INFORMATION TO USERS

This material was produced from a microfilm copy of the original document. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the original submitted.

The following explanation of techniques is provided to help you understand markings or patterns which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting thru an image and duplicating adjacent pages to insure you complete continuity.

2. When an image on the film is obliterated with a large round black mark, it is an indication that the photographer suspected that the copy may have moved during exposure and thus cause a blurred image. You will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., was part of the material being photographed the photographer followed a definite method in "sectioning" the material. It is customary to begin photoing at the upper left hand corner of a large sheet and to continue photoing from left to right in equal sections with a small overlap. If necessary, sectioning is continued again — beginning below the first row and continuing on until complete.

4. The majority of users indicate that the textual content is of greatest value, however, a somewhat higher quality reproduction could be made from "photographs" if essential to the understanding of the dissertation. Silver prints of "photographs" may be ordered at additional charge by writing the Order Department, giving the catalog number, title, author and specific pages you wish reproduced.

5. PLEASE NOTE: Some pages may have indistinct print. Filmed as received.

University Microfilms International
300 North Zeeb Road
Ann Arbor, Michigan 48106 USA
St. John's Road, Tyler's Green
High Wycombe, Bucks, England HP10 8HR

RICE UNIVERSITY

INITIATION OF DNA REPLICATION IN SP01-INFECTED *BACILLUS SUBTILIS*

by

JEFFREY GLASSBERG

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

THESIS DIRECTOR'S SIGNATURE:

Charles Stewart

HOUSTON, TEXAS

DECEMBER, 1976

ACKNOWLEDGMENTS

I would like to take this opportunity to thank some of the people who directly contributed to this thesis.

Without the enthusiastic and able technical assistance of Michelle Franck, this work probably would still be six months from completion.

Encouraging and stimulating conversations with Jim Cregg and Ray Slomiany, contributed to the feeling of excitement when the work was going well and lessened the discouragement when it was not.

My debt to Charles Stewart is large. Not only did he provide the conceptual framework in which these experiments were designed and interpreted, he also provided an environment in which my intellect, creativity, and perhaps most importantly, my soul, could flourish.

I also would like to express my gratitude towards the original *Satyrium liparops* (as exotic now as then) who first piqued my interest in arcane endeavors.

Lastly, I would like to thank J.V. Scott for personal communications very much prior to publication.

TABLE OF CONTENTS

GENERAL INTRODUCTION

In 1953, Watson and Crick (1953a) published a model for the structure of a DNA molecule, in which was implicit a conceptually simple mode of reproduction. Although there were some early suggestions that DNA might replicate by some type of self assembly process (Watson and Crick, 1953b), it soon became apparent that special enzymes were necessary for DNA replication (Kornberg *et al.*, 1956).

In a number of bacterial and viral systems, conditional-lethal mutants have been isolated in which DNA synthesis is affected (Edgar and Lielausis, 1964; Kohiyama *et al.*, 1966; Mendelson and Gross, 1967). The use of such mutants, in conjunction with biochemical analyses has enabled the elucidation of the roles of several proteins in the replication process, especially of proteins involved in the polymerization process *per se* (see Kornberg, 1974, for review).

Less is known about the roles of proteins needed for the initiation of DNA replication. Mutants which are probably affected in such proteins have been isolated and identified in a relatively small number of systems, i.e., *Escherichia coli* (Hirota *et al.*, 1970; Carl, 1970; Wechsler and Gross, 1971; Beyersmann *et al.*, 1974; Wada and Yura, 1974), *Bacillus subtilis* (Karamata and Gross, 1970; White and Sueoka, 1973; Laurent and Vannier, 1973; Riva *et al.*, 1975), the single-stranded coli-phages (Francke and Ray, 1971; Francke and Ray, 1972; Lin and Pratt, 1972; Henry and Knippers, 1974), coli-phage P2 (Geisselsoder, 1976), Simian Virus 40 (Tegtmeyer, 1972), and adenovirus type 5 (Van der Vliet and Sussenbach, 1975).

The research which comprises this thesis was undertaken with the coal of isolating and identifying temperature-sensitive mutants of bacteriophage SP01, which are defective specifically in the initiation of SP01 DNA replication.

