally restrict the growth of either mutant alone. If the mutants are affected

TABLE 2. Effect of age of culture on PBS2-induced cell killing

Time[a] (min)	Viable counts[b] (CFU/ml)	Survivors[c] (CFU/ml)	Survival (%)	Phage-producing colonies (%)
20	5.9×10^7	5.4×10^6	9.2	54
60	8.9×10^7	7.8×10^6	8.8	64
90	2.2×10^8	4.5×10^7	20.1	64
120	4.0×10^8	1.7×10^8	42.5	12
150	4.3×10^8	3.5×10^8	81.4	13

[a] Time of infection after dilution of cells

[b] Viable counts at the time of infection

[c] Viable counts at 20 min after infection

in different functions, then each will make up for the defect in the other, resulting in at least partial restoration of phage growth. However, if the mutants are defective in the same function, no such complementation will occur and no phage growth will be observed. Benzer (134) suggested that the functional units of the chromosome, thus revealed, be termed cistrons. Furthermore, biochemical and genetic studies which followed have shown that complementation can occur at the level of a single protein. This has been called intra-cistronic complementation, although by the earlier definition it is a contradiction in terms. Cistron is therefore an ambivalent designation, unless the molecular nature of a given function has been defined. Generally, however, genetic classifications are made before the nature of a given function is determined. In light of this, the results of complementation tests in this thesis have been used to classify mutants into functional units called complementation groups, in advance of further information.

On the basis of results from spot complementation tests, the 119 suppressor-sensitive mutants were assigned to 39 complementation groups (Table 3). As has been observed in other phage systems (98,109) there was an unequal distribution of mutants between complementation groups.

Two putative mutants were found to be wild-type during purification. Mutants SFU7, SFU45, SNG17, and SNG43 produced some lysis on control plates and thus could not be assigned to complementation groups using spot tests. The preparation of high titre lysates with mutants SFU22, SFU33, and SFU38 proved to be difficult and therefore they were also not assigned to groups.

TABLE 3. Complementation groups of PBS2

Complementation groups	Mutants
1	SFU1;SNG38
2	SFU3,69
3	SFU4,17
4	SFU5;SNG30
5	SFU8,53,14,26;SNG14
6	SFU9,6,61,62,25,41; SNG34
7	SFU10,41,47,48;SNG40
8	SFU12
9	SFU13,34;SNG32
10	SFU49,19,20,23,26,28,29, 30,32,35,39,42,43,46,54, 56,57,63,64,65,68;SNG2, 4,12,24,36
11	SFU50;SNG7,20,44,49
12	SFU58;SNG13,33
13	SFU59,27,37;SNG19,37,39
14	SFU60
15	SFU18
16	SFU55(15,31);(SNG45)
17	SFU66,52;SNG8,27,31
18	SFU21;SNG35(16)
19	SFU24
20	SFU44,SNG23,41

TABLE 3. (continued)

Complementation groups	Mutants
21	SFU16
22	SNG1
23	SNG3
24	SNG5,(46)
25	SNG6,22
26	SNG9
27	SNG10
28	SNG11,18
29	SNG15
30	SNG21,42
31	SNG25
32	SNG28
33	SNG29
34	SNG47
35	SNG48
36	SFU36
37	SFU67
38	SFU70
39	SFU51

Assignments in parentheses are tentative.

In order to establish the reliability of spot complementation tests, quantitative tests in liquid medium were carried out with certain mutant pairs. This, in turn, required that individual mutants would produce low yields of phage in cultures of the nonsuppressing strain. Complementation would then be indicated by a significant increase in phage yields in culture infected with two different mutants relative to cultures infected with either of the two. Actual burst size of suppressor-sensitive mutants in nonsuppressing cultures cannot be determined since no assessment of originally infected cells is possible. Therefore, results are best expressed in relation to wild-type phage yield.

Relative phage yields of several mutants were determined with strain JB0128 as host by the technique described for quantitative complementation in Materials and Methods, except that cultures were diluted to 2×10^7 cells/ml prior to infection. Table 4 gives the results expressed as phage yields in the final dilution tube. The yields of all mutants tested were found to be extremely low relative to that of wild-type PBS2. Complementation tests were done with these mutants at the same cell concentration and the results are recorded in Table 5. Mutants assigned to different groups using spot tests produced phage yields comparable to the wild-type, while those assigned to the same group gave no complementation.

In order to facilitate the characterization of the phage functions affected in the mutants isolated, representative mutants for each of the 39 groups were selected for further study. These have been

TABLE 4. Phage yields in mutant-infected nonsuppressing cells

Phage	Group	Phage yield (PFU/ml)	Relative yield
PBS2	-	5.4×10^5	100
SFU4	3	1.3×10^2	0.02
SFU5	4	2.5×10^3	0.46
SFU6	6	5.6×10^3	1.04
SFU9	6	4.2×10^3	0.78
SFU61	6	3.6×10^3	0.67
SFU10	7	4.0×10^2	0.07

TABLE 5. Complementation tests with suppressor-sensitive mutants of PBS2

Mutants	SFU4	SFU5	SFU6	SFU9	SFU61	SFU10
SFU4	0.02	98	42	51	–	54
SFU5		0.46	76	106	–	72
SFU6			1.04	<0[a]	–	30
SFU9[w]				0.78	<0[a]	57
SFU61					0.70	–
SFU10						0.07

[a]Sum of control yields exceed experimental yield

–: Not performed

listed in Table 6, together with typical reversion frequencies.

Characterization of mutants

The classification of a large number of conditional lethal bacteriophage mutants can be facilitated by techniques designed to test major phage functions. Phage-induced DNA synthesis has been shown in various systems to require a variety of phage specific proteins (98,113). The induction of cell lysis is usually effected by a few phage genes at most. However, in some instances, the ability of phage to lyse cultures or to carry out 'late' (post-replicative) functions depends on the normal procession of DNA replication (135,136). It was therefore presumed that defects in a number of PBS2 functions might be manifested as anomolous DNA synthesis and/or cell lysis.

Effect of PBS2 on host DNA synthesis: Since PBS2 contains uracil in its DNA in place of thymine, the effect of phage infection on host DNA synthesis may be determined by following changes in ^{3}H-dThd incorporation into acid-insoluble material. This was done in an uninfected and PBS2 infected culture of JB0128 (Fig. 1). PBS2 infection resulted in a significant decrease in the rate of ^{3}H-dThd incorporation between the 12th and 15th minutes of infection. If PBS2 infection shuts off host DNA synthesis, the residual incorporation may be due to continuing DNA synthesis in the uninfected and phage-carrying cells in the culture. Alternatively, the process of infection may reduce the rate of uptake of ^{3}H-dThd into the cells. Results of this and other experiments to be presented will support the conclusion that PBS2 does shut off host DNA synthesis.

TABLE 6. A list of representative suppressor-sensitive mutants of PBS2

Complementation group	Mutants	Reversion frequency
1	SFU1	$<1.4 \times 10^{-8}$
2	SFU3	1.4×10^{-7}
3	SFU4	1.0×10^{-7}
4	SFU5	4.0×10^{-8}
5	SFU8	3.0×10^{-8}
6	SFU9	7.5×10^{-7}
7	SFU10	1.1×10^{-8}
8	SFU12	1.1×10^{-5}
9	SFU13	7.0×10^{-8}
10	SFU49	9.0×10^{-7}
11	SFU50	2.0×10^{-7}
12	SFU58	2.0×10^{-7}
13	SFU59	1.3×10^{-6}
14	SFU60	$<1.0 \times 10^{-8}$
15	SFU18	4.1×10^{-7}
16	SFU55	$<2.0 \times 10^{-9}$
17	SFU66	1.6×10^{-7}
18	SFU21	2.5×10^{-7}
19	SFU24	4.6×10^{-7}
20	SFU44	1.6×10^{-6}

TABLE 6. (continued)

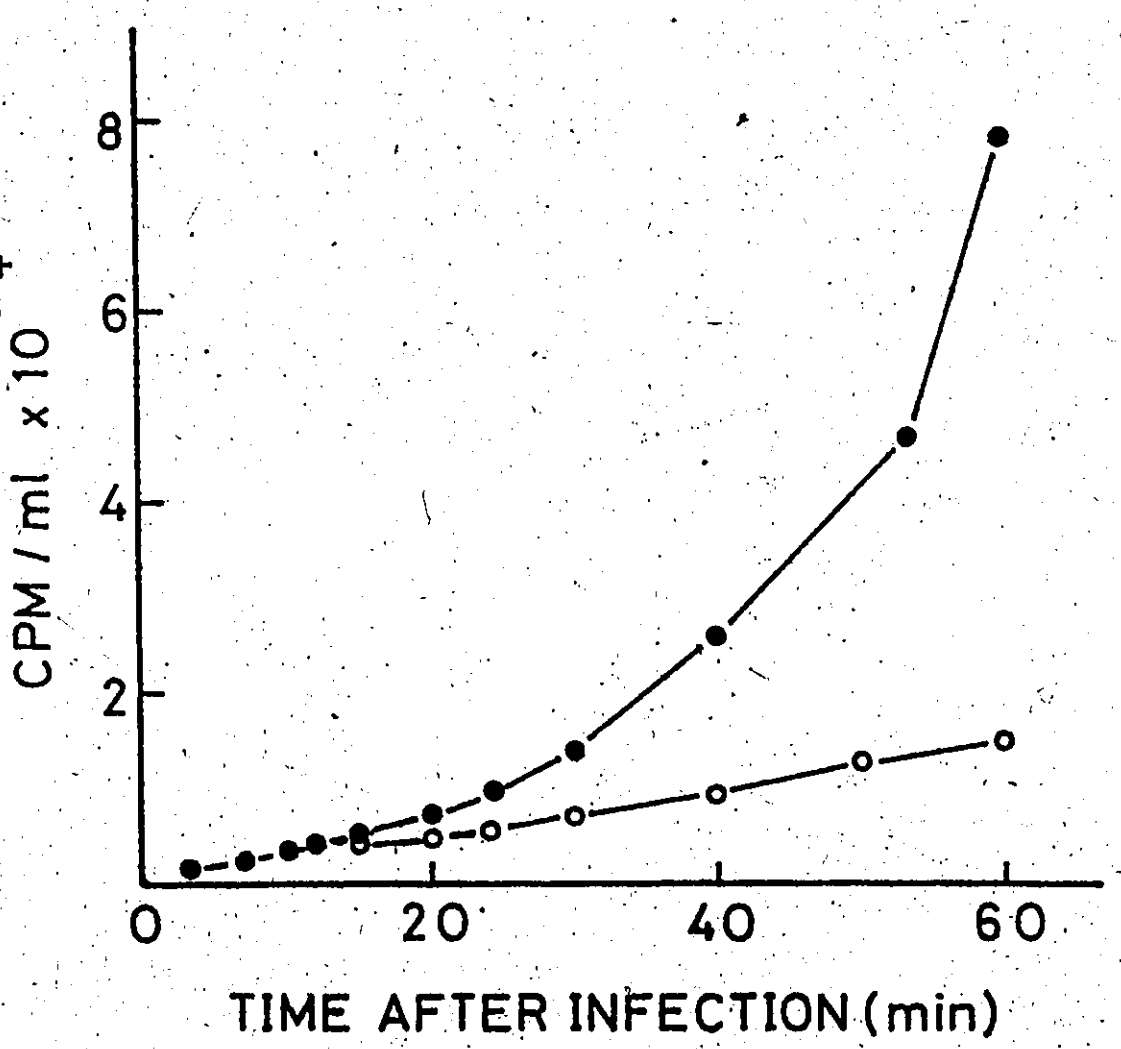
Complementation group	Mutants	Reversion frequency
21	SFU16	5.0×10^{-6}
22	SNG1	1.5×10^{-6}
23	SNG3	2.6×10^{-7}
24	SNG5	1.2×10^{-7}
25	SNG6	7.0×10^{-7}
26	SNG9	7.5×10^{-8}
27	SNG10	1.0×10^{-7}
28	SNG11	6.0×10^{-6}
29	SNG15	$<1.2 \times 10^{-8}$
30	SNG21	1.0×10^{-6}
31	SNG25	1.0×10^{-6}
32	SNG28	1.5×10^{-7}
33	SNG29	1.0×10^{-6}
34	SNG47	1.6×10^{-7}
35	SNG48	7.0×10^{-5}
36	SFU36	1.5×10^{-6}
37	SFU67	3.3×10^{-5}
38	SFU70	1.3×10^{-6}
39	SFU51	8.0×10^{-8}

FIGURE 1. Incorporation of ^{3}H-dThd into TCA-insoluble material in PBS2-infected cells

Parallel early exponential cultures of JB0128 in PA (3-4 x 10^{7} cells/ml) were continuously labelled with ^{3}H-dThd (5 µCi/ml). One culture was infected with PBS2 at an MOI of 5-10. Samples (0.1 ml) were withdrawn at appropriate times and radioactivities in TCA-insoluble material were determined.

●: Uninfected cells

○: PBS2-infected cells.


8
6
4
2
CPM / ml x 10^-4
0
20
40
60
TIME AFTER INFECTION (min)

Effect of PBS2 on host RNA synthesis: Price and Frabotta (79) reported that PBS2 infection results in the complete cessation of net RNA synthesis. Rima and Takahashi (80) found that although PBS1 production was insensitive to rifamycin, the rates of RNA and DNA synthesis, as measured by ^{3}H-Urd incorporation in the presence of the drug, were reduced to about 3% of control values. Experiments with ^{3}H-dCyd and DNA-affected PBS2 mutants isolated in this study showed that phage DNA synthesis was actually the same in treated and untreated cultures. These authors concluded that rifamycin either specifically inhibits the uptake of ^{3}H-Urd from the medium or that the drug affects the specific activity of the ^{3}H-UTP pool in some other way.

In order to examine the effects of PBS2 on host RNA synthesis and to study both RNA and DNA synthesis in rifamycin-treated, infected cells, incorporation experiments were carried out with the mutant pyrG1dck4 as host. This strain is unable to produce cytidine nucleotides from the uridine nucleotide precursor, which normally occurs through amination. It also lacks a cytidine deaminase which normally converts Cyd and dCyd to Urd and dUrd respectively. Finally, this mutant lacks dCyd kinase which normally provides for the uptake of dCyd from the medium into deoxycytidine nucleotides in B. subtilis. Therefore, it requires Cyd for growth, which is metabolized via Cyd kinase and so the dCTP pool in this strain is totally derived from ribonucleotide reduction of the appropriate cytidine nucleotide precursor.

The incorporation of ^{3}H-Cyd into RNA and DNA in uninfected and PBS2-infected cells is illustrated in Fig. 2. Although there was some

FIGURE 2. Incorporation of ^{3}H-Cyd into RNA and DNA in PBS2-infected cells

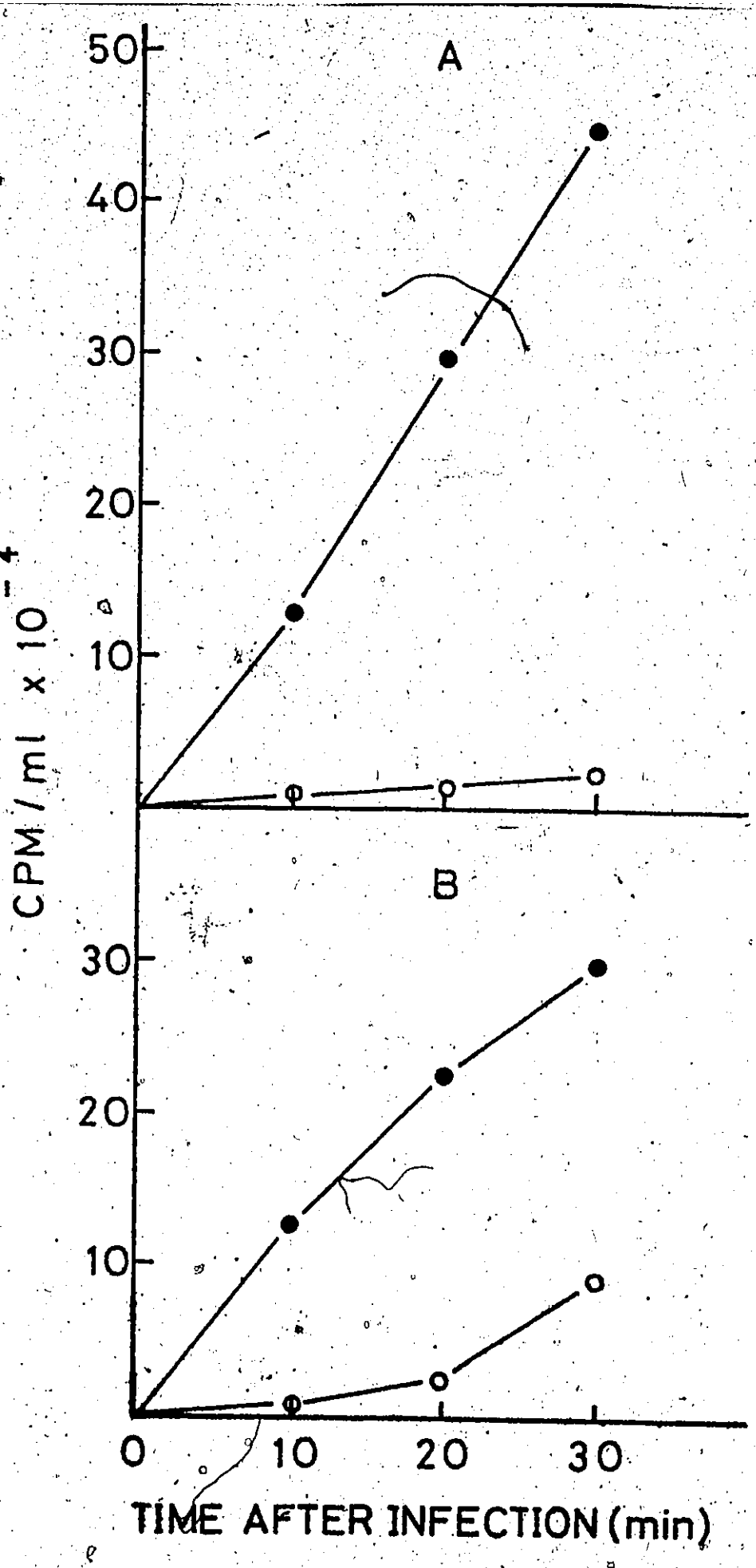
A culture of pyrGldck/4 was grown to about 2 x 10^{8} cells/ml. The culture was infected with PBS2 at an MOI of 10 and ^{3}H-Cyd was added at 2.5 µCi/ml. At various times after infection, 0.1 ml samples were withdrawn to measure incorporation of radioactivity into RNA and DNA.