SP01 is attractive as an experimental system for the study of DNA replication because (1) its DNA is easily separable from the host DNA due to buoyant-density differences, (2) marker rescue experiments in the *B. subtilis* system are very sensitive and easy to perform, and (3) SP01 lacks a well-developed superinfection exclusion system.

Before proceeding to the Results, I would like to briefly review what is known about (1) bacteriophage SP01, and (2) about the initiation of DNA replication. Hopefully, this will suggest possible functions for the SP01 proteins affected in the temperature-sensitive initiation mutants.

Bacteriophage SP01

SP01 belongs to a group of large *B. subtilis*-phages whose DNA contains hydroxymethyluracil in place of thymine (Kallen *et al.*, 1962). For a recent review of SP01 and other *B. subtilis* phages, see Hemphill and Whiteley (1975).

A genetic study of SP01 was begun by Okubo *et al.* (1972). They classified 120 SP01 suppressor-sensitive mutants into 36 cistrons on the basis of complementation tests. Recombination analysis showed that 34 of these cistrons could be arranged in a linear genetic map, although the additivity of recombination distances was not good. Two cistrons, 35 and 36, could not be placed on the genetic map because

mutations in these cistrons gave high recombination values with all other mutations, and with each other. As with other bacteriophage, e.g., T4 (Matthews, 1971) and T7 (Studier, 1972), the genes needed for DNA replication were found to be clustered--in this case on the arbitrarily designated right-half end of the SP01 genetic map.

Nine SP01 cistrons, 21-23 and 27-32, were found to be necessary for viral DNA replication (Okubo *et al.*, 1972). Suppressor-sensitive mutants in cistron 29 are deficient in hydroxymethyltransferase activity (Okubo *et al.*, 1972) and extracts of some cistron 29 temperature-sensitive mutants exhibit temperature-sensitive hydroxymethyltransferase activity *in vitro* (James Cregg, personal communication). Cistron 28 codes for a transcriptional control protein that modifies the host RNA polymerase, allowing new SP01 cistrons to be transcribed (Duffy *et al.*, 1975; Fox *et al.*, 1976). SP01 is also known to code for a new DNA polymerase of 122,000 molecular weight (Yehle and Ganesan, 1973) but the genetic locus of this protein is, as yet, unknown.

SP01 DNA synthesis begins about 10 minutes post-infection (at 37 C). Immediately thereafter, the DNA is found complexed with RNA and protein in a form, termed VF, that sediments 4-6 times as fast as mature SP01 DNA (Levner and Cozzarelli, 1972). At about 20 minutes post-infection, the VF DNA is converted to "F" DNA which sediments about 3 times as rapidly as mature DNA. The VF DNA can be converted to F DNA *in vitro* by pronase treatment. This latter result suggests that the VF DNA may have the same number of phage equivalents as the

F DNA but because of an association with other cellular structures be in a more compact form. The conversion of VF to F DNA *in vivo* depends upon phage protein synthesis since the addition of chloramphenicol or infection with a cistron 34 mutant (deficient in late protein synthesis, Fujita *et al.*, 1971), blocks the conversion (Levner, 1972).

Initiation of DNA Replication

A. Membrane attachment.

Jacob *et al.* (1963) first postulated that DNA might be attached to the cell membrane as a means of properly segregating the two daughter molecules formed as a result of DNA replication. The association of bacterial and viral DNAs with the cell membrane has since been reported in many systems and appears to be a general phenomenon (see Klein and Bonhoeffer, 1972, and Siegel and Schaecter, 1973, for reviews). Recently, Portalier and Worcel (1976) showed that UV treatment of *E. coli* could link a membrane protein of about 80,000 daltons to *E. coli* DNA. This protein could be the same as an 80,000 dalton *E. coli* protein the synthesis of which correlated with the initiation of a round of replication (Gudas *et al.*, 1976).

The fact that the replication of DNA appears to be associated with the cell membrane implies that this association is either essential, or confers a selective advantage on the system in which it is employed. One possibility is that membrane attachment is necessary for the initiation of replication *in vivo*. Consistent with this idea are the reports that in *E. coli* (Parker and Glaser, 1974), *B. subtilis*

(O'Sullivan and Sueoka, 1972), coli-phage T4 (Marsh *et al.*, 1971) and coli-phage T7 (Helland and Nygaard, 1975) the DNA near an origin of replication is preferentially associated with the cell membrane. Other observations consistent with the membrane playing a role in initiation are that (1) some dnaA mutants of *E. coli* (defective in initiation) have altered compositions of membrane proteins (Shapiro *et al.*, 1970; Siccardi *et al.*, 1971), (2) an inhibitor of unsaturated fatty acid synthesis blocks replication after two doubling times and increases the temperature-sensitivity of temperature-sensitive mutants defective in initiation (Fralick and Lark, 1973) and (3) cold-sensitive suppressors of heat-sensitive dnaA mutations are defective in nuclear organization, chromosome segregation or septation (Wechsler and Zdziencka, 1975) suggesting an interaction between dnaA protein and cell membrane or wall.

Membrane attachment is certainly *not* essential in all systems, at least *in vitro*, since both the conversion of ØX174 single-strand DNA to replicative form DNA (Schekman *et al.*, 1975) and replicative form replication (Eisenberg *et al.*, 1976) both have been reconstituted *in vitro* using purified proteins with no membrane fraction. However, the *in vitro* environment is obviously different from that *in vivo*. Membrane attachment may prove to be essential in the latter.

B. Nicking

In order to replicate double-stranded DNA, the DNA double helix must be unwound. A nick in one of the strands may be required

to permit this unwinding to occur. If the synthesis of one of the DNA strands is initiated by extending an existing 3' end, then a nick is needed to generate the free 3' hydroxyl group. The above considerations lead to the possibility that at least some initiation proteins will have DNA nicking activity. Such proteins have been found, the best characterized example being the ØX174 gene A protein. This protein which is required for ØX174 replicative form DNA replication, nicks the ØX174 viral strand at a specific site within gene A and replication initiates from this region (Francke and Ray, 1972; Henry and Knippers, 1974; Baas *et al.*, 1976). Geisselsoder (1976) has shown that the A gene of bacteriophage P2 is required for the *in vivo* production of a strand-specific nick, while the lambda replication genes O and P are thought to have endonuclease activity (Shuster and Weissbach, 1969; Freifelder and Kirschner, 1971). To date, a nicking activity is the only known activity which can be ascribed to any protein known to function specifically in initiation.

C. RNA priming

Since no known DNA polymerase can initiate a *de novo* DNA chain *in vitro*, the question arose as to how such chains were initiated *in vivo*. In 1971 Brutlag *et al.* showed that coli-phage M13 single-strand to replicative form DNA replication, both *in vivo* and *in vitro*, was sensitive to the RNA polymerase inhibitor rifampicin. Subsequent work demonstrated that an RNA transcript could serve as a primer for DNA synthesis (see Kornberg, 1976, for review). Almost all experiments demonstrating an RNA primer for DNA synthesis involve

either very small DNA molecules, i.e., small viruses or plasmids, or nascent DNA fragments (Okazaki fragments). Messer *et al.* (1975) reported that they could isolate a DNA-RNA co-polymer from *E. coli*. They termed the RNA moiety of this co-polymer origin-RNA (o-RNA) and showed that the synthesis of o-RNA was dependent upon functional *E. coli* dnaA gene product but not upon dnaC product. Consistent with the idea that dnaA mutants are deficient in a step prior to RNA priming while dnaC mutants are deficient in a later initiation step, are the results of Hiraga and Saitoh (1974). They found that after shifting temperature-sensitive dnaA mutants from restrictive to permissive temperature that replication was still sensitive to rifampicin while under the same conditions temperature-sensitive dnaC mutants exhibited replication that was rifampicin insensitive.

Hopefully, the mutants whose isolation and characterization are described in this thesis, will allow one to determine which of the SP01-coded proteins function in some of these initiation steps and how these functions are regulated.

MATERIALS AND METHODS

Abbreviations

cs, cold-sensitive; DO, replication-deficient (DNA-negative); HA, hydroxylamine; hs, heat-sensitive; MOI, multiplicity of infection; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; PFU, plaque-forming unit; p.i., post-infection; sup, suppressor; sus, suppressor-sensitive; ts, temperature-sensitive.