Panel A: Uninfected cells

Panel B: PBS2-infected cells

●: CPM in RNA

○: CPM in DNA


A
B
50
40
30
20
10
30
20
10
0
10
20
30
CPM / ml x 10⁻⁴
TIME AFTER INFECTION (min)

reduction in net RNA synthesis in infected cells, an appreciable amount of RNA was synthesized during the first 30 minutes of infection. The results shown in Fig. 2 suggest that the net RNA synthesis in PBS2-infected cultures cannot be accounted for solely by uninfected and/or phage-carrying cells. It is possible that PBS2 does shut off host RNA synthesis and induces net synthesis of phage-specific RNA. To test this possibility, the effect of rifamycin on this RNA was studied. The experiment in Fig. 3 was similar to the previous one except that after the time of infection, cells were pre-labelled for 5 min with ^{3}H-Cyd and then exposed to rifamycin. This was done to avoid an effect of rifamycin on the specific activities of the cytidine and deoxycytidine nucleotide pools. Incorporation of ^{3}H-Cyd into DNA was unaffected by rifamycin whereas net RNA synthesis was completely inhibited by the drug. It therefore appears that net host RNA synthesis continues in PBS2-infected cells during the first 30 minutes of infection.

PBS2 DNA synthesis

Figure 4 shows that incorporation of ^{3}H-dCyd into acid insoluble material, when added to the medium after the apparent arrest of host DNA synthesis, was significantly smaller in phage-infected cells than in the uninfected control. Since the results obtained earlier show (Fig. 1) that at least 20% of the incorporation of label into DNA infected cells may be due to host DNA synthesis, the identification of PBS2 mutants defective in DNA synthesis might prove very difficult using a continuous labelling technique. The results in Table 7 were obtained by following

FIGURE 3. Effect of rifamycin on net RNA synthesis in PBS2-infected cells

A culture of pyrGldck 4 was grown as described in Fig. 2, infected with PBS2 at an MOI of 10 and ^{3}H-Cyd was added at 2.5 µCi/ml 10 min after infection. Rifamycin (20 µg/ml) was added to one culture 15 min after infection. At various times thereafter, 0.1 ml samples were withdrawn to measure incorporation of ^{3}H-Cyd into RNA and DNA.

Panel A: No rifamycin added

Panel B: Rifamycin added

O: CPM in RNA

●: CPM in DNA

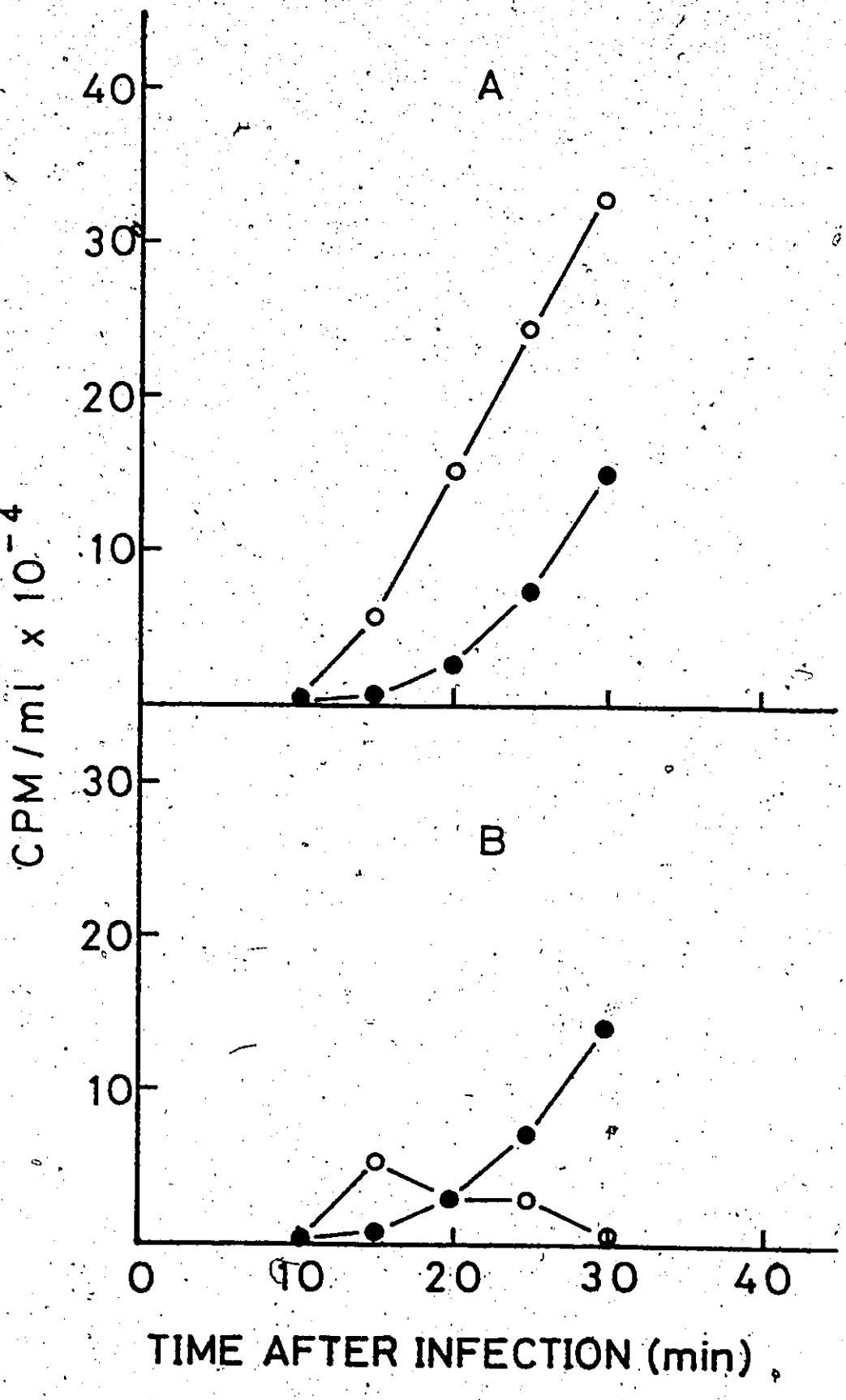

A
B
40
30
20
10
30
20
10
0
10
20
30
40
CPM / ml x 10⁻⁴
TIME AFTER INFECTION (min)

FIGURE 4. Continuous incorporation of ^{3}H-Cyd into TCA-insoluble material into PBS2-infected and uninfected cells

An early exponential culture (3-4 x 10^{7} cells/ml) of JB0128 was divided into two flasks, one of which was infected with PBS2 at an MOI of 10. At the 15th min of infection, each culture was diluted 2-fold in PA containing ^{3}H-dCyd (5 µCi/ml). At various times thereafter, 0.1 ml samples were withdrawn to determine radioactivities in the TCA-insoluble fraction.

●: Uninfected cells

○: PBS2-infected cells

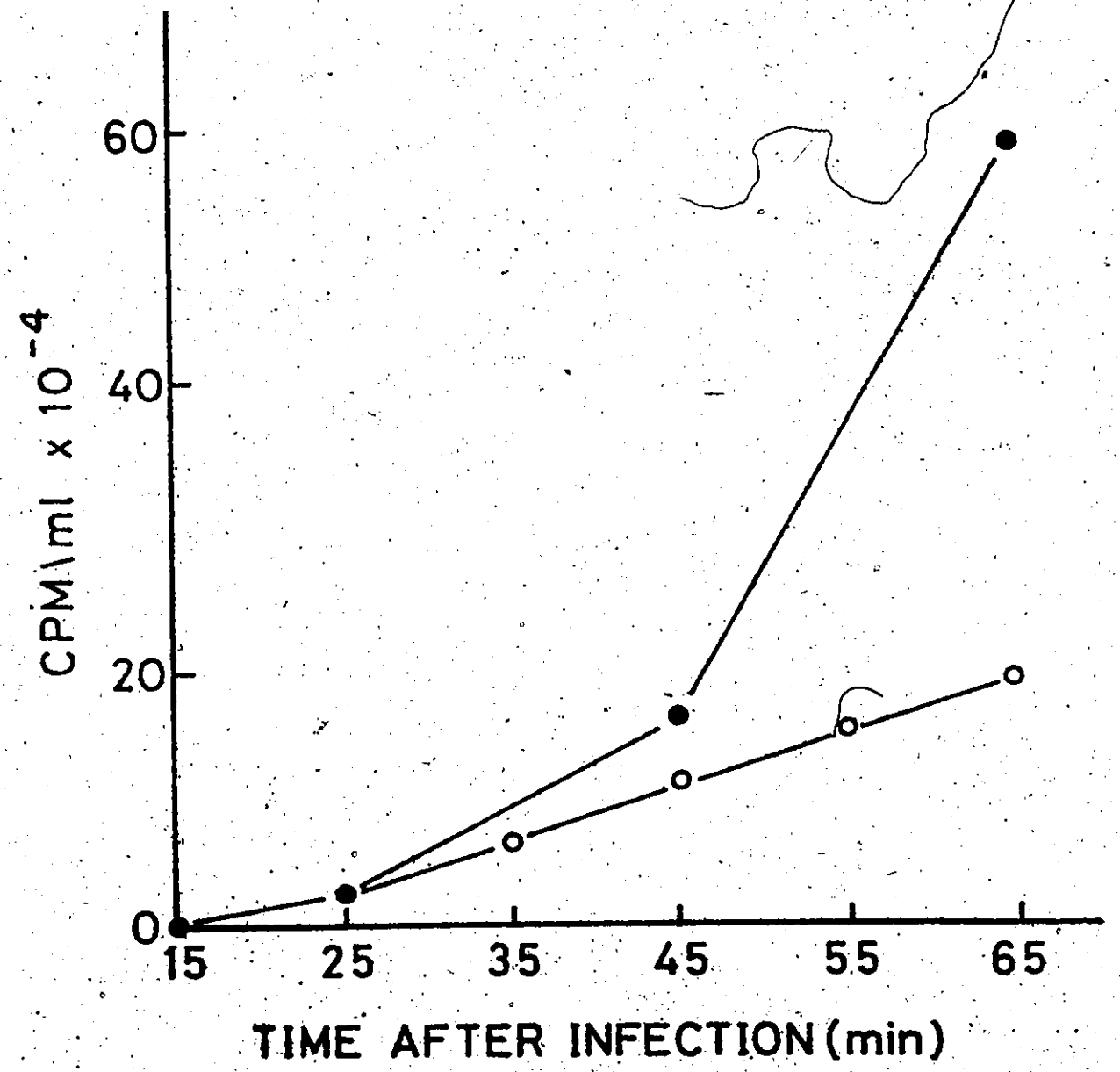

60
40
20
0
CPM\ml x 10⁻⁴
15
25
35
45
55
65
TIME AFTER INFECTION (min)

TABLE 7. Incorporation of ^{3}H-dCyd into TCA-insoluble material in mutant-infected cells

Phage	Complementation group	Relative incorporation
PBS2	-	100
SFU5	4	45
SFU8	5	98
SFU9	6	48
SFU10	7	76
SFU12	8	95
SFU49	10	39
SFU50	11	36
SFU58	12	126
SNG1	22	48
SNG3	23	115
SNG5	24	67
SNG6	25	109
SNG9	26	60
SFU67	37	77

the continuous incorporation of ^{3}H-dCyd into TCA-insoluble material from the 15th to the 45th min of infection. Total incorporation in mutant-infected cells was compared to that in cells infected with wild-type phage during the same period. Of 14 mutants tested, 5 resulted in the incorporation of less than 50% as much label as wild-type infection. None of these results suggest gross deficiency in DNA synthesis. This however may be due to significant residual host-specific incorporation in mutant-infected cells.

This problem was overcome by determining rates of incorporation of ^{3}H-dCyd into DNA rather than total incorporation. It was found that apparent rates of DNA synthesis in phage-infected cells were greater than in uninfected cells from the 10th to the 30th min of infection (Fig. 5). It was therefore possible to test the mutants for deficiencies in DNA synthesis by comparing the rate of incorporation of ^{3}H-dCyd into DNA from the 20th to the 25th min of infection to that of the wild-type. Since under these conditions, wild-type-infected cells incorporate ^{3}H-dCyd at more than twice the rate of uninfected cells, contribution of residual host-specific incorporation in infected cells could be considerably reduced. Ten mutants incorporated ^{3}H-dCyd at 30% or less of the wild-type rate at the 20th min of infection (Table 8), indicating that these mutants may be affected in DNA synthesis. It was possible that defects in DNA synthesis revealed by this technique related to the time after infection chosen for the experiment and not necessarily to the whole course of infection.

It was found, in the course of this work, that ^{3}H-dCyd incorporation into DNA in PBS2-infected cells continues in the presence of rifamycin

FIGURE 5. Rates of incorporation of ^{3}H-dCyd into DNA in PBS2-infected and uninfected cells

An early exponential culture (3-4 x 10^{7} cells/ml) of JB0128 was divided into two flasks, one of which was infected with PBS2 at an MOI of 10. At various times after infection, 0.5 ml samples were diluted 2-fold into PA containing ^{3}H-dCyd (2.5 µCi/ml). After 5 min, 0.25 ml samples were withdrawn and incorporation of ^{3}H-dCyd into alkali-stable, TCA-insoluble material was determined. Points on the figure represent the start of the 5 min pulse.

●: Uninfected cells

○: PBS2-infected cells

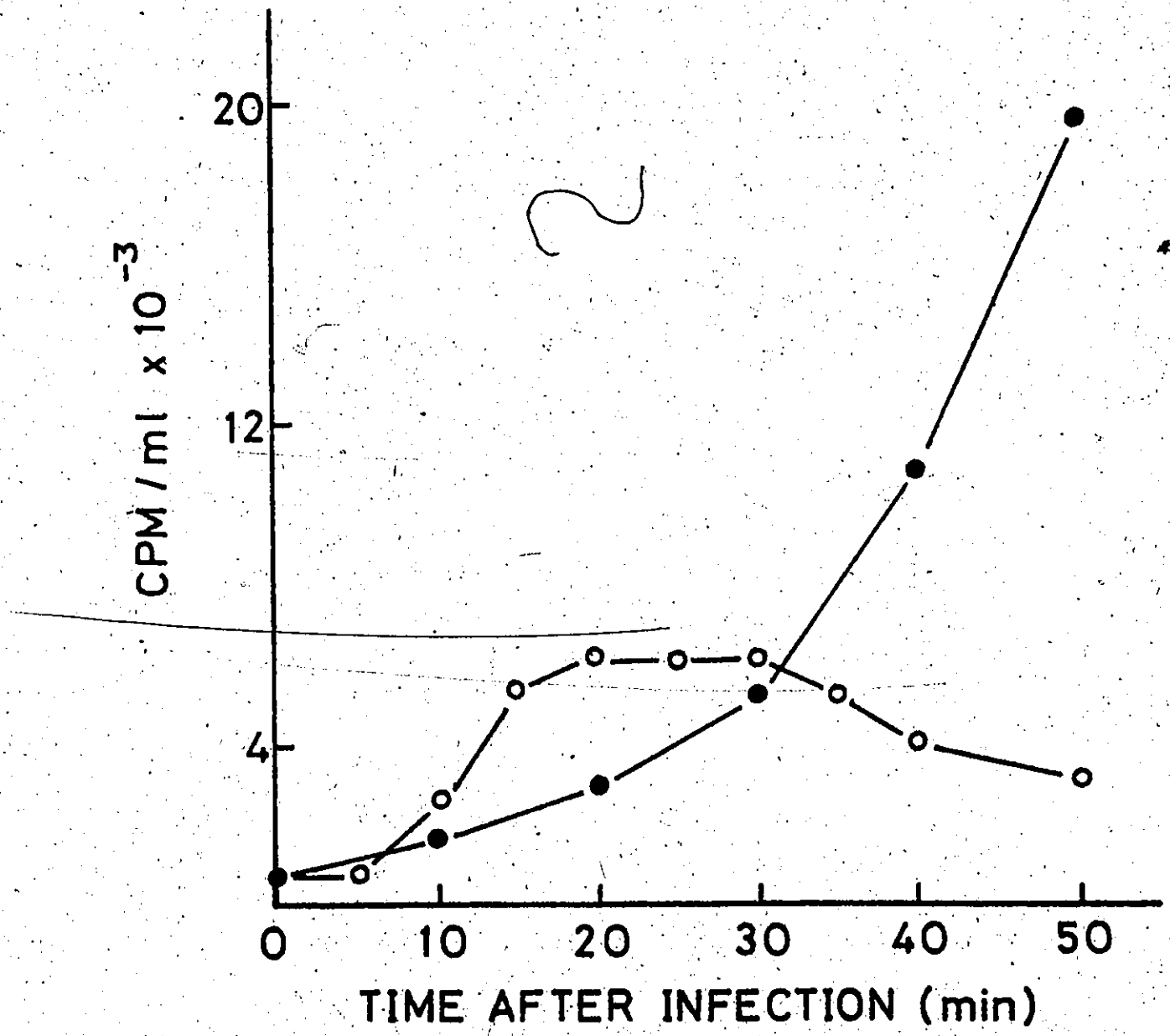

20
12
4
CPM / ml x 10^-3
0
10
20
30
40
50
TIME AFTER INFECTION (min)

TABLE 8. Incorporation of ^{3}H-dCyd into DNA in mutant-infected cells

Phage	Complementation group	Relative incorporation
PBS2	-	100
uninfected	-	34
SFU1	1	25
SFU3	2	12
SFU4	3	99
SFU5	4	8
SFU8	5	98
SFU9	6	30
SFU10	7	100
SFU12	8	81
SFU13	9	81
SFU49	10	7
SFU50	11	7
SFU58	12	129
SFU59	13	114
SFU60	14	131
SFU18	15	110
SFU55	16	109
SFU66	17	59
SFU21	18	125
SFU24	19	104

TABLE 8. (continued)

Phage	Complementation group	Relative incorporation
SFU44	20	152
SFU16	21	157
SNG1	22	8
SNG3	23	77
SNG5	24	14
SNG6	25	123
SNG9	26	Not done
SNG10	27	63
SNG11	28	71
SNG15	29	102
SNG21	30	106
SNG25	31	125
SNG28	32	151
SNG29	33	74
SNG47	34	10
SNG48	35	123
SFU36	36	10
SFU67	37	Not done
SFU70	38	141
SFU51	39	93

while being inhibited in uninfected cells. This observation enabled us to investigate phage-induced DNA synthesis in infected cultures. In the presence of rifamycin, residual host DNA synthesis in infected cells should be minimized. Experiments were conducted in which cells were pre-treated with rifamycin, infected and continuously labelled with ^{3}H-Cyd from the 5th min of infection. Figure 6 shows that the DNA-affected mutants are deficient in phage DNA synthesis throughout the course of infection.

The DNA-affected mutant phenotypes identified in pulse labelling experiments (Table 8) were not clearly detectable in continuous labelling experiments (Table 7). However, when infected cells were continuously labelled in the presence of rifamycin (Fig. 6), DNA-affected phenotypes were clearly distinguishable. These results are consistant with the conclusion that productive PBS2 infection results in shut-off of host DNA synthesis. Considerable residual host-specific DNA synthesis occurs in infected cultures, presumably due to the presence of uninfected and/or phage-carrying cells. If this interpretation is correct, the results in Table 8 indicate that all DNA-affected mutants except perhaps SFU1 and SFU9 shut-off host DNA synthesis. The fact that the DNA synthesis in SFU1 and SFU9 infected cells is resistant to rifamycin (Fig. 6) suggests that they are both capable of shutting-off host DNA synthesis and carrying out considerable phage-specific DNA synthesis.

PBS2-induced host cell lysis: To test the suppressor-sensitive mutants for their ability to induce host cell lysis, a simple reproducible procedure

FIGURE 6. DNA synthesis in infected, rifamycin-treated cells

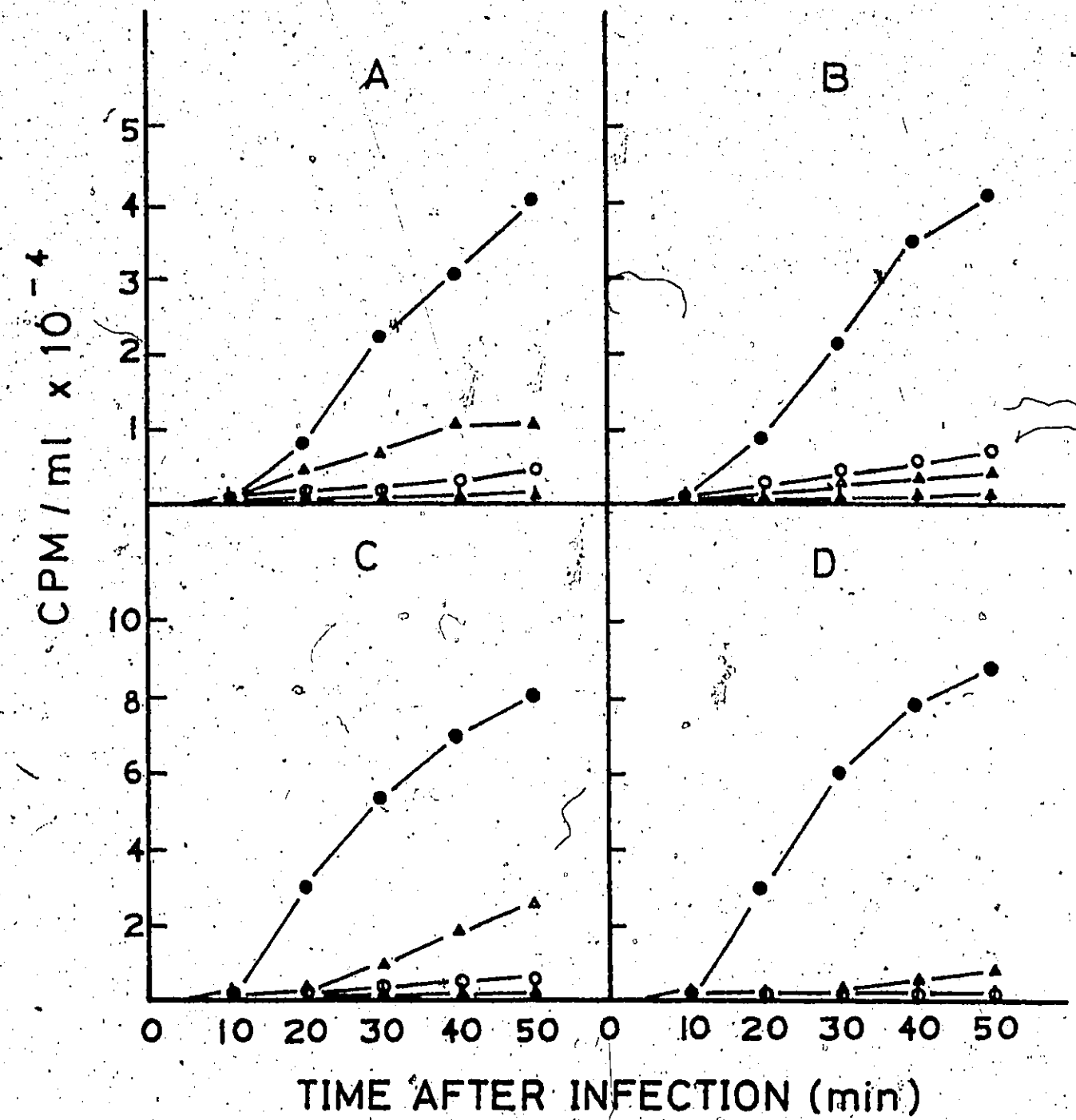
Early exponential cultures of JB0128 ($3-4 \times 10^7$ cells/ml) were treated with rifamycin at 20 µg/ml for 2 min prior to infection. Five minutes after infection, ^{3}H-dCyd was added to the cultures at 2.5 µCi/ml and incorporations of label into alkali-stable, TCA-insoluble material were determined at appropriate times.

Panel A:
- ●: PBS2-infected cells
- O: Uninfected cells
- ▲: SFU50-infected cells
- Δ: SFU9-infected cells

Panel B:
- ●: PBS2-infected cells
- O: SFU2-infected cells
- ▲: SNG5-infected cells
- Δ: SFU5-infected cells

Panel C:
- ●: PBS2-infected cells
- ▲: SFU49-infected cells
- Δ: SFU1-infected cells
- O: uninfected cells

Panel D:
- ●: PBS2-infected cells
- O: SNG1-infected cells
- ▲: SNG47-infected cells


A
B
C
D
CPM / ml x 10⁻⁴
5
4
3
2
1
10
8
6
4
2
0
10
20
30
40
50
TIME AFTER INFECTION (min)

for observing this phenomenon in wild-type PBS2-infected cultures of JB0128 was established. Figure 7 illustrates a typical result using this procedure, comparing PBS2-infected and uninfected cells. The onset of lysis was usually observed between the 40th-45th min of infection and was complete by the 60th-70th min in wild-type PBS2-infected cultures. This temporal pattern of lysis is consistent with the results of single step growth experiments.

The representative mutants were tested in this manner for the ability to induce lysis in early exponential cultures of JB0128. As shown in Table 9, eight mutants which were previously shown to be affected in DNA synthesis, were also unable to lyse host cells. The other DNA-affected mutants SFU1 and SFU9 were found to be normal with respect to cell lysis. Mutants SFU60 and SNG15, which are apparently able to induce DNA synthesis were also incapable of cell lysis.

It was then considered that either or both of SFU60 and SNG15 might be defective solely in the ability to induce lysis and not in the intracellular production of phage. To investigate this possibility, an experiment was carried out in which phage yields in infected cultures were compared to yields after the cells were artifically lysed. A 4 h culture of JB0128 was diluted 10-fold in fresh PA and grown to 10^8 cells/ml. Cells were infected with the appropriate phage at an MOI of 10, and after 15 min for adsorption, cells were collected by centrifugation and resuspended in 1/10 volume of prewarmed PA. Incubation was continued until lysis occurred in the wild-type-infected culture and the contents of each flask were assayed for PFU on the suppressing host, JB0130. The

FIGURE 7. PBS2-induced host cell lysis

An early exponential culture (3-4 x 10^7 cells/ml) of GBO128 in PA was divided into two flasks, one of which was infected with PBS2 at an MOI of 10. Incubation was continued and at various times after infection, turbidity in Klett units was determined.

●: Uninfected cells

O: PBS2-infected cells

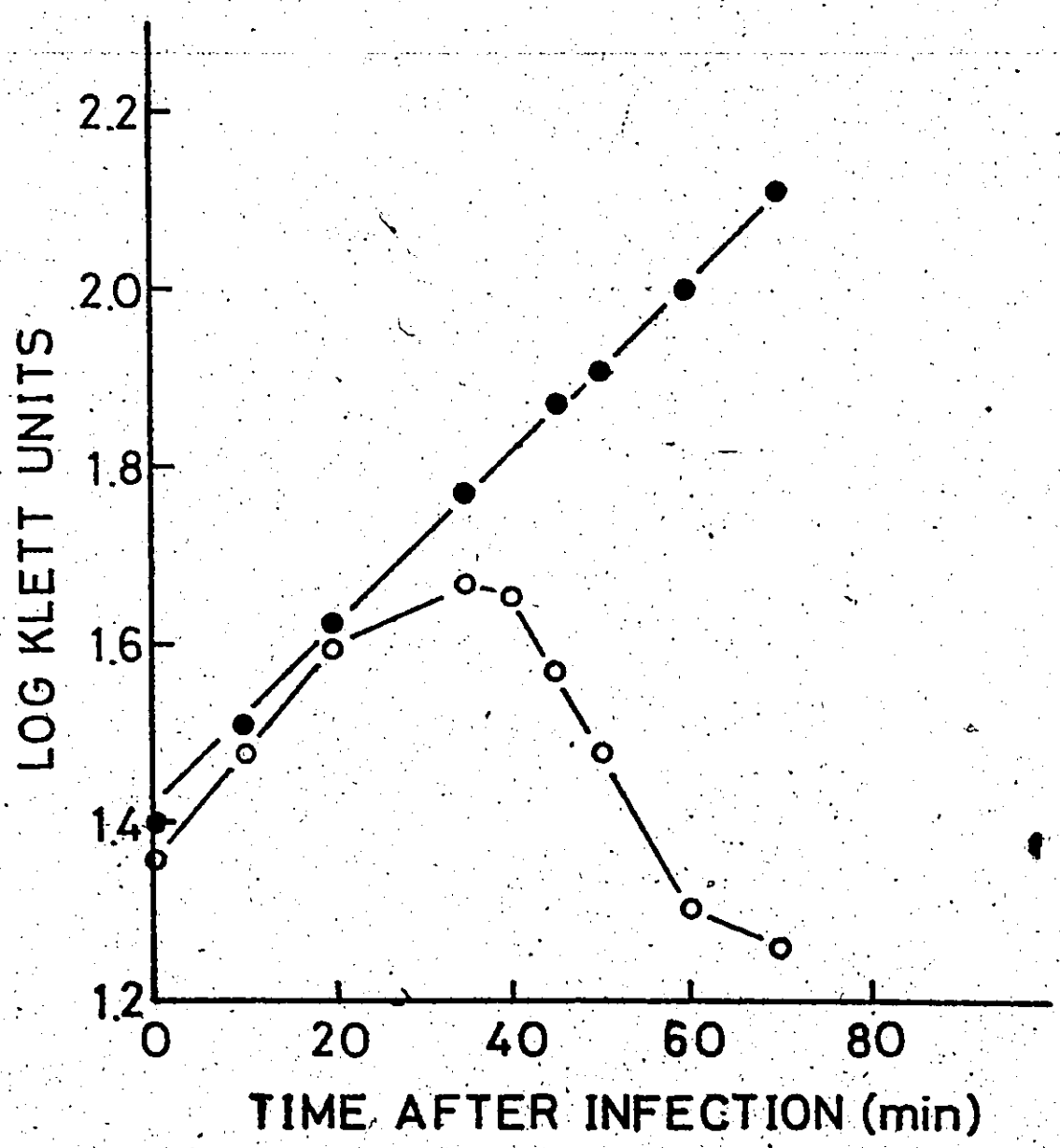

LOG KLETT UNITS
2.2
2.0
1.8
1.6
1.4
1.2
0
20
40
60
80
TIME AFTER INFECTION (min)

TABLE 9. Ability of mutants to induce cell lysis

Phage	Complementation group	DNA synthesis[a]	Lysis[b]
SFU1	1	25	+
SFU3	2	12	-
SFU5	4	8	-
SFU9	6	30	+
SFU49	10	7	-
SFU50	11	7	[illegible]
SFU60	14	131	-
SNG1	22	8	-
SNG5	24	14	-
SNG15	29	102	-
SNG47	34	10	-
SFU36	36	10	-

[a] Data from Table 8

[b] +: Lysis; -: No lysis

cultures were then treated with lysozyme (250 µg/ml) for 20 min and again assayed on JB0130. Table 10 shows that the mutant SFU50, which is deficient in phage-induced DNA synthesis and in host cell lysis was similarly incapable of phage production. Mutants SFU60 and SNG15 were capable of producing significant amounts of phage, although yields were less than 10% that of the wild-type. Following lysozyme treatment, the relative phage yield in the SFU50-infected cultures remained unchanged, whereas both the SFU60 and SNG15-infected cultures contained phage titres comparable to the wild-type-infected control. It is therefore concluded that these two mutants contain defects in protein(s) involved in the lytic function and are not affected in intracellular phage development.

TABLE 10. Phage yields of mutants affected in lysis

Phage	Untreated (PFU/ml)	Relative yield	Lysozyme treated (PFU/ml)	Relative yield
PBS2	9.3×10^9	100	1.5×10^{10}	100
SFU50	1.3×10^6	0.01	1.8×10^6	0.01
SFU60	6.5×10^8	7.0	1.2×10^{10}	80
SNG15	4.8×10^8	5.2	3.0×10^{10}	200

CHARACTERIZATION OF DNA-AFFECTED MUTANTS

One of the methods for obtaining information on conditional lethal mutants with altered physiological phenotypes involves determining their ability to carry out specific functions which may be essential for viability. Some mutants may actually exert pleitropic effects, causing a number of different phage functions to be affected. Examples of this would be maturation defective mutants of phage T4 (113), or N gene mutants of phage lambda (137). With these possibilities in mind, the mutants of ΦPBS2 which were found to be affected in phage DNA synthesis were examined for their ability to perform a number of PBS2-related functions in the nonsuppressing host.

Cell killing

It was earlier shown that PBS2 infection results in the loss of colony-forming ability in the majority of infected cells (Table 2). To examine cell killing by mutants, four hour cultures of JB0128 were diluted 10-fold in fresh PA and grown to about 10^8 cells/ml. The fraction of surviving cells was obtained by plating cells on TBB agar before and 20 min after infection (Table 11). The results show clearly that all of the DNA-affected mutants are capable of killing infected cells.

dCTP deaminase

Tomita and Takahashi (42) proposed that the PBS1 induced dCTP deaminase in infected cells produces a major pathway for the synthesis

of dUTP, a presumed precursor for phage DNA. If this is so, phage mutants which have lost the ability to induce dCTP deaminase might be unable to synthesize DNA since dTTP is presumably depleted in infected cells. Crude extracts of PBS2 infected cells of JB0128 were prepared at 30 min after infection and assayed for dCTP deaminase activity. Figure 8 shows a time course of one such assay at 37C. No activity was detected in extracts from uninfected cells whereas extracts from PBS2-infected cells deaminated dCTP linearly for 15 min. The specific activity for dCTP deaminase in this extract was calculated to be 50 nmoles/min/mg protein. Using the same technique, specific activities of dCTP deaminase in extracts of nonsuppressing cells infected with various mutants were determined. All of the DNA-affected mutants induced dCTP deaminase in nonsuppressing cells (Table 11).

Deoxyribonuclease inhibition

It was found that B. subtilis contains an endonuclease specific for native, uracil-containing DNA (25). Tomita and Takahashi (45) showed that a heat stable protein which inhibits this enzyme is induced by PBS1 at 20 min after infection. In the present study it was considered that a phage mutant which is unable to induce this inhibitor might appear phenotypically as a DNA-affected mutant.

Crude extracts of PBS2 and mutant-infected cells were prepared at 30 min after infection and assayed for the nuclease activity. The results in Table 11 are expressed as percent of the CPM in ^{3}H-Urd labelled PBS2 DNA which became acid soluble. It can be seen from the data that PBS2

FIGURE 8. Spectrophotometric assay of PBS2-induced dCTP deaminase

To obtain crude extracts, cells of JB0128 were harvested at 30 min after infection, resuspended in 1/20 volume of tris-chloride buffer (25 mM, pH 7.5) and treated twice with a French press. Assays of dCTP deaminase were carried out with 0.05 ml of crude extract. The extracts of PBS2-infected and uninfected cells contained 3.4 and 3.5 mg protein/ml respectively. Deamination of dCTP was followed by measuring the decrease in absorbance at 290 nm (A_{290}).