Bacterial strains

Bacillus subtilis CB-10 has been described by Nester and Lederberg (1961) as SB-1. CB-312, 313, and 314 were kindly provided by M. Mandel and were described by Tevethia *et al.* (1974) as strains 129, 151 and 135, respectively. CB-10 is sup^-, his^-, trp^-; CB-312 is sup^-, his^-, met^-; CB-313 is sup 3^+, and CB-314 is sup 1^+, his^-, met^-. CB-312, 313 and 314 are otherwise isogenic. CB-312 was our standard host strain while CB-313 was used to grow most of the sus phage mutants. Some of the sus mutants grew only on CB-314 (see Appendix). CB-327 was created by transforming CB-312 to prototrophy. CB-327 grows at 20 C while the prototrophic CB-10 does not.

Phage strains

All sus mutants were generously provided by E.P. Geiduschek and have been previously described (Okubo *et al.*, 1972). For frequently used mutants, a nomenclature has been adopted in which the strain designation is initiated by a prefix indicating phenotype (sus, hs or cs) followed by a number indicating the cistron affected, followed

by the number assigned to that particular mutant. For example, hs 30-2 is a heat-sensitive, cistron 30 mutant designated #2. Table I shows the correspondence between this nomenclature and the original designations of the mutant.

Media used

Bacterial and phage growth was generally in VY. Platings used TBAB bottom and top agar. These media, the deuterated medium, and Spizizen's minimal medium, have been described (Stewart *et al.*, 1971; Stewart 1969; Spizizen, 1958). Deuterated algal extract and deuterated sugars were gifts of H. Crespi.

Spot-test complementation

The procedure was as described by Okubo *et al.* (1972) except that plates were incubated at restrictive temperature, i.e., 43 C for hs or 20 C for cs mutants. The spot-test proved to be unsatisfactory for the majority of cs mutants, giving very ambiguous results. Most of the cs mutants were therefore assigned to cistrons on the basis of complementations in liquid. Since the results of these complementations were also often ambiguous, only about half of the cs mutants have been assigned to known cistrons.

Complementation in liquid of hs mutants

A 0.5 ml aliquot of CB-312, growing at 43 C and at a klett of 70, was added to a tube already containing 0.1 ml of each of the two mutant lysates to be tested (MOI about 5:1 for each phage). Incubation was continued at 43 C and at 10 min p.i., 0.1 ml of a 100-fold

TABLE I. Phage Mutants Used

Designation in this work	Original designation	Reference
sus1-1	F12	Okubo *et al.*, (1972)
sus2-1	F47	"
sus3-1	F37	"
sus11-1	F6	"
sus13-1	O75	"
sus13-2	HA7	"
sus14-1	F11	"
sus21-1	N6	"
sus21-2	F2	"
sus22-1	F30	"
sus23-1	N5	"
sus23-2	F20	"
sus27-1	HA20	"
sus28-1	F28	"
sus29-1	F13	"
sus30-1	F26	"
sus30-2	O52	"
sus31-1	F1	"
sus32-1	F38	"
sus33-1	F14	"
sus34-1	F4	"
sus35-1	F24	"
sus36-1	N34	"
hs22-1	G132	This laboratory
hs22-2	G256	"
hs23-1	G112	"
hs23-2	G249	"
hs29-1	G149	"
hs29-2	G187	"
hs29-3	G298	"
hs30-1	G133	"
hs30-2	G253	"
hs31-1	G168	"
hs31-2	G259	"
hs32-1	G143	"
hs32-2	G210	"
cs21-1	IIID	"
cs22-1	W237	"
cs28-1	W271	"

dilution of anti-SP01 antiserum was added. At 16 min p.i., the culture was diluted 10,000-fold into fresh VY, prewarmed to 43 C. At 120 min p.i., lysates were plated on CB-312 and the plates incubated at 20 C.

Assay for DNA synthesis

Unless stated otherwise, 0.5 ml of the culture to be assayed was added to a tube containing 0.1 ml, μCi/ml [6]-^{3}H-uridine and incubated with shaking at the indicated temperature for the indicated length of time. The incorporation of ^{3}H-uridine was stopped by placing the assay tube on ice and adding 0.6 ml of 2N KOH. The treatment of samples after KOH addition was essentially as described previously (Stewart *et al.*, 1972). In brief, RNA was digested by incubation for about 18 hr at 37 C, HCl was added, and the samples were then precipitated with tri-chloro acetic acid and filtered through 0.45 μ millipore filters. Filters were dried and counted in a Packard Tri-carb Scintillation Counter, using toluene-based scintillation fluid.