●: PBS2-infected cell extract

○: Uninfected cell extract

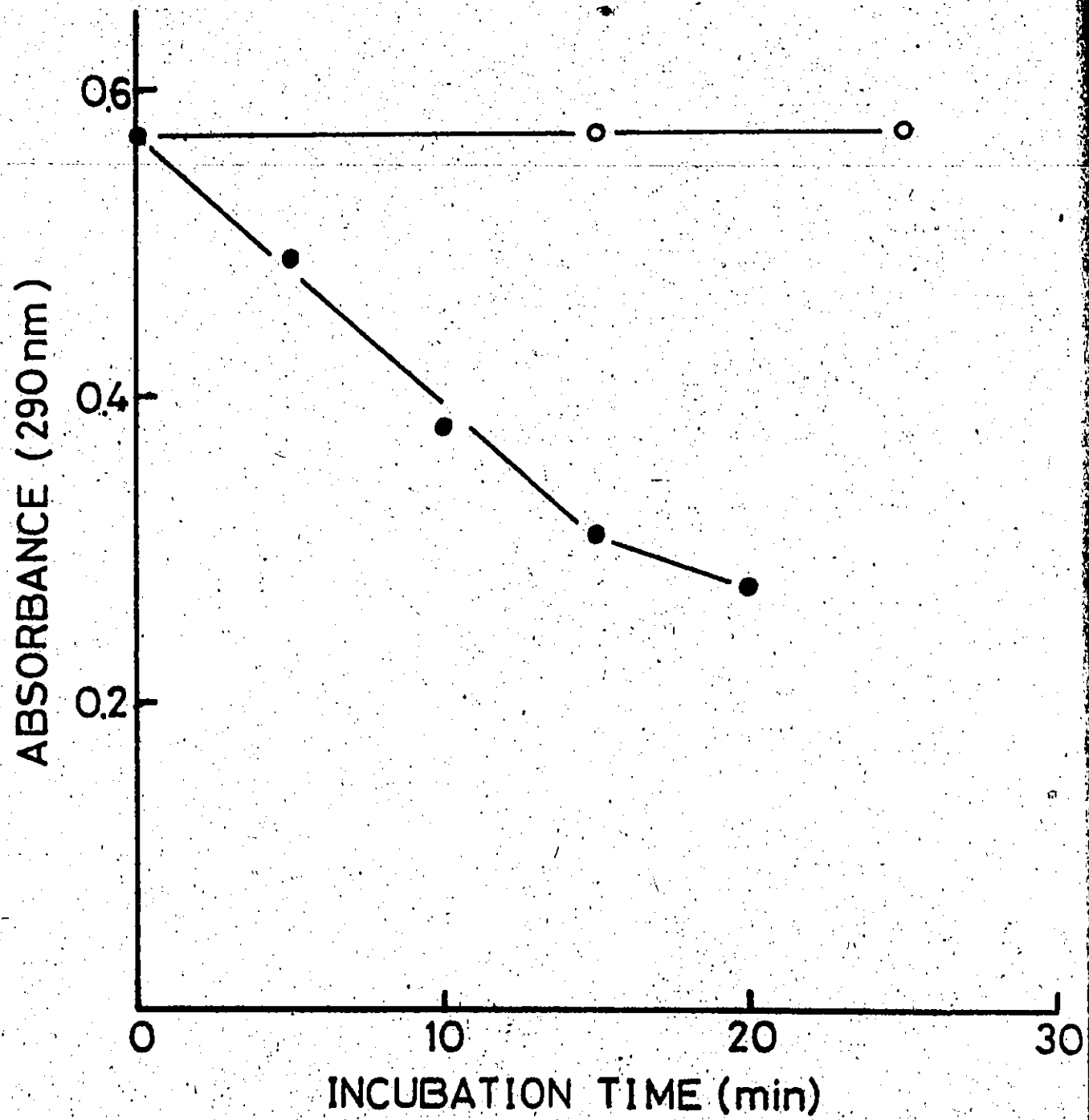

0.6
0.4
0.2
ABSORBANCE (290nm)
0
10
20
30
INCUBATION TIME (min)

TABLE 11. Characterization of DNA-affected mutants

Phage	Complementation group	Survival[a] of infected cells	dCTP[b] deaminase	DNase[c] activity	DNA[d] polymerase
Uninfected	-	-	0	69.0	11
PBS2	-	8	31	1.6	100
SFU1	1	23	26	1.5	87
SFU3	2	11	33	1.0	190
SFU5	4	11	15	1.2	233
SFU9	6	21	43	1.2	124
SFU49	10	4	32	1.8	5
SFU50	11	8	34	1.6	5
SHG1	22	9	47	1.1	156
SHG5	24	16	32	1.2	224
SHG47	34	19	53	2.1	56
SFU36	39	8	Not tested	Not tested	9

TABLE 11. (continued)

[a]Percent survival of infected cells at 20 min after infection.

[b]Specific activity expressed as nmoles/min/mg protein.

[c]Percent of CPM ^{3}H-Urd-labelled substrate rendered TCA soluble in 30 min at 37C.

[d]Specific activity relative to PBS2-infected cell extracts. The average of 4 experiments was 15,369 CPM/min/mg in PBS2-infected extracts.

and all the DNA-affected mutants are capable of inducing the inhibitor of this nuclease.

DNA polymerase

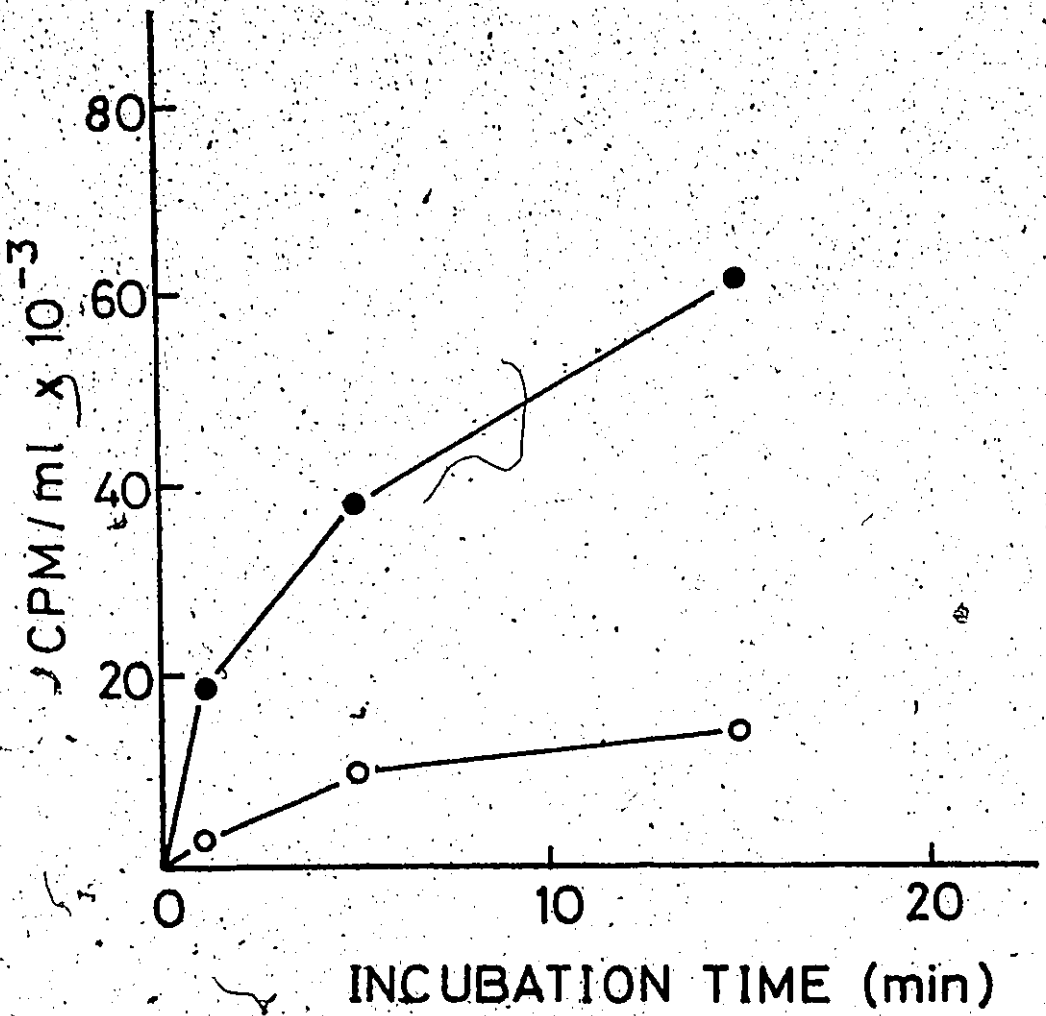
PBS2, like some other B. subtilis phages, is resistant to 6-(p-hydroxyphenylazo)-uracil, which inhibits the host DNA polymerase III, and induces a new DNA polymerizing activity in infected cells (70). If this new enzyme is involved in the replication of PBS2 DNA, then a mutation inactivating it would lead to conditional lethality as in the case of gene 43 mutants of phage T4 (54,55). Figure 9 shows the results of DNA polymerase assays with crude extracts of PBS2-infected and uninfected nonsuppressing cells. In this case, the specific activity of DNA polymerase in the uninfected cell extract was about 15% that of the infected cell extract. However, under the above conditions, the initial rate of reaction in PBS2-infected cell extracts was not proportional to protein concentration. Therefore, to test the mutants for the ability to induce DNA polymerase activity, infected cells were harvested as in Fig. 9 and resuspended in 1/20 volume of 25 mM tris-chloride (pH 7.5), rather than 1/100 volume, to reduce the protein concentration. These were then extracted and assayed as described in Materials and Methods. Protein concentrations in extracts prepared in this way were usually 2-4 mg/ml. The initial velocity of reaction with crude extracts of wild-type PBS2-infected cells was found to be proportional to protein concentration in the range of 0.02-0.2 mg/ml in the incubation mixture. The specific activities of DNA polymerase in crude extracts of mutant-infected cells harvested

FIGURE 9. DNA polymerase activities in PBS2-infected and uninfected cells

PBS2-infected and uninfected cells of JB0128 were harvested at 40 min after infection and resuspended in 1/100 volum of tris-chloride buffer (10 mM, pH 7.5). Lysozyme extracts were prepared and dialyzed as described in Materials and Methods. The incubation mixtures contained: dCTP, 4 mM; ^{3}H-dCTP (22.6 Ci/mMole), 2.8 µCi/ml; 50 µg of heat denatured calf thymus DNA; crude extract corresponding to 0.63 mg protein in the case of infected cells and 0.71 mg in the uninfected control, in a final volume of 0.29 ml.

●: PBS2-infected cell extract

○: Uninfected cell extract


80
60
40
20
0
10
20
CPM/ml x 10⁻³
INCUBATION TIME (min)

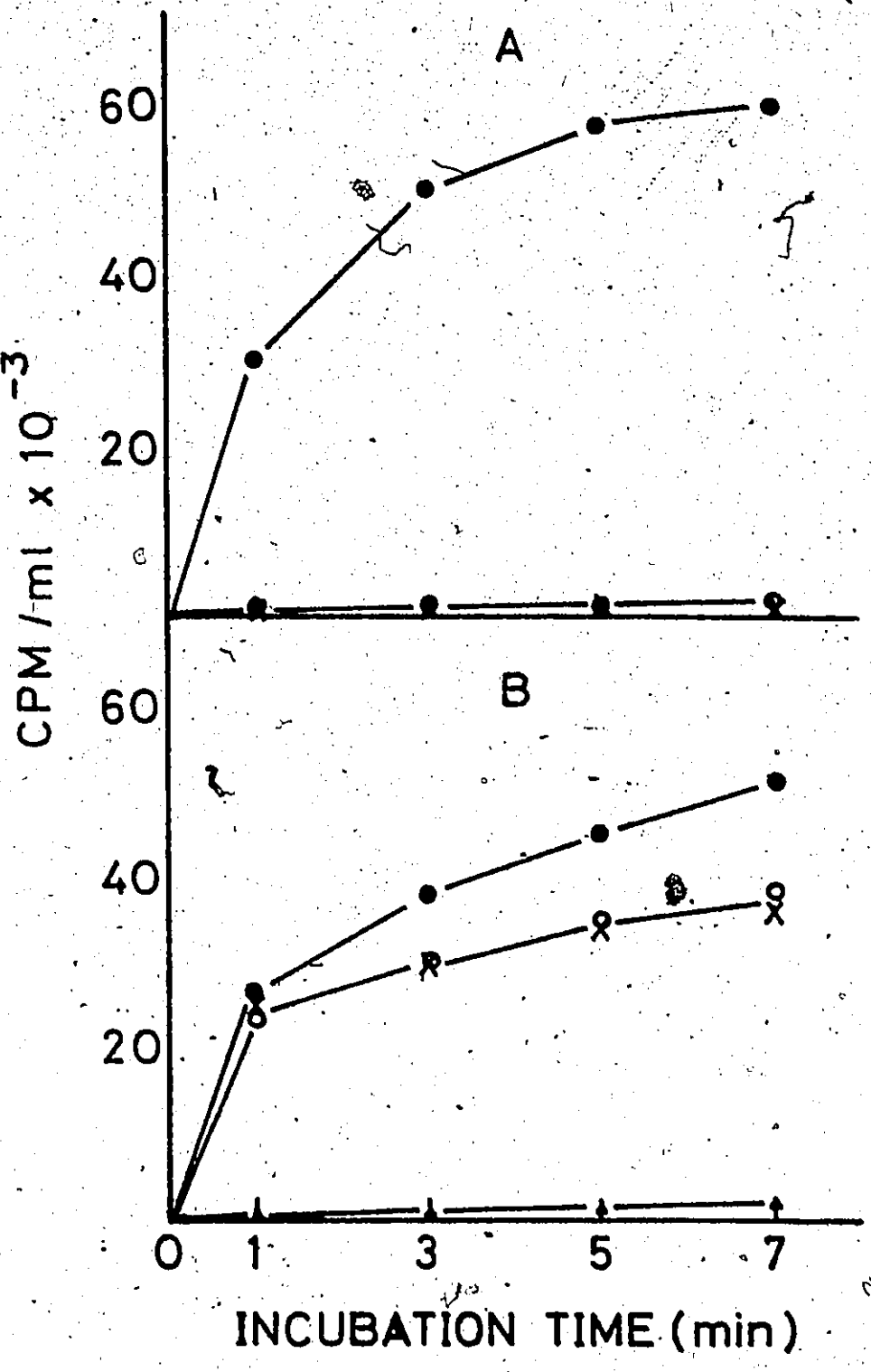
at the 40th min after infection were determined (final protein concentrations were 0.1-0.2 mg/ml) and compared to those in PBS2-infected and uninfected extracts of JB0128 (Table 11). Three of the mutants, SFU49, SFU50, and SFU36 were unable to induce DNA polymerase activity above the level found in uninfected cells. In a separate experiment, it was found that the initial rate of reaction of PBS2-infected cell extracts prepared this way was only 50% dependent on exogenous DNA. That being the case, it was possible that the low DNA polymerase activities in one or more of these three mutants reflected a lack of endogenous template rather than of enzyme. When extracts were prepared from PBS2-infected cells using a French press rather than lysozyme, the initial rate of reaction in the absence of exogenous DNA, was less than 10% that of the reaction mixture containing 100 µg of calf thymus DNA. The results of DNA polymerase assays with PBS2 and mutant-infected cell extracts prepared this way are shown in Fig. 10. Panel A shows that extracts of SFU49 and SFU50-infected cells still exhibited very low DNA polymerase activity. Panel B shows that no diffusible inhibitor was present in extracts of SFU49 or SFU50-infected cells since mixed extracts did not affect the initial rate of DNA polymerase activity of the PBS2-infected extract. Moreover, when added together, the SFU49 and SFU50-infected extracts exhibited no complementation. Complementation would have been expected if these mutants affect different proteins of a reassociable homo- or heteromultimeric enzyme, or in fact, two physically separate proteins which together are responsible for DNA synthesis in vitro. The mutant SFU36 was not included in these experiments since there were indications that it might actually belong to the same complementation group as SFU49.

FIGURE 10. DNA polymerase activities in mutant-infected cells

Infected cells were harvested at 40 min after infection, resuspended in 1/20 volume tris-chloride buffer (25 mM, pH 7.5) and extracted with a French press. Incubation mixtures contained: dGTP, 20 µM; dCTP, 20 µM; dATP, 2 µM; ^{3}H-dATP (16 Ci/mmole), 1 µCi; dUTP, 1 mM; 100 µg of heat denatured calf thymus DNA; tris-chloride buffer (pH 8.5) 64 mM; and 0.02 ml of crude extract in a final volume of 0.3 ml. At appropriate times after the addition of crude extract, 0.05 ml samples were withdrawn and TCA-insoluble counts on membrane filters were determined.

Panel A:
- ●: PBS2-infected cell extract
- O: SFU49-infected cell extract
- X: SFU50-infected cell extract

Panel B:
- ●: PBS2-infected cell extract
- O: PBS2-infected + SFU49-infected cell extract
- X: PBS2-infected + SFU50-infected cell extract
- ▲: SFU49-infected + SFU50-infected cell extract


A
B
60
40
20
60
40
20
CPM/ml x 10⁻³
0
1
3
5
7
INCUBATION TIME (min)

Experiments with toluenized cells

Moses and Richardson (138) reported that toluenized cells of E. coli were capable of synthesizing DNA in the presence of the appropriate deoxynucleoside triphosphates. This synthesis was stimulated by ATP and did not require exogenous DNA. They also found that a form of repair synthesis, requiring functional DNA polymerase I, could be distinguished from apparent semiconservative replicative synthesis. Matsushita et al. (139) later showed that semiconservative synthesis of biologically active DNA occurs in toluenized B. subtilis cells. Their results indicated that this replicative synthesis requires ATP.

Lavi and Marcus (140) demonstrated that the arrest of host DNA synthesis which is normally observed in B. subtilis during phage Øe infection, was manifested in toluenized preparations of infected cells. The virulent phage Øe, which contains hydroxymethyluracil in its DNA in place of thymine, was unable to incorporate ^{3}H-dTTP into phage DNA in infected toluenized cells. However, a mutant capable of arresting host DNA synthesis but lacking the phage-induced enzyme, dTTPase, was able to do so. This mutant had previously been shown to incorporate some thymine into phage DNA in vivo.

Recent studies with phage T4 showed that replication of phage DNA occurs in toluenized preparations of infected E. coli cells (141,142,143). This synthesis was lacking in cells infected with DNA-affected phage mutants of T4. The results of Elliott et al. (141) indicated that host DNA replication which is shut off in vivo, may proceed in T4 infected toluenized cells. Although T4 DNA contains hydroxymethylcytosine, dCTP was found to be

efficiently incorporated into toluenized cells. In light of these new techniques for studying DNA replication, further studies were undertaken to examine DNA synthesis in PBS2 and mutant-infected B. subtilis cells.