Mutagenesis

Hydroxylamine mutagenesis was basically as described by Tessman (1968). An SP01 wt lysate in 6 ml of 10^{-3} M EDTA was incubated at 37 C for 24 hr with 4 ml of 1 M hydroxylamine. MNNG mutagenesis was performed as described by Kahan (1966). Cultures of *B. subtilis* CB-312 were combined with 1 μg MNNG per ml immediately before infection with phage.

Preparation of ^{3}H-labelled phage lysates

CB-312, growing at either 30 (for hs mutants) or 37 C (for cs mutants) was infected with the appropriate mutant phage at a MOI of about 10:1. Shortly thereafter, [6]-^{3}H-uridine was added to about 10 μCi/ml. After lysis, the culture was centrifuged at 3000 x g for 10 min. The supernatant fraction was discarded and the pellet resuspended overnight in one-fifth volume of deuterated medium.

Preparation of deuterated B. subtilis

B. subtilis CB-327 was grown for about 16 hr at 37 C with aeration in deuterated medium. Aliquots of this culture were frozen in liquid nitrogen and were used to inoculate fresh deuterated medium.

Density transfer--temperature shift experiments

For hs mutants, deuterated CB-327 was grown at 37 C to a cell density of about 1.2 x 10^{8} cells/ml in deuterated medium. The culture was shifted to 43 C for 10 min and then infected at a multiplicity of infection of about 3:1 with the appropriate hs mutant (light density, ^{3}H-labelled, and resuspended in deuterated medium). At 25 min p.i., 5 ml aliquots of the infected culture were transferred to 250 ml flasks, precooled to 20 C. The above protocol served to partially synchronize the initiation of DNA replication. At various times after the shift to 20 C, DNA replication was stopped either by pouring the culture into flasks already at 43 C (restrictive temperature) and incubating for 15 min before pouring onto the Spizizen's minimal medium ice.

For cs mutants, the procedure was basically the same except that 20 C was restrictive and 37 C permissive. 150 min after infection at 20 C, 5 ml aliquots were shifted to 37 C and then stopped at various times as before.

The DNA extraction procedure was essentially that of Okun (1969) as modified by Stewart *et al.* (1972). Cells were washed three times, frozen and thawed, and then lysed with egg white lysozyme. After successive treatment with RNase and pronase, samples were made 1% with respect to Sarkosyl and incubated at 37 C for 30 min. After overnight incubation with sodium perchlorate, they were dialyzed against two changes of SSC (0.15 M NaCl, 0.15 M sodium citrate).

Shearing of DNA

DNA was sheared by passing three times through a 30 gauge hypodermic needle (Harris-Warrick *et al.*, 1976).

Equilibrium CsCl centrifugation

The procedure for equilibrium CsCl centrifugation was as described by Stewart (1969) with the following modificatins. Centrifugation was in a Beckman L2-65B, using a type 65 rotor at 32,000 rpm for about 66 hr. Each polyallomer tube contained 4.1 ml sample plus water, plus 5.55 g CsCl, yielding a density of about 1.731 g/cm^3. Ten-drop fractions (about 0.11 ml) were collected from the bottom. Fractions were assayed for radioactivity by placing 10 µl aliquots on Whatman glass fiber filters, drying, adding toluene-based scintillation fluid, and counting in a Packard Tri-

carb Scintillation Counter.

Marker rescue

The concentration, in each gradient fraction, of DNA carrying specific genetic markers was determined by a marker rescue assay. This was performed in a manner similar to that described by Green (1966). Each pair of adjacent fractions from the CsCl gradient was pooled. 0.45 ml of competent cells was added to a bovine serum albumin coated (to prevent the DNA from sticking to the glass) culture tube already containing 10 µl of one of the pooled fractions. The culture tube was incubated at 37 C with shaking for 4.5 min and then superinfected with 0.1 ml of an SP01 mutant carrying a sus mutation in the cistron of interest (giving a multiplicity of infection of roughly 5:1). Incubation was continued for another 10 min and the culture was then plated on a CB-10 (sup^-) lawn (three plates per culture tube using 0.2, 0.2 and 0.1 ml of the sample, respectively). Plates were incubated at 30 C for about 20 hr and plaque-forming units were counted. CB-10 was used as the bacterial lawn because, unlike CB-312, it was insensitive to the concentrations of CsCl used. With the DNA concentrations used, there was no measureable transfecting activity, so plaque formation depended upon recombination between the sus^- genome and DNA carrying its sus^+ allele.

Calculation of percent replication

For each CsCl gradient it was determined, by inspection of the ^{3}H-cpm profile, which fractions constituted the light, hybrid and heavy regions of the gradient. For any given marker, the activity

in each region was determined as follows: (1) the numbers of PFUs on the three plates for each fraction were summed, (2) from this sum was subtracted the total number of PFUs on the comparable three reversion control plates, and (3) the values from step 2, for all fractions in a given region, e.g., the light region, were summed. Percent replication was then calculated as described by Copeland (1975), using the formula $\frac{HL/2}{LL + HL/2} \times 100\%$, where HL represents the total activity for a given marker in the hybrid region and LL represents the total activity in the light region. To compute the percent of twice replicated DNA, the formula $\frac{HH/2}{LL + HL/2} \times 100\%$ was used, where HH represents the total activity in the heavy region of the gradient. This formula is valid only if no DNA has been trice-replicated.

Chemicals

[6]-^{3}H-uridine was purchased from New England Nuclear (Boston). RNase, pronase, and lysozyme were purchased from Calbiochem (San Diego).

SECTION I: A Screen for the Isolation of Heat- and Cold-sensitive, Replication-deficient Mutants of SP01

Introduction

When I began this work, the existing SP01 mutant collection consisted of a large set of sus mutants (Okubo *et al.*, 1972). Since my intended experiments on initiation required ts mutants, my first step was to isolate such mutants. This section describes: (1) an elaboration and modification of a screening procedure first used by Gurdon (cited in Edgar and Lielausis, 1964) and later by Talavera *et al.* (1971), which permitted the ready isolation of ts mutants in at least eight of the nine known SP01 DO cistrons.

Results

General Screening Procedures

Initially, I looked for tsDO strains by the traditional replica streaking method. An SP01 lysate was mutagenized with hydroxylamine. After further segregation growth in CB-312 at 20 C, the lysate was plated on CB-312 at 20 C. Plaques were picked and streaked onto two plates in replica pattern. One plate was incubated at 20 C, the other at 43 C. Thirty-one of 1620 strains showed temperature-sensitive growth. Of these 31 strains, only three proved to be replication-deficient--two affected in cistron 30 and one in cistron 31. Based on the above results, it was calculated that approximately 700,000 plaques would have to be tested in order to have a 95% probability of finding at least one ts mutation in each of the 9 known DO cistrons.

I next tried looking for ts mutants among revertants of sus DO mutants. Although the search was far from exhaustive, the fact that I found no ts mutants among more than 600 revertants of 20 different sus mutations was discouraging.

Finally, in conjunction with Raymond Slomiany, I developed the following screening procedure. Its use depends upon the fact that the time of lysis of SP01-infected *B. subtilis* is generally dependent upon the extent of SP01 DNA replication. (Table II shows that, for a series of replication intermediate mutants, the rate of lysis varies with the rate of DNA synthesis. With DO mutants, lysis occurs either very slowly, or not at all.)

For the isolation of hsDO mutants, a culture of *B. subtilis* CB-312, growing exponentially at 43 C, was infected with mutagenized SP01 at a multiplicity of about 1. Eight minutes later, a 0.8 ml aliquot of the culture was added to 0.2 ml of anti-SP01 antiserum, pre-warmed to 43 C. Incubation was continued until 60 min post-infection (p.i.). Since the normal time of lysis at 43 C is about 35 min p.i., cells infected with phage that replicated their DNA at 43 C should have lysed before 60 min p.i., and any liberated phage particles should have been inactivated by the anti-SP01 antiserum. In contrast, cells infected with replication-deficient phage should have remained intact.