It was found that infection of either JB0128 or the DNA polymerase I deficient strain, 168 polA by PBS2 stimulated the incorporation of ^{3}H-dATP into acid insoluble material several fold in toluenized cells (Fig. 11). Although the experiments illustrated in Fig. 11 (A and B) were done by two slightly different methods, PBS2 stimulated incorporation similarly in each case. DNA synthesis in infected cells treated with toluene was generally linear for 7-15 min. The reaction mixtures became clear thereafter, indicating cell lysis. When ATP was omitted from the reaction mixture, the rate of incorporation of ^{3}H-dATP into infected JB0128 cells treated with toluene was reduced by 75% while that in uninfected toluenized cells was reduced by 55%.

If the DNA synthesis in toluenized infected cells was due to phage replicative synthesis, some of the DNA-affected mutants, at least, would be expected to be deficient in this in vitro system. Figure 12 shows the rates of DNA synthesis in cells of 168 polA harvested and toluenized at various times after infection. It can be seen that the rate of ^{3}H-dATP incorporation in PBS2-infected cells increased approximately 5-fold in 30 min while that in uninfected cells increased 2-3 fold over the same period. However, the rate of incorporation in cells infected with the mutant SFU5 decreased considerably during the same period. Earlier results presented in Fig. 6 showed that SFU5-infected cells treated with rifamycin incorporated very little ^{3}H-dCyd into phage DNA

FIGURE 11. DNA synthesis in toluenized cells

Panel A: A four hour culture of JB0128 was diluted 10-fold in fresh PA and grown to 10^8 cells/ml. The culture was divided into two flasks, one of which was infected with PBS2 at an MOI of 5-10. Twenty min after infection, the cells were harvested, washed, and resuspended in 1/15 volume of 50 mM tris-chloride, 80 mM KCl, 5 mM $MgCl_2$, pH 7.5, containing 1% toluene. Cells were kept on ice for 15 min with intermittant agitation (144), after which they were diluted $\frac{1}{2}$ in the reaction mixture. The reaction mixture contained the following (final concentration): tris-chloride buffer (pH 7.5), 50 mM; $MgCl_2$, 5 mM; KCl, 80 mM; dATP, 2.6 µM; dUTP, dGTP, dCTP, 20 µM each; ^{3}H-dATP, 10 µCi/ml; ATP, 1 mM. Samples corresponding to 3.8×10^7 cells (0.05 ml) were added to cold 10% TCA at appropriate intervals, washed onto membrane filters and assayed for TCA-insoluble counts.

●: PBS2-infected cells

○: Uninfected cells

Panel B: Cells of 168 polA were grown and infected as above and harvested at 20 min after infection. These were washed and resuspended in the same buffer as above without toluene (uninfected cells were harvested at 0 min). Toluene was added to 1% and cells were agitated for 1 min according to the procedure for Lavi and Marcus (140). Assays were as above excpet that the dATP concentration was 2.3 µM and ^{3}H-dATP was at 5 µCi/ml. Samples corresponding to 2.5×10^7 cells (0.05 ml)

were withdrawn at appropriate times and assayed as in Panel A.

●: PBS2-infected cells

○: Uninfected cells

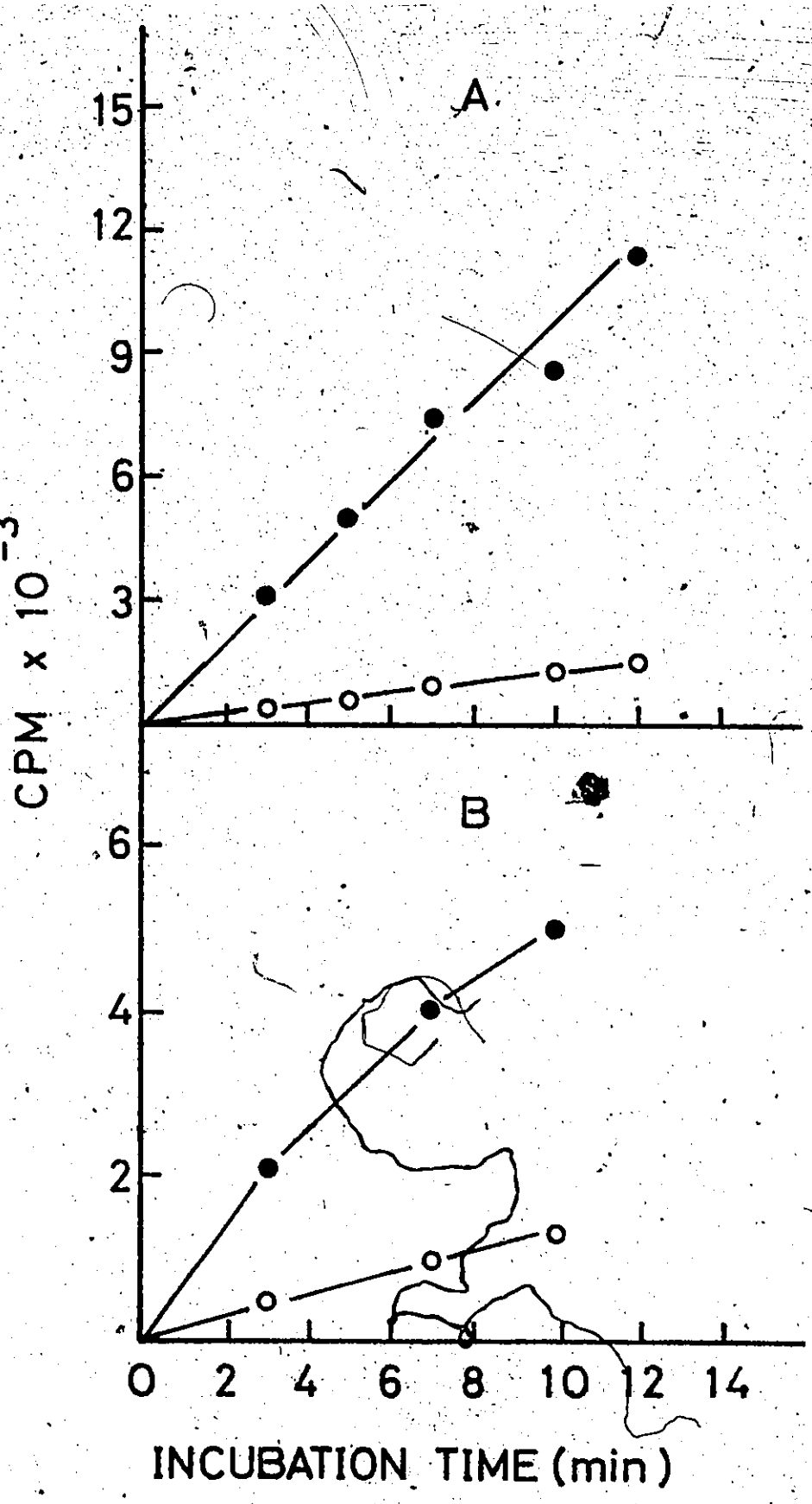

A
B
15
12
9
6
3
6
4
2
0
2
4
6
8
10
12
14
CPM x 10⁻³
INCUBATION TIME (min)

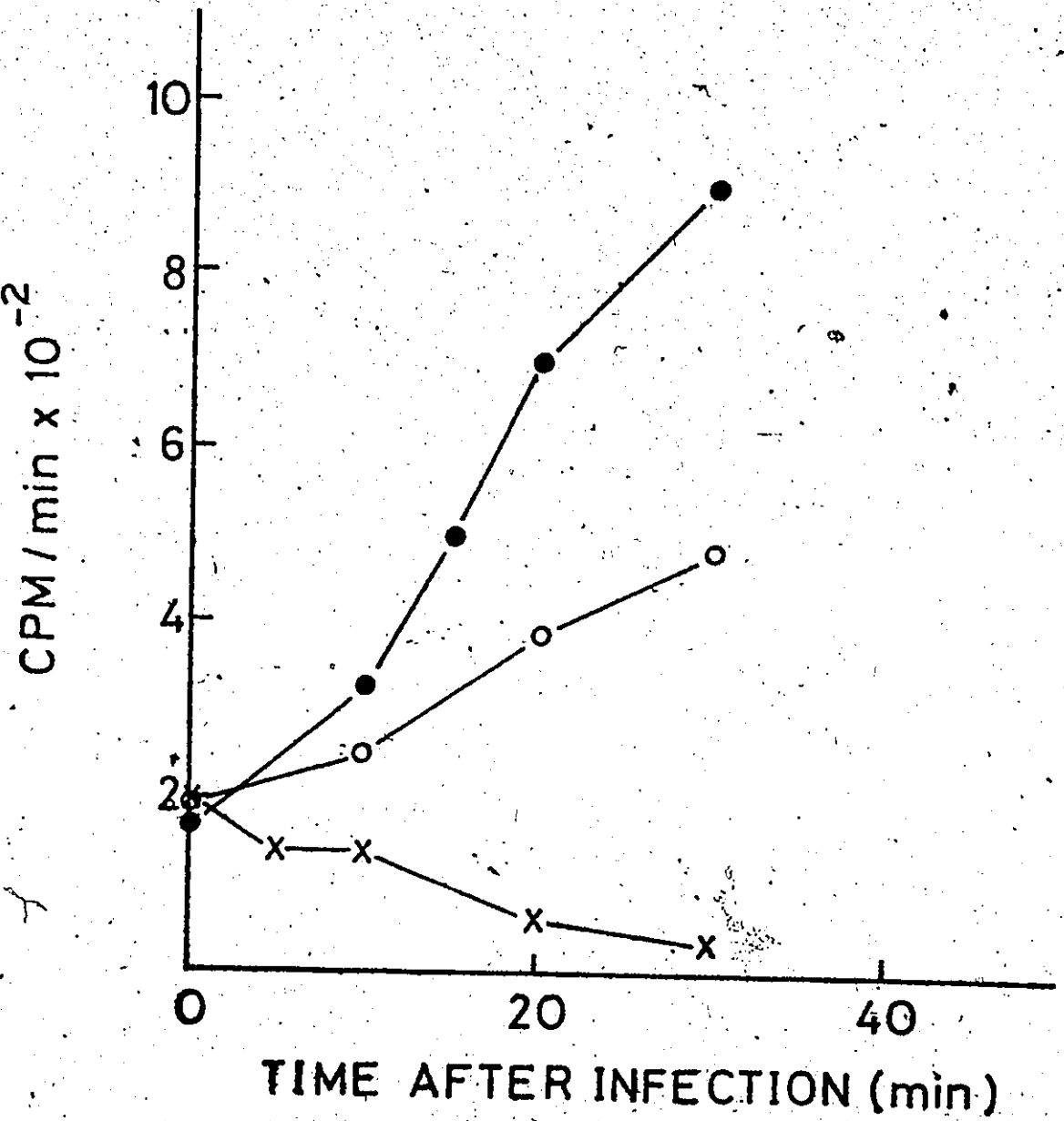
FIGURE 12. Rate of DNA synthesis in toluenized cells at different times after infection

Cells of 168 polA were grown and infected as in Fig. 11 and harvested at various times after infection. Cells were then toluenized and assayed as in Fig. 11B.

●: PBS2-infected cells

X: SFU5-infected cells

O: Uninfected cells


10
8
6
4
2
CPM / min x 10^{-2}
0
20
40
TIME AFTER INFECTION (min)

as compared to wild-type PBS2-infected cells. It was found in the present experiment that this defect is expressed in toluenized cells as well. Furthermore, it was observed that the decreased incorporation rate in SFU5-infected cells was less than that in the uninfected control, indicating that phage-induced shut-off of host DNA synthesis is manifested in this system. It might be argued that the low rate of incorporation in SFU5-infected cells merely reflects the lack of endogenous phage DNA since this mutant induced very little DNA synthesis in vivo (Fig. 6). If this is the case, it would mean that the stimulation of incorporation in PBS2-infected cells represents synthesis requiring phage DNA.

In order to determine whether other DNA-affected mutants are similarly incapable of DNA synthesis, a number of mutants were tested with JB0128 and/or 168 polA as host. Cells were grown for 4 h in PA, diluted 10-fold in fresh PA and grown to 10^8 cells/ml. The cells were infected, harvested at 20 min after infection (0 min for uninfected cells), washed and assayed as described in Fig. 12. As shown in Table 12, SFU49 and SFU50-infected cells incorporated ^{3}H-dATP at lower rates than did uninfected cells. However, the rates of incorporation with SFU1 and SFU9 were higher than uninfected cells. These two mutants were shown (Fig. 6) to be able to incorporate 20-30% as much ^{3}H-dCyd into DNA as wild-type PBS2 in the presence of rifamycin. This limited ability to synthesize phage DNA may be reflected in toluenized cells as indicated in Table 12.

There is some evidence that toluenized cells may become permeable to at least some proteins (138). However, attempts to enhance incorporation into mutant-infected toluenized cells by the addition of French press extracts

TABLE 12. Rate of DNA synthesis in mutant-infected toluenized cells

Phage	Complementation group	Relative rate of ^{3}H-dATP incorporation	
		JB0128	168 polA
PBS2		100	100
Uninfected		11	23
SFU1	1	22	Not done
SFU5	4	Not done	8
SFU9	6	18	Not done
SFU49	10	7	Not done
SFU50	11	8	13

of cells infected with wild-type PBS2 produced no positive results. It is probable that such complementation experiments would require temperature-sensitive mutants or an in vitro replication system to which exogenous DNA could be added.

Preliminary experiments indicated that PBS2-infected toluenized cells incorporated ^{3}H-dTTP into DNA when dTTP completely replaced dUTP in the reaction mixture. An experiment was then designed to investigate whether the PBS2 replicative apparatus is involved in ^{3}H-dTTP incorporation. Cells of JB0128 were grown and infected as in Fig. 11, and harvested for assay at 20 min after infection (0 min for uninfected cells). Toluenized cells (as in Fig. 11, Panel B) were assayed with the standard reaction mixture in which the ^{3}H-dATP concentration was 1.9 µCi/ml or with one in which dUTP was completely replaced by 2 uM dTTP with ^{3}H-dTTP (2.5 µCi/ml) as the radioactive precursor. In the latter mixture the dATP concentration was 20 µM. Rates of incorporation of ^{3}H-dATP and ^{3}H-dTTP into DNA, corrected for differences in specific activities are shown in Table [illegible]. With the dUTP-containing reaction mixture, PBS2-infected cells incorporated label almost 10 times faster than uninfected cells while the rates in SFU49 and SFU50-infected cells were lower than that in uninfected cells. When dTTP replaced dUTP and ^{3}H-dTTP was the radioactive precursor, PBS2-infected cells still incorporated label 7 times faster than uninfected cells and the rates in SFU49 and SFU50-infected cells were still lower than that in uninfected cells. These data suggest that the phage replication apparatus is capable of incorporating dTTP in place of dUTP, presumably during replication of phage DNA. The data

TABLE 13. PBS2-induced ^{3}H-dTTP incorporation into DNA in toluenized cells

Phage	Rate of incorporation[a] (CPM/min/6 x 10^{7} cells)	
	^{3}H-dATP	^{3}H-dTTP
Uninfected	56	94
PBS2	521	620
SFU49	35	32
SFU50	41	31

[a]Corrected for the difference in ^{3}H-dATP and ^{3}H-dTTP specific activities

further support the conclusion that phage-induced shut-off of host DNA synthesis is manifested in toluenized cells.

GENETICS

Complementation tests

Mutants in ten complementation groups were found to be affected in phage-induced DNA synthesis (Table 8). SFU36, SFU49, and SFU50 were unable to induce a phage-related DNA polymerising activity (Table 11). Since these ten DNA-affected mutants had previously been classified qualitatively by spot tests (Table 3) before recombination analysis could be undertaken a more rigorous assessment of complementation was required. A major interest in this investigation was to establish whether the DNA polymerase-deficient mutants, SFU36, SFU49, and SFU50 belong to different genetic loci.

Quantitative complementation tests were carried out for all pair-wise combinations of the ten mutants (Table 14). Results are expressed as total phage yields relative to that in the wild-type PBS2-infected culture with an appropriate correction for singly infected controls. Thus, in single infections of JOBL28 with DNA-affected mutants, phage yields were 0.1-1.1% of the wild-type. However, in complementing pairwise infections, corrected relative yields were 20-71% of the wild-type. Co-infection with the noncomplementing pair, SFU36 and SFU49 resulted in a phage yield only 0.2% of the wild-type. In three experiments, the average burst size in wild-type PBS2-infected cultures was 48. By comparison, the culture co-infected with SFU36 and SFU49 produced an average of less than 0.1 particle/cell. It can be seen from the data that all mutants except SFU36 and SFU49 belong to different groups.

TABLE 14. Quantitative complementation tests with DNA-affected mutants

Mutant	SFU1	SFU3	SFU5	SFU9	SFU36	SFU49	SFU50	SNG1	SNG5	SNG47
SFU1	0.009	0.26	0.28	0.33	0.55	0.33	0.31	0.36	0.56	0.39
SFU3		0.001	0.34	0.39	0.42	0.20	0.42	0.46	0.40	0.30
SFU5			0.005	0.71	0.68	0.47	0.30	0.30	0.24	0.25
SFU9				0.002	0.32	0.33	0.50	0.31	0.57	0.43
SFU36					0.001	0.002	0.28	0.42	0.55	0.47
SFU49						0.001	0.20	0.35	0.36	0.25
SFU50							0.001	0.39	0.40	0.31
SNG1								0.011	0.33	0.53
SNG5									0.001	0.41
SNG47										0.001

Relative yields for single mutants are averages of 2 experiments.

The mutant SFU36, previously assigned to group 36 (Table 3) was unable to complement SFU49 and was therefore reassigned to group 10. Since these mutants were all found to be affected in DNA synthesis (Fig. 6), it can be concluded that of 38 complementation groups identified in this study, at least 9 affect phage DNA replication. Results of the complementation tests showed that SFU1 and SFU9 which are phenotypically similar yet distinct from mutants in other groups (Table 9) are themselves involved in different functions.

It is of particular interest to note that, whereas SFU36 and SFU49 were found to be in the same group, both of these mutants were able to complement SFU50. It could be argued that SFU50 affects the same protein as SFU36 and SFU49, but is capable of strong intracistronic complementation of each of these. This possibility may be explored further either by biochemical analysis of the phage-induced DNA polymerase or by constructing a genetic map of PBS2.