The culture was now diluted 100-fold, to dilute the antiserum, and 0.1 ml aliquots were plated on a *B. subtilis* lawn. These plates were incubated at 20 C. A phage which did not replicate its DNA at 43 C, but that was able to replicate at 20 C, could now produce a

TABLE II. Time of lysis *vs.* extent of DNA replication in SP01-infected *B. subtilis* at 20°C[a]

Phage	Time post-infection at which a cell density of 2.4×10^8 cells/ml was reached (min.)	TCA-precipitable cpm incorporated ($\times 10^{-2}$)
wt	298	581
susll-1	290	547
susl4-1	422	219
susl2-1	480	110
sus2-1	498	36

[a]CB-312 was grown to a cell density of about 3×10^8 cells/ml at 37°C and then shifted to 20°C for one hour. The culture was distributed in 5 ml aliquots to flasks already containing various phage lysates (M.O.I. of about two). At 1.5 hours post-infection (p.i.), a one ml aliquot from each flask was removed to a tube container 0.05 ml of 4 µc/ml 6 ^{3}H uridine. The assay tubes were incubated at 20°C until four hours p.i., at which time the incorporation was stopped by the addition of 0.5 ml of 2N KOH. Samples were then treated and counted as described in Materials and Methods. Lysis was followed turbidimetrically. The table presents the time at which lysis had proceeded far enough to reduce the cell density to about 2.4×10^8 cells/ml.

burst and eventually a plaque. Plaques were then picked and replica streaked at 43 and 20 C to determine heat-sensitivity. Strains showing an acceptable degree of heat-sensitivity were complemented by spot-tests (see Materials and Methods) against a set of SP01 sus mutants to assign them to cistrons.

The procedure for the isolation of csDO mutants was basically the same except that 37 C was permissive and 20 C was restrictive. Also, since there is a much longer latent period at 20 C (about 5 hr), the time sequence of the procedure was modified accordingly.

Using these procedures I isolated 106 heat-sensitive and R. Slomiany isolated 44 cold-sensitive mutants that are sufficiently temperature-sensitive to be of use. Table III shows that this screening procedure yielded a 56-fold increase in the proportion of hsDO mutants, and at least a 9-fold increase in csDO's (no csDO's were detected without the screen). However, the most useful feature of the screen was that virtually all ts mutants isolated were replication-deficient, greatly decreasing the work required in preparing lysates, and testing for complementation and replication-deficiency.

Cistron-specific Screening Procedure

It is possible to modify the above procedure to select for mutants affected in specific cistrons. The procedure is the same as before except that along with the mutagenized SP01 wt phage, one adds at a high multiplicity (about 20:1) a mutant carrying a sus mutation in the desired cistron. Since the phage carrying the sus mutation should complement almost all mutants except those affected in the desired cistron, mutants affected in this cistron should be

TABLE III. Comparison of mutagenized phage populations before and after the general screening procedure.

	Before screen	After screen
Percent heat-sensitive	1.7	10.0
Percent hs which are hs DO	10.0	95.0
Percent hs DO	0.17	9.5
Percent cold-sensitive	0.17	0.34
Percent cs which are cs DO	<17	75.0
Percent cs DO	< 0.03	0.25

virtually the only ones to survive the screen.

This screen has been used successfully for several of the SP01 D0 cistrons. Among hs mutants derived by the general screen from one mutagenized lysate, only one of 84 was affected in cistron 23. Using this same lysate with a cistron-specific screen for cistron 23, one of the two ts mutants isolated was affected in this cistron. Of 22 hs mutants derived by the general screen from another mutagenized lysate, none failed to complement sus27-1. After a cistron 27 specific screen on the same lysate, six of the ten hs mutants surviving the screen failed to complement sus27-1. (These mutants may not be affected in cistron 27, however, see Appendix).

Discussion

By using a screening procedure which makes use of the replication-dependence of lysis, I have been able to select for SP01 tsD0 mutants. A variation of this screening procedure allows one to select tsD0 mutants affected in a specific cistron of interest if a sus mutant in the cistron is available (with appropriate modifications, the reverse should also be possible). The use of these screening procedures has enabled us to isolate more than 150 tsD0 mutants affected in at least 8 of the 9 known SP01 D0 cistrons. These procedures could be applicable to any viral system in which lysis of the host cell is dependent upon viral DNA replication. In some cases, although lysis may not be naturally dependent upon replication, it may be possible to construct a system in which such a dependence is created. For instance, if the appearance of a parti-

cular cell-surface antigen in an animal virus system is dependent upon viral DNA replication, as in vaccinia virus infection (Weintraub and Dales, 1974), then one may be able to use a combination of antiserum and complement to lyse only those cells in which there has been viral DNA replication. The cistron-specific screen, which I have used successfully to isolate mutants in several cistrons, should also be applicable to other systems.