Two-factor crosses of DNA-affected mutants

Once a collection of phage mutants has been characterized phenotypically and classified according to functional identity, they become more amenable to further genetic analysis. With the appropriate techniques, mutants may be mapped according to their relative positions on the genome. When this has been done, further experiments such as transformation may enable us to determine the approximate physical location of a particular mutation within the chromosome. The ordering of phage mutants on a genetic map involves the use of two and three- factor

genetic crosses. These, combined with information from complementation tests, would reduce a potentially enormous number of experiments to a more manageable quantity. The assumptions implicit in recombination analysis of most phages are essentially those expounded by Visconti and Delbruck (92) to account for the many observations made with the mutants of E. coli phage T4. A number of questions may be answered at least in part, by constructing a genetic map. One of these is whether or not similar phage functions are clustered together on the chromosome. Organization at this level has numerous implications for the regulation of expression of phage functions during infection. Also, mutants whose genetic identities are under question may be classified with greater confidence once relative positions have been established. Finally, the linearity or circularity of the genome may be established and this may provide information on the processes of replication, recombination or phage head formation.

A number of two-factor crosses of DNA-affected mutants were performed as outlined in Materials and Methods. The results are presented in Table 15 as percent recombination. The superscripts refer to the number of times crosses were performed. To give an indication of variation in these crosses, coefficients of variation were calculated for a number of crosses and listed in Table 16. In experiments with rII mutants of phage T4, Edgar (145) obtained values for this parameter of 0.12-0.30. In two-factor crosses of temperature-sensitive mutants of PBS2, Herrington (122) obtained coefficients varying from 0.19 to 0.64. The average for ten crosses with ts mutants was 0.36 whereas the average for the nine crosses presented in Table 16 was 0.26.

TABLE 15. Two-factor crosses with DNA-affected mutants[a]

Mutant	SFU1	SFU3	SFU5	SFU9	SFU49	SFU50	SNG1	SNG5	SNG47	SFU36
SFU1	-	19.9^{6}	-	12.3^{6}	16.4^{3}	9.1^{6}	7.8^{2}	12.3^{3}	-	-
SFU3		-	10.6^{4}	19.9^{8}	4.2^{7}	18.8^{9}	10.2^{2}	8.9^{3}	2.1^{2}	-
SFU5			-	2.8^{1}	4.2^{1}	0.94^{4}	-	-	3.8^{1}	5.4^{1}
SFU9				-	6.9^{4}	2.9^{4}	-	-	-	-
SFU49					-	7.1^{5}	19.2^{1}	17.1^{1}	-	0.01^{1}
SFU50						-	7.9^{5}	9.1^{6}	7.0^{1}	6.5^{1}
SNG1							-	11.5^{3}	-	-
SNG5								-	12.7^{2}	-
SNG47									-	-
SFU36										-

[a]Superscripts represent number of times crosses were performed.

TABLE 16. Variation in crosses

Crosses	Mean recombination frequency	Number of crosses	Standard deviation	Coefficient of variation[a]
SFU1 x SFU3	19.9	6	5.7	0.28
SFU1 x SFU9	12.3	6	2.4	0.20
SFU1 x SFU49	16.4	3	1.8	0.11
SFU1 x SFU50	9.1	6	3.7	0.41
SFU3 x SFU9	19.9	8	7.1	0.36
SFU3 x SFU49	4.2	7	1.4	0.33
SFU3 x SFU50	18.8	9	6.1	0.32
SFU50 x SFU5	0.94	4	0.13	0.14
SFU50 x SFU9	2.9	4	0.52	0.18
SFU50 x SFU49	7.1	5	2.2	0.31

[a]Calculated as: $\frac{\text{standard deviation}}{\text{mean recombination frequency}}$

The results of these experiments provide for a linear arrangement of the DNA-affected mutants as shown in Fig. 13. The mutants are arranged according to recombination frequency, approximately to scale, revealing their possible order on the phage genome. The non-additivity of the values may be explained, in part, by variation as well as by theoretical considerations to be discussed later. A number of interesting observations can be made regarding this arrangement. Firstly, the mutants SFU36 and SFU49 which were found to belong to the same complementation group (Table 14) appear to be very close together. The mutant SFU50 is distant from both of these and at least one mutant, SFU9 lies between the two groups. Should this order prove to be correct, it would rule out the possibility that SFU36, SFU49, and SFU50, none of which induces the phage-related DNA polymerase (Table 11), affect the different regions of a homomultimeric enzyme. In addition, saturation appears to occur somewhere between 10 and 20% recombination since additivity is worst when distances compared are 10% or more. Recombination frequencies from crosses involving SNG1 and/or SNG5 cannot be reconciled with the order presented. Nor does it appear that any simple arrangement can be made to accommodate them in a linear map compatible with the other data. The best explanation, at the present, is that both SNG1 and SNG5 are not closely linked to the other DNA-affected mutants or to each other. This proposal would also mean that the sequence in Fig. 13, although saturated in terms of recombination frequency, represents only a portion of the whole genome.

The task at this stage was to attempt to verify the basic map

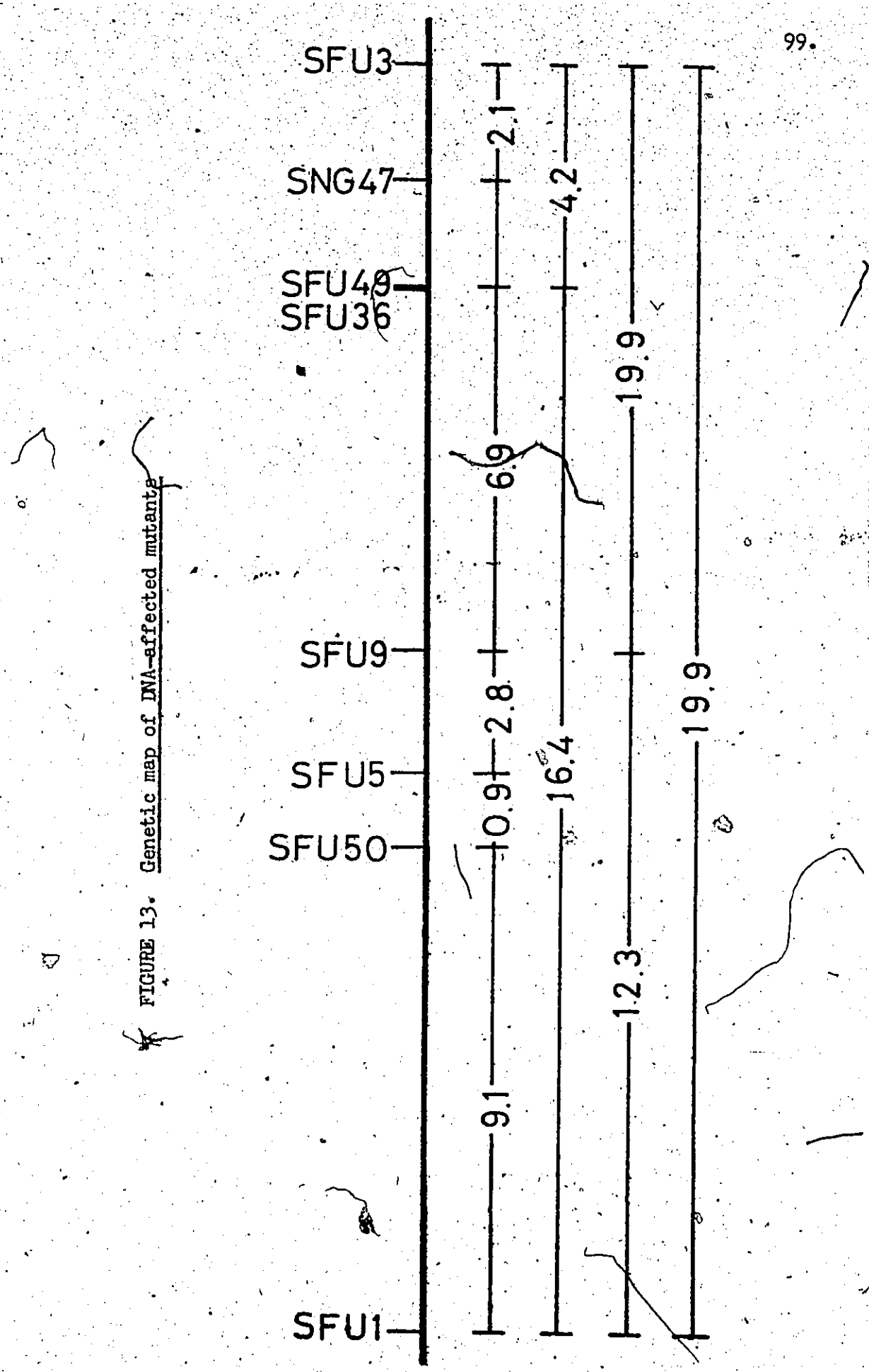


FIGURE 13. Genetic map of DNA-affected mutants

order suggested in Fig. 13 by three-factor crosses.

To carry out three-factor crosses, a number of suppressor-sensitive double mutants were isolated from the progeny of two-factor crosses. These were then used in three-factor crosses with other suppressor-sensitive DNA-affected mutants to test the genetic map order suggested in Fig. 13. Three-factor crosses represent a major tool for ordering mutants which are linearly related to one another. They are based on the idea that, when a mutant lies between two other mutants, the frequency of double recombinants expected is approximately the product of the recombination frequencies for the two genetic intervals. Comparison of the results from three-factor crosses presented in Table 17 with those for two-factor crosses (Table 15) indicates a general compatibility with the proposed order. Mutants SFU1 and SFU3 appear to be the terminal markers of those tested.

The results are also consistent with the proposition that both SFU9 and SFU49 lie between SFU50 and SFU3. Also, the cross SFU3SFU9 x SFU49 provides further evidence that SFU9 lies between SFU50 and SFU49.

The interference index i (146) is a parameter which can indicate correlations between recombination in two genetic regions. If recombination in one region enhances the frequency of a second event in another region, negative interference is said to occur. Negative interference is characteristic of bacteriophage crosses (146). The magnitudes of the interference index for the crosses in Table 17 are comparable to those indicating low negative interference in other phage systems (147,148). The basic order of the mutants proposed in Fig. 13

TABLE 17. Three-factor crosses with DNA-affected mutants

Parent 1	Parent 2	sus+ recombinants (%)		Interference index (i)[c]
		Expected[a]	Observed[b]	
SFU1SFU3	SFU9	2.0	2.4^{2}	1.2
SFU1SFU3	SFU49	0.7	1.6^{1}	2.2
SFU1SFU3	SFU50	1.7	2.8^{4}	1.6
SFU3SFU50	SFU5	0.1	0.8^{1}	8.0
SFU3SFU50	SFU9	0.6	1.4^{1}	2.3
SFU3SFU50	SFU49	0.3	1.2^{1}	4.0
SFU3SFU49	SFU49	0.3	1.3^{2}	4.3

[a] Refers to the frequency of double recombinants expected if the mutation in parent 2 lies between those in parent 1 based on the frequencies of single recombinants from Table 15.

[b] Represents the observed frequencies of sus+ recombinants with superscripts referring to the number of crosses.

[c] Calculated as: $\frac{\text{expected frequency of sus}^{+}\text{ recombinants}}{\text{observed frequency of sus}^{+}\text{ recombinants}}$

is therefore compatible with two and three-factor cross data and may further be examined by the use of linkage tests.

Linkage tests

Streisinger and Bruce (93) proposed operational criteria for linkage of bacteriophage markers, which are independent of the mechanism of recombination or mode of mating. This is necessary since linkage in the classical sense of non-independent assortment of alleles, is not applicable to bacteriophage, especially since the outcome of a single phage cross represents a dynamic population of mating and recombining genomes. Their method basically involves measuring the frequency with which an allele of an unselected marker appears among single recombinants selected for the other two loci.

In order to test the order proposed in Fig. 13, a marker was required, whose position relative to at least one sus marker was known, which could act as an unselected marker in the appropriate crosses. Since there were no plaque-morphology or host-range mutants of PBS2 available, a number of temperature-sensitive mutants of the phage, isolated by Herrington (122) were examined for adaptability to this technique. The mutant chosen was THA31, which grew well in B. subtilis at 30C but not at 45C. In order to minimize expression of the mutation, crosses involving this marker were carried out at 30C.

Two-factor crosses between THA31 and a number of the DNA-affected mutants were performed as described in Materials and Methods. The results in Table 18 show that THA31 can best be situated to the left of

TABLE 18. Crosses for mapping of THA31

Cross	Recombination frequency (%)	
	at 30C	at 37C
THA31 x SFU1	7.3^{2}	-
THA31 x SFU1SFU50	7.6^{1}	-
THA31 x SFU50	15.5^{2}	-
THA31 x SFU3	12.5^{1}	
SFU1 x SFU3	13.1^{2}	19.9^{6}
SFU50 x SFU1	4.7^{2}	9.1^{6}
SFU50 x SFU3	8.2^{3}	18.8^{9}
SFU50 x SFU49	4.8^{1}	7.1^{5}

Superscripts indicate the number of times crosses were performed.

SFU1 on the genetic map. Another feature of these crosses was the general reduction of recombination frequencies when compared to those at 37C. The basic order however appears to be the same and has been represented in Fig. 14.

To facilitate experiments to determine linkage, attempts were made to isolate ts-sus double mutants. The double mutant, THA31SFU50 was obtained and its genotype was ascertained by spot complementation tests. This mutant was then used in linkage tests by carrying out standard crosses at 30C with other suppressor-sensitive mutants. Wild-type recombinants for the sus loci were selected for by plating the progeny of the crosses on JB0128 at 30C for single plaques. These were then tested for temperature sensitivity by picking onto plates seeded with JB0128 cells which were then incubated overnight at 45C. These were scored for lysis the following morning and the frequencies of the ts^+ allele among the sus^+ recombinants were calculated. If the order in Fig. 4 is correct, the frequencies of ts^+ allele among sus^+ recombinants from crosses involving mutants which lie to the right of SFU50 should be greater than 0.5. Conversely, the frequencies of the ts^+ allele among sus^+ recombinants from crosses involving mutants which lie between SFU50 and THA31 should be less than 0.5. The results of these crosses are in Table 19. The genotypes for the three loci being tested in each cross are schematically presented, + referring to wild-type, according to their proposed order. It can be seen that these results are consistent with the order shown in Fig. 14, and, also suggest that the mutant SFU5 lies to the right of SFU50. This means that at least two complementation groups represented by SFU5 and SFU9 lie between SFU50 and SFU49.

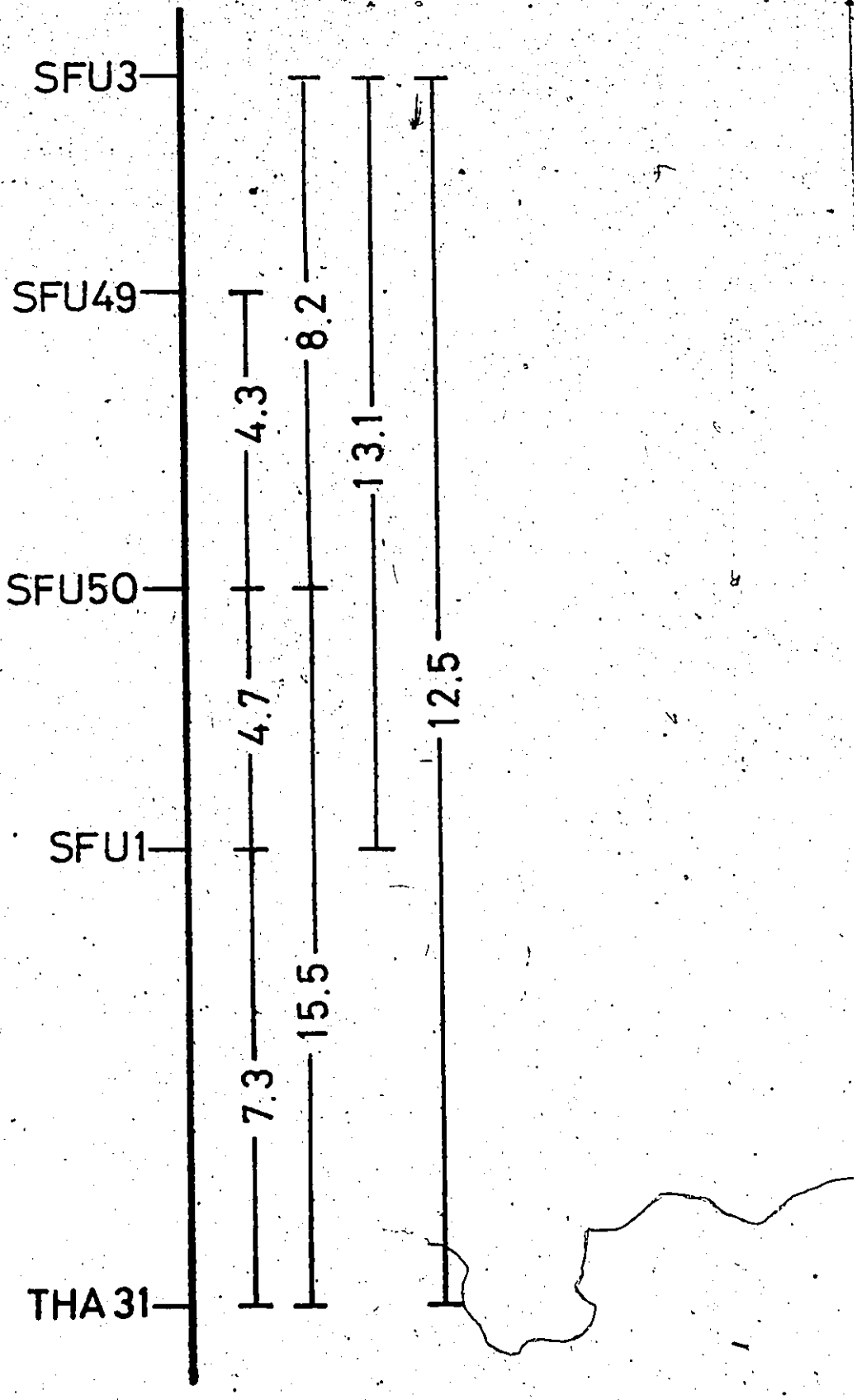


FIGURE 14. A map showing the relative position of THA31

TABLE 19. Linkage tests

Cross	Parent 2	Frequency of *ts*$^+$ allele[a]
1	THA31 SFU50 +	+ + SFU3	0.80
2	THA31 + SFU50	+ SFU1 +	0.28
3	THA31 SFU50 +	+ + SFU5	0.61
4	THA31 SFU50 +	+ + SFU9	0.71
5	THA31 SFU50 +	+ + SFU49	0.74

[a] Calculated as: $\frac{\text{number of } ts^+ \text{ progeny}}{\text{total progeny tested}}$

One hundred *sus*$^+$ recombinants were tested from cross 1 and more than 230 were tested for each of the other crosses

Additional mapping

The genetic map established by comparison of results from two and three-factor crosses and linkage tests should serve as a basis for the determination of the positions of the other complementation groups.

Results of additional crosses listed in Table 20 indicate that the lysis-deficient mutants, SFU60 and SNG15, lie close together and to the right of SFU3 on the map proposed in Figs. 13 and 14. The DNA-affected mutants, SNG1 and SNG5 were not found to be close to these markers. However, two-factor crosses at 30C with THA31 suggest that SNG5 may lie on the same side of the map as this temperature-sensitive mutant (Fig. 15).

Other two-factor crosses were carried out to determine the positions of mutants which are not affected in phage-induced DNA synthesis (Table 8) and host cell lysis. It may therefore be inferred that they are affected in various functions required for the normal morphological development of the phage. A composite genetic map based on previous results and these crosses is shown in Fig. 15. The positions proposed in this final scheme should be amenable to further anlaysis with the techniques used herein.

Further analysis by two-factor crosses revealed that two other mutants, SFU21 and SFU55 appear to lie outside of markers SFU4 and SFU3. Also, the mutant SFU18 has been found to lie beyond the region bounded by SFU1 and SFU3.

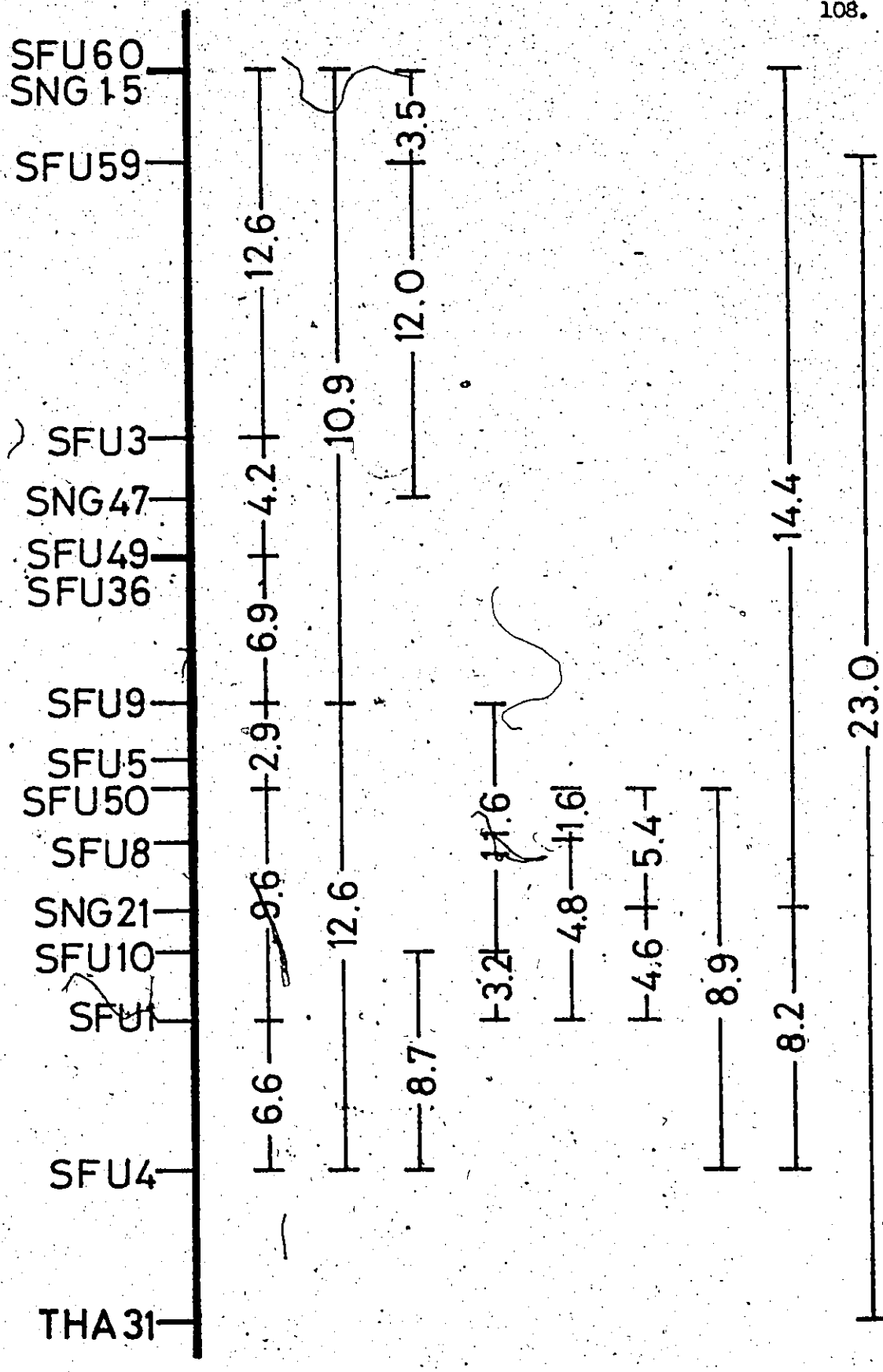


FIGURE 15. Composite genetic map of PBS2

TABLE 20. Mapping of lysis-defective mutants

Cross	Recombination frequency (%)
SFU60 x SFU1	12.8^{6}
SFU60 x SFU50	10.8^{5}
SFU60 x SFU9	10.9^{2}
SFU60 x SFU49	10.4^{2}
SFU60 x SFU3	12.6^{5}
SFU50SFU60 x SFU1	6.1^{3}
SFU50SFU60 x SFU3	4.7^{3}
SFU60 x SNG15	0.3^{2}
SFU60 x SNG1	8.4^{3}
SFU60 x SNG5	9.9^{4}
SFU50SFU60 x SNG1	8.7^{1}
SFU50SFU60 x SNG5	7.8^{1}
THA31 x SNG1[a]	9.8^{1}
THA31 x SNG5[a]	5.1^{1}

[a]Performed at 30C

Superscripts refer to the number of times crosses were performed.

PBS2 transformation

The technique of bacteriophage transformation, adapted by Herrington and Takahashi (130) to PBS2 should provide further means for arranging the genetic map of the phage and particularly for determining the relationship between recombination frequency and physical distance in the PBS2 chromosome.

Preliminary experiments were done to determine whether transformation of suppressor-sensitive mutants was possible. The suppressor-carrying host JB0130 was not competent for transformation, however, subsequent experiments with the nonsuppressing host, SB202 were successful. Transformation of SFU50 was found to be dependent on DNA concentration up to about 3 μg/ml (Fig. 16), Since SFU50 synthesizes little or no phage DNA it must be concluded that fragments of wild-type phage of DNA taken up during transformation are capable of complementing the affected function and/or recombining in the absence of DNA replication. The frequencies of transformants of a number of single and double mutants were determined and are shown in Table 21. The DNA concentration in these experiments was 0.5 μg/ml and MOI was approximately 1.0. As expected, at this limiting DNA concentration, double mutants were transformed at significantly lower frequencies than single mutants. Frequencies of transformation were much lower than those obtained for temperature-sensitive mutants (149) presumably because these experiments were performed in the restrictive host. Reliable quantitation for physical mapping will probably require a suppressor-carrying host which is competent for transformation and perhaps the use of mutants carrying an unselected marker.

FIGURE 16. Effect of DNA concentration on transformation of SFU50

Competent cells of SB202 were infected with SFU50 at an MOI of 1.0 and DNA was added at the time of infection. The yields of wild-type transformants were determined with nonsuppressing JB0128 as host.

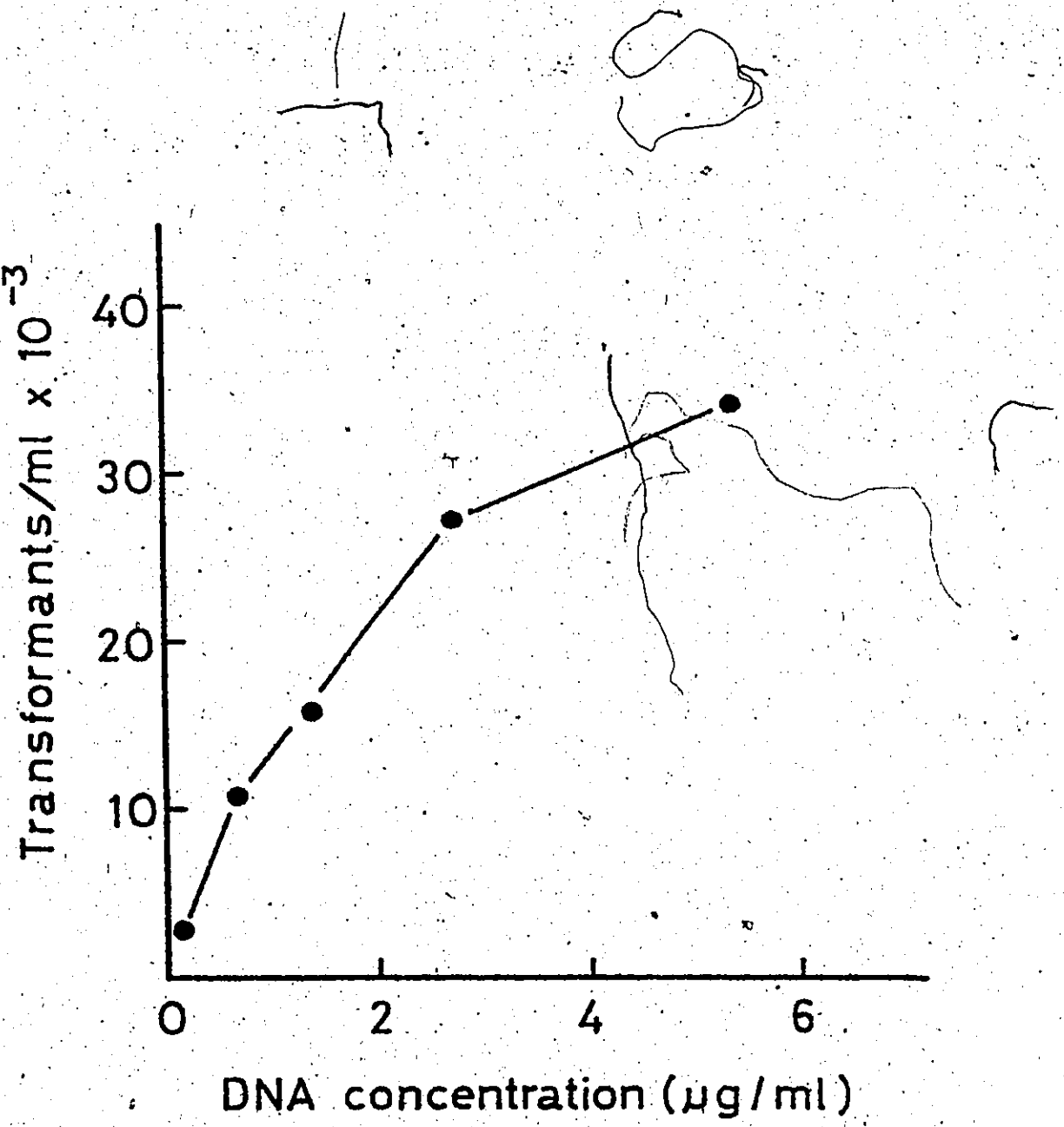

Transformants/ml x 10^{-3}
40
30
20
10
0
2
4
6
DNA concentration (µg/ml)

TABLE 21. Transformation of suppressor-sensitive mutants

Mutant	Wild-type transformants (PFU/ml)
SFU1	3.2×10^5
SFU3	6.0×10^4
SFU9	1.4×10^5
SFU50	2.7×10^4
SFU1SFU3	1.4×10^2
SFU1SFU50	2.6×10^2
SFU3SFU50	9.9×10^2
SFU3SFU9	1.8×10^2

No plaques were obtained when DNA was omitted.

DISCUSSION

Mutagenesis

The uridine analogue, FdUrd is known to affect DNA synthesis by inhibiting thymidylate synthetase (150). Bacteriophage PBS2, which contains uracil instead of thymine in its DNA was shown by Lozeron and Szybalski (30) to incorporate FdUrd into DNA. Not surprisingly, Herrington and Takahashi (121) found that ts mutants can be induced in PBS2 by this analogue, presumably in a similar fashion to those induced in other phage systems by the thymidine analogue BdUrd (151). In the present study, suppressor-sensitive mutants were induced at a frequency of 0.6% by FdUrd treatment.

The mutagen NG is an alkylating agent and a carcinogen, which is thought to induce mutations at the replication fork (152). In this study, suppressor-sensitive mutants were induced at a frequency of 1% by NG. These results are comparable to those of Herrington and Takahashi (121) for the isolation of temperature-sensitive mutants of PBS2.

In order for a gene to mutate to suppressor sensitivity, it must be essential for viability under the conditions of selection. Therefore nonessential phage functions, which may be numerous in a large bacteriophage such as PBS2, would not be observed in the mutant collection. The frequency of sus mutations in essential functions depends on the number of suppressible amino acid changes possible which can seriously affect the activity of a given protein. It may also be viewed as a reflection

of the number and type of mutable sites. Benzer (154) has shown that the frequencies of mutation within a cistron at various mutable sites may vary drastically with the mutagen used.

In the present study, 119 sus mutants of PBS2 isolated with NG and FdUrd were found to represent 39 complementation groups based on spot tests (Table 3). SFU36 was subsequently found to belong to group 10 (Table 14). As expected from the above considerations, the distribution of mutants among the groups was not uniform. Group 10 was the largest of the complementation groups, containing 28 mutants. Further data on the internal genetics of this region may prove helpful in assessing the significance of this result. The large number of mutants obtained in this group may reflect the size and/or mutability of the cistron it represents. This is of interest since mutants in group 10 do not induce the phage-related DNA polymerase activity (Table 11).

As a matter of perspective it is meaningful to consider the portion of the coding capacity of PBS2 represented by these 38 groups. The molecular weight of the PBS2 chromosome is in the range of $1.6\text{-}1.9 \times 10^8$. This is enough genetic material for about 200-300 cistrons coding for polypeptides of 300-500 amino acids each. Provided that no intracistronic complementation is found with the sus mutants, the 38 groups identified herein may represent approximately 10-20% of the coding capacity of the PBS2 genome.

Further analysis of these mutants should help to resolve the question of whether JB0130 carries a missense or a nonsense suppressor. If these phage mutants are missense, then intracistronic complementation

should be expected in at least some of the groups (111). Tevethia et al. (116) examined the apparently pleitropic effects of two suppressors in B. subtilis. These suppressors were sup-1 and sup-3, originally isolated by Okubo and Yanagida (114) and Georgopoulus (115) respectively. The properties of strains carrying these mutations suggested to Tevethia et al. (116) that both are nonsense suppressors. By analogy to the E. coli system, they tentatively suggested that sup-1 and sup-3 are ochre and amber suppressors respectively. The suppressor carrying strain used in this study, JB0130 was also obtained from Okubo. Its low sporulation frequency and growth rate compared with its parent strain JB0128, indicated that it may carry the sup1 mutation.

It has been observed in the course of this work that protein synthesis in PBS2-infected cells can be measured in the presence of rifamycin, which totally inhibits incorporation of ^{3}H-histidine into uninfected cells within 10 min (data not presented). Consequently, exclusive radioactive labelling of PBS2-specific proteins and their identification by chromotography should be possible. Application of this to mutant-infected JB0130 and JB0128 cultures should indicate whether polypeptide fragments, caused by premature chain termination, occur in the nonsuppressing host.

Host DNA and RNA synthesis

Since uracil completely replaces thymine in PBS2 DNA, any incorporation of ^{3}H-dThd into DNA in infected cultures should be due to host-specific DNA synthesis. Experiments shown in Fig. 1 were carried

out to determine the incorporation of ^{3}H-dThd into DNA in PBS2-infected and uninfected cells of JB0128. According to the data, PBS2 apparently arrested host DNA synthesis at 10-12 min after infection although incorporation at a greatly reduced rate continued throughout the course of the experiment. The residual incorporation in infected cells can be explained as DNA synthesis in uninfected and phage-carrying cells in the culture.

Contrary to the conclusion of Price and Frabotta (79), the results in Fig. 2 do not suggest that PBS2 infection results in the shut-off of net RNA synthesis. It is possible that net host RNA synthesis ceases and is replaced by net PBS2 RNA synthesis shortly after infection. This interpretation is unlikely in view of the results of the experiment illustrated in Fig. 3. In this experiment, infected cells of pyrG1dck4 were briefly labelled with ^{3}H-Cyd and then treated with rifamycin. This resulted in both RNA and DNA being labelled through the intracellular CTP pool and allowed equilibration of this pool with ^{3}H-Cyd in the medium. This equilibration was necessary to overcome the effect of rifamycin on incorporation of labelled nucleoside observed by Rima and Takahashi (80) in PBS1-infected cells. Figure 3 shows that rifamycin did not affect the incorporation of ^{3}H-Cyd into DNA but totally inhibited its net incorporation into RNA in PBS2-infected cells. Since PBS2 is resistant to rifamycin, it seems likely that the net RNA synthesized in infected cells is host specific.

PBS2-induced DNA synthesis

The rates of incorporation of ^{3}H-dCyd into DNA in PBS2-infected and uninfected JB0128 cells were determined in pulse labelling experiments (Fig. 5). Under the conditions used, phage DNA synthesis began at 10-15 min after infection and reached a maximum rate at the 20th min. In similar experiments, mutant-infected cells were pulse-labelled from the 20th min to the 25th min of infection, during which time the rate of DNA synthesis in uninfected cells is much less than in cells infected with wild-type PBS2. Ten of the mutants tested were found to be affected in DNA synthesis (Tables 8 and 9). Continuous labelling in the presence of rifamycin, which inhibits host DNA synthesis, showed that these mutants are affected in the ability to synthesize DNA throughout the course of infection (Fig. 6). Furthermore, each of the ten DNA-affected mutants was able to effect shut-off of host DNA synthesis. Earlier experiments showed that continuous labelling in mutant-infected cells in the absence of rifamycin does not reveal defects in DNA synthesis very clearly (Table 7). These results can be explained by assuming that uninfected and phage-carrying cells in infected cultures produce a high background incorporation which is host specific and which is decreased drastically in the presence of rifamycin.

The DNA-affected mutants appear to comprise three phenotypic classes (Fig. 6). Mutants SFU1 and SFU9 are both capable of considerable phage-induced DNA synthesis and can produce host cell lysis as well (Table 9). Apparent DNA synthesis in cells infected with SFU3 and SNG5 increases somewhat with time but is still much lower than that in cells

infected with wild-type phage. Mutants SFU5, SFU49, SFU50, SNG1, and SNG47 are apparently capable of little or no phage DNA synthesis. In the latter two cases, no host lysis occurs (Table 9).

PBS2-induced cell lysis

Another characteristic of PBS2-infection amenable to simple observation is host lysis, which allows the release of progeny phage into the medium. In the case of phage T4 for E. coli, at least two genes are known to be directly involved in the lytic process (135). In the same phage, visible lysis does not occur in the absence of phage DNA synthesis although phage-induced lysozyme activity may appear intracellularly (154). Phage SP82 for B. subtilis was shown to require at least one host-specific function for normal lysis to occur (135,158). In the present study, it was found that in cells of JB0128 infected with PBS2, the onset of lysis occurs at about 40 min after infection (Fig. 7). Among representative mutants tested, ten were found to be deficient in the ability to induce cell lysis (Table 9). Of these, eight were previously found to be affected in phage DNA synthesis. Two mutants, SFU60 and SNG15 were apparently normal in this respect but were unable to induce lysis. A subsequent experiment (Table 10) showed that low but significant yields of infective phage occurred in JB0128 cells infected with these latter two mutants in contrast to those infected with the non-lysing, DNA-affected mutant, SFU50. When cultures infected with these mutants were lysed artifically with lysozyme, titres of infective phage in the SFU60 and SNG15-infected cultures increased to levels comparable

to the wild-type while the titre in the SFU50-infected culture was only 0.01% of the wild-type control. This was taken as a clear indication that SFU60 and SNG 15 harbour mutations affecting the lytic process rather than some stages in PBS2 development. Presumably, the low levels of extracellular phage normally produced by these mutants in nonsuppressing cells are insufficient to form plaques in the standard assay.

An interesting preliminary observation was that plaques of SFU60 or SNG15 picked onto plates seeded with cells of JB0128 did not produce lysis when plates were incubated overnight at 30C but did when incubation was at 45C. The reason for this is unknown but it may involve activation of altered enzymes at the higher temperature or lysis by defective enzyme(s) due to a change in relative growth rates of phage and host.

DNA-affected mutants

A number of new functions have been shown to be induced in *B. subtilis* following infection with PBS1 and PBS2 (25,39,42,43,45,62,83). The DNA-affected sus mutants were examined for their capacity to carry out some of these functions in the nonsuppressing host. It was also considered, at the outset, that certain mutants might exhibit pleitropic effects.

All of the DNA-affected mutants were found to be able to kill infected cells (Table 11) despite the fact that seven of them could not produce cell lysis (Table 9). In the case of phage T4, it is known that the events which occur at the cell membrane during the infection

process can have internal metabolic ramifications in the absence of phage DNA injection (155). The fact that all the DNA-affected PBS2 mutants were found to carry out phage-specific functions (Table 11) indicates that they are all capable of adsorption to and injection of DNA into the host.

Tomita and Takahashi (42) first described a novel enzyme, dCTP deaminase, induced after PBS1 infection of B. subtilis. They suggested that this enzyme might provide a major pathway for dUTP synthesis as a precursor for phage DNA. Similar activities have since been observed in uninfected E. coli and S. typhimurium (132,165). If the enzyme is essential for dUTP synthesis in PBS1 and PBS2-infected cells, a phage mutant lacking the activity would be expected to be lethal. The results in Table 11 show that all the DNA-affected mutants induce dCTP deaminase after infection. Recently, Rima and Takahashi (156) observed that dCTP deaminase contributes only in part to dUTP synthesis in PBS1-infected cells. They also found that a novel host enzyme, dCDP deaminase and ribonucleotide reductase are also involved in dUTP synthesis. Thus, a mutant lacking dCTP deaminase may not be lethal unless the enzyme is otherwise involved in phage development. Experiments with phage T4 suggest that a phage induced enzyme dCMP hydroxymethylase performs a replicative function other than its catalytic one (157).

Tomita and Takahashi (25,45) discovered an endonucleolytic DNase in uninfected B. subtilis cells, which is specific for native uracil-containing DNA. This enzyme may effect PBS1 and PBS2 DNA in vivo since the same authors also found that the phage induces a heat stable

protein inhibitor of DNase. It seems likely that a phage mutant lacking this inhibitor would appear to be defective in DNA synthesis. However, all of the mutants tested (Table 11) were able to inhibit the DNase after infection. It is possible that this nuclease may be partly responsible for the persistance of uninfected cells in PBS1 and PBS2 infected cultures. It would therefore be useful to obtain host mutants affected in this DNase as well as phage mutants lacking the inhibitor in order to study the regulation of the carrier state. The maintenance of this state probably involves complex regulation which at present is poorly understood.

Phage T4 induces a new DNA polymerase specified by gene 43, which is essential for phage DNA replication (54,55). A number of other E. coli and B. subtilis phages have also been shown to induce new DNA polymerase activities. Price and Cook (62) observed a new DNA polymerase activity in PBS2-infected B. subtilis cells. This activity is distinct from DNA polymerase I of the host since it was also observed after infection of a polA mutant. Crude extracts of JB0128 cells infected with seven of the DNA-affected mutants were found to contain the phage-induced DNA polymerase activity (Table 11). However, SFU36, SFU49, and SFU50-infected cell extracts had low DNA polymerase activities, comparable to that in uninfected cell extracts. In a later experiment, two of these, SFU49 and SFU36 were found to belong to the same complementation group while SFU50 was able to complement both of them (Table 14).

One plausible explanation of these results may be that these apparent DNA polymerase mutants all belong to one cistron which codes for

a homomultimeric enzyme. If this were the case, at least some pairs of mutants would exhibit intracistronic complementation, provided they are not nonsense mutants. Another possibility is that these mutants are located in two different cistrons which either produce a heteromultimeric DNA polymerase or produce two proteins which, although physically separate, are both responsible for the observed activity. For each of these cases, in vitro complementation would be a possibility, but its absence alone would not rule out any of them. It can be seen in Fig. 10 that attempts to observe in vitro complementation of SFU49 and SFU50-infected cell extracts were unsuccessful. Moreover, the possibility that either of these mutants produces an inhibitor of the wild-type activity was excluded. There are at least two other simple interpretations of the results which have not been eliminated. The first of these is that either or both of SFU49 and SFU50 may belong to regulatory cistrons normally required for the synthesis of activation of a phage-specific DNA polymerase. The second is that the new DNA polymerase is host-specific and is merely activated or modified by phage functions altered in SFU49 and SFU50. However, since the phage is resistant to rifamycin, adequate levels of this enzyme would have to exist in cells at the time of infection.

The genetic data to be discussed provide strong support for a model which would have the mutations in SFU49 and SFU50 located in different cistrons. It should be noted that the phage DNA polymerases purified to date are composed of single polypeptide chains (56,59,60). However, DNA polymerase III of E. coli, which is thought to be the replicative enzyme, is apparently homomultimeric in its active form (159). In addition, the

active form requires a non-catalytic protein co-polymerase for activity (159). DNA polymerase III of B. subtilis, which is also essential for replication, is inhibited by 6-(p-hydroxyphenylazo)-uracil (66). Phages for B. subtilis which induce new DNA polymerase activities are resistant to this drug (70). However, the simple conclusion that replication of these phages does not require host DNA polymerase III is not warranted. Recently Lavi et al. (160) observed that the related phages SPO1 and Øe, which induce new DNA polymerase activities, are not capable of growth at the restrictive temperature in a host mutant with a temperature sensitive DNA polymerase III. Temperature shift experiments showed that SPO1 and Øe require active host DNA polymerase III throughout the replication stage. These authors suggested that the new DNA polymerase activity in SPO1 and Øe-infected cells is actually host DNA polymerase III which is modified by a phage-induced protein which confers resistance to 6-(p-hydroxyphenylazo)-uracil on the enzyme and at the same time effects the arrest of host DNA synthesis. It would be interesting to determine whether PBS2 also requires host DNA polymerase III in view of the fact that the phage contains uracil in place of thymine in its DNA, whereas SPO1 and Øe have hydroxymethyluracil, which is a thymine analogue in their DNA. However, the observation that SFU49 and SFU50 are capable of shutting-off host DNA synthesis would complicate the application of the model of Lavi et al. to these mutants.

It has recently been observed that PBS2-infected cells contain a new RNA polymerase resistant to rifamycin (83). A study of conditional lethal mutants for this enzyme should facilitate an explanation of why PBS2 infection is resistant to rifamycin even when cells are pre-treated

with the drug. If this enzyme is responsible for a major portion of phage specific RNA synthesis, a mutant for this enzyme should affect many different phage functions. Such a pleitropic effect has not been observed for any of the mutants tested herein.

Toluenized cell experiments

DNA synthesis in toluenized cells of E. coli and B. subtilis has many of the properties of normal semi-conservative DNA replication (139). Recently, replicative phage DNA synthesis has been observed in T4-infected toluenized E. coli cells (141,142,143). In the present study, it was found that PBS2 infection stimulates several-fold the synthesis of DNA in toluenized B. subtilis cells (Fig. 11). The PBS2-induced synthesis of DNA in toluenized infected cells was 75% dependent on added ATP. Incorporation into toluenized cells further reflected the in vivo situation in that the DNA-affected mutants were deficient in DNA synthesis but apparently shut-off host DNA synthesis.

It is known that toluenized B. subtilis cells are permeable to small proteins (161). Nevertheless, in vitro complementation with this system may be difficult due to a lack of endogenous DNA in cells infected with DNA-affected mutants. Complementation tests for identification and purification of proteins involved in replication require an in vitro replication system to which endogenous DNA can be added. One such system was devised by Barry (162) for the replication of phage T4 DNA.

It was observed that PBS2-infection also stimulated the incorporation of ^{3}H-dTTP into DNA in toluenized cells. This stimulation

was absent in SFU49 and SFU50-infected cells (Table 12). This suggests that the phage replicative apparatus is capable of synthesizing thymine-containing DNA. If this incorporation is into phage DNA, then PBS2 particles containing thymine in their DNA may be obtainable. This observation also suggests that the in vivo exclusion of thymine from PBS2 DNA is due to the phage induced dTTPase (43) and dTMPase (39) rather than some discrimination within the replicative apparatus. A mutant of phage Øe which has up to 20% of its normal pyrimidine, hydroxymethyluracil replaced by thymine has been isolated (68).

Genetics

Quantitative complementation tests showed that the ten mutants affected in DNA synthesis are actually in nine different complementation groups, with SFU36 being reassigned to group 10 (Table 14). This test is a powerful indicator of complementation between sus mutants of PBS2 to a degree unattainable with ts mutants of Herrington (122). This is attributable to the fact that the burst size of the wild-type in the nonsuppressing host is generally 30-50 at 37C, whereas Herrington (122) found that the burst size of the wild-type at the restrictive temperature ranged from 2.5 to 32 with an average of only 9.2. This latter condition created difficulties since burst sizes of ts mutants were sometimes as high as 1.8 and as a result, a somewhat arbitrary criterion of complementation was chosen. This problem was not encountered in this study since phage yields in single sus mutant infections were 1% or less of wild-type yields while complementing pairs produced yields 20% or

more of the wild-type in the nonsuppressing host.

In order to determine whether the DNA-affected mutants are clustered together on the PBS2 chromosome , two-factor genetic crosses were carried out (Table 15). The mutants were then arranged on a linear map which best accounts for the recombination data (Fig. 13). Two mutants, SNG1 and SNG5/did not appear to be closely linked to each other or to the other markers. Saturation or loss of additivity apparently became significant at recombination frequencies greater than 8%.

This tentative map was further examined by three-factor crosses using a variety of sus double mutants (Table 17). Deviations from calculated double recombination frequencies were indicative of low negative interference such as has been observed with phages T4 and lambda (147,148). The results support the basic order in Fig. 13 in which SFU1 and SFU3 are terminal markers with SFU49 lying between SFU50 and SFU3. The important question to be asked in these experiments is whether or not any complementation groups lie between SFU49 and SFU50, which are unable to induce the phage-related DNA polymerase activity.

The best method of ordering SFU5, SFU50, SFU9, and SFU49, the internal markers, was the use of linkage tests similar to those devised by Streisinger and Bruce (93). A ts mutant, THA31, isolated by Herrington (122) was used as an unselected marker in these experiments. The position of THA31 relative to some of the markers was first determined by two-factor crosses (Fig. 14). The double mutant SFU50THA31 was isolated and crossed with the appropriate mutants. The results in Table 19 are compatible with the suggested position of THA31 and indicate

that SFU5, SFU9, and SFU49 all lie to the right of SFU50. If this is the case, the best fit for all the foregoing genetic data requires that SFU5 and SFU9 lie between SFU50 and SFU49. Since these mutants represent four different complementation groups (Table 17), then the SFU50 and SFU49 mutations must lie in different cistrons, both of which affect the appearance of the phage-induced DNA polymerase activity. If either of these cistrons serves a regulatory role, it may specifically involve the DNA polymerase since mutations in them do not affect other phage-related functions (Table 11).

Extension of the genetic map should facilitate determination of linearity or circularity of the PBS2 genome. One feature of the present map is that mutants affected in post-replicative functions lie on either side of mutants affected in DNA replication. If PBS2 regulates RNA transcription in a sequential manner as observed in other phage systems (137,163), then late RNA transcription would proceed in two directions along the chromosome.

Further recombination studies should help reveal the extent to which PBS2 genetics can be explained by the model of Visconti and Delbruck (92). This model, which is frequently applied to phage systems, was devised to explain the genetics of phage T4. Recombination in other phage systems may differ from that in T4 as in the case for phage lambda (106). Moreover, the size of intracellular mating pools as well as the number of mating events possible for each chromosome may vary for different phages.

The difficulty in mapping SNG1 and SNG5 may mean that they are

actually distant from known markers. However, mutations in some genes involved in DNA synthesis in other systems have been shown to affect recombination frequencies (164) and this possibility should not be overlooked with mutants of PBS2.

The fact that transformation of DNA-affected mutants was observed in the nonsuppressing host (Table 21) suggests that either recombination can occur in the absence of replication or that transforming DNA fragments are capable of functioning prior to recombination. Resolution of this question may help to explain the absence of transfection by PBS2. Transformation of mutants in a suppressor-carrying host which is competent for transformation should serve to test the genetic map. If JB0130 carries the sup-1 mutation, then according to the findings of Tevethia et al. (126), a sup-3 host might be useful for such experiments. These authors found that supl-carrying strains are not competent for transformation whereas sup-3 apparently has no affect on competence development. Mapping by transformation has been used with success for phage Ø105 for B. subtilis and could also help in establishing a relationship between genetic and physical distances on the PBS2 chromosome.

SUMMARY

PBS2 infection results in the shut-off of host DNA synthesis 12 to 15 min after infection of the nonsuppressing host, JB0128. Some residual host-specific DNA synthesis occurs in infected cultures, which is attributable to uninfected and phage carrying cells. Phage-induced DNA synthesis begins at about 10 min and reaches a maximum 20 min after infection of JB0128. Incorporation of ^{3}H-dCyd into DNA in PBS2-infected, rifamycin-treated cells continues whereas it is inhibited in uninfected cells treated with the drug.

Contrary to the observation of Price and Frabotta (79), net RNA does not cease in PBS2-infected cells. The net RNA synthesis in infected cells is probably host-specific since it is completely inhibited by rifamycin. If has not been determined whether host mRNA synthesis continues in PBS2-infected cells.

One hundred and nineteen sus mutants of PBS2 have been isolated and assigned to thirty-eight complementation groups. Mutants in nine of the groups are affected in the ability to synthesize phage DNA. Seven of these DNA-affected mutants are also unable to induce lysis in the restrictive host. The DNA-affected mutants SFU1 and SFU9 which are capable of synthesizing some phage DNA are normal with respect to lysis induction. Mutants of SFU60 and SNG15 are unable to lyse the restrictive host although normal levels of infective phage are produced intracellularly.

All of the DNA-affected mutants are capable of cell killing, shutting off host DNA synthesis and induction of dCTP deaminase and the DNase inhibitor, after infection of the restrictive host. Mutants SFU49 and SFU50, which have been shown quantitatively to belong to two distinct complementation groups, are both unable to induce the phage-related DNA polymerase activity in the restrictive host. Crude extracts from cells infected with these mutants do not complement one another in vitro, nor do they inhibit the wild type phage DNA polymerase activity. Genetic data indicates that SFU49 and SFU50 lie in two different cistrons, i.e., affect two different proteins, since at least two other complementation groups lie between them on the phage genome.

The genetic data are consistent with a linear arrangement of the mutants tested although no conclusion may be drawn regarding the configuration of the complete genome. The DNA-affected mutants are not all clustered together on the genetic map. A number of mutants tested are not closely linked to the present genetic map or each other, indicating that a considerable portion of the phage genome is still unrepresented. Suppressor-sensitive mutants are superior to ts mutants for genetic experiments with PBS2 since they provide much more reliable complementation data. Preliminary experiments indicate that transformation of sus mutants is possible. This should facilitate any effort of relating genetic information to the physical structure of the genome.

Phage-induced DNA synthesis requiring ATP occurs in PBS2-infected toluenized cells. Host DNA synthesis is apparently shut-off in infected, toluenized cells. Mutants affected in phage DNA synthesis in vivo are

similarly deficient in toluenized, infected cells of the restrictive host. The phage replication apparatus is capable of utilizing dTTP as a precursor for DNA synthesis indicating that elimination of thymine from PBS2 DNA *in vivo* may depend largely on the phage induced dTTPase (42) and dTMPase (39).

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