SECTION II: A Preliminary Characterization of the Mutants Isolated

Introduction

Having isolated a series of ts replication-deficient mutants, I then proceeded to assign these mutants to the known SP01 complementation groups and to characterize their ability to synthesize DNA following a shift from permissive to restrictive temperature.

Results

Choice of mutants for further study.

Where possible, two independent mutations in each D0 cistron were selected for further characterization. After having been tentatively assigned to cistrons on the basis of spot-test complementations, the selected mutants were re-tested by complementation in liquid. Table IV shows the results of the liquid complementations. All mutations used had reversion frequencies between 10^{-5} and 10^{-7} and thus are presumably single-site mutations. If the two mutants affected in a particular cistron were isolated from the same lysate then independent origin was established by a recombination analysis (see legend of Table IV for procedure).

Temperature-shift experiments.

One can imagine two general types of initiator mutants: one type affecting only initiation of the first round of replication, and another affecting initiation of each round of replication. These should be distinguishable from each other, and from some other sorts of D0 mutants, by the following temperature-shift experi-

TABLE IV. Liquid complementation of selected mutants[a]

Mutant	Burst size
cs21-1	0.33
sus21-1	<0.05
cs21-1 x sus21-1	0.31
cs21-1 x sus30-1	2.62
hs22-2	2.4
hs22-1	3.2
sus22-1	<0.05
hs22-1 x hs22-2	4.5
hs22-1 x sus22-1	6.2
hs22-1 x hs30-1	27
hs23-1	0.59
hs23-2	0.15
sus23-1	<0.05
hs23-1 x hs23-2	0.30
hs23-1 x sus23-1	0.18
hs23-2 x hs30-2	12
hs29-1	0.03
hs29-2	0.21
hs29-3	0.90
hs29-1 x hs29-2	0.32
hs29-1 x hs29-3	0.90
hs29-2 x hs23-1	8.2
hs30-1	2.1
hs30-2	1.4
sus30-1	<0.05
hs30-1 x hs30-2	1.8
hs30-1 x sus30-1	0.8
hs30-1 x hs22-1	27
hs31-1	3.4
hs31-2	2.5
sus31-1	<0.05
hs31-1 x hs31-2	4.0
hs31-1 x sus31-1	2.0
sus31-1 x hs22-2	36
hs32-1	0.06
hs32-2	0.1
sus32-1	<0.05
hs32-2 x hs32-1	0.08
hs32-2 x sus32-1	0.22
hs32-1 x hs23-2	7.5
wt (43°)	13.8
wt (20°)	6.4

[a]For crosses involving hs mutants, CB-312 growing exponentially at 43°C was infected with each phage at a M.O.I. of 5. At ten min. p.i., anti-SP01 antiserum was added. At 16 min. p.i., infected cultures were diluted 1000-fold into fresh VY, pre-warmed to 43°C. At 75 min. p.i., lysates were plated on CB-312 at 20°C.

For crosses involving cs mutants, CB-313 growing exponentially at 20°C was infected with each phage at a M.O.I. of 5. At 30 min. p.i., anti-SP01 antiserum was added. At 60 min. p.i., infected cultures were diluted 1000-fold into fresh VY, pre-cooled to 20°C. At 11 hrs. p.i., lysates were plated on CB-313 at 37°C.

The data for cs28-1 are not shown. The combination of complementation and recombination data suggests that this mutant is in cistron 28, but this assignment is only tentative due to ambiguities in the data.

ment: Cells are infected at permissive temperature. After replication has begun, the infected cells are shifted to restrictive temperature, and the rate of DNA synthesis is measured. On the basis of their behavior following this temperature-shift, one can divide the mutants into three classes: I. Mutants which continue DNA synthesis, II. Mutants which slowly stop DNA synthesis, and III. Mutants which abruptly stop DNA synthesis. A "first-round only" initiation mutants should fall into class I while a mutants affecting initiation of each round should fall into class III. (Under the assay conditions used, the completion of only one round of replication would appear to be an abrupt stop).

The results of these temperature-shift experiments are shown in Figures 1 and 2 and Table V. Cs23-1 and hs29-3 fall into class I, hs23-1, hs23-2 and hs29-1 into class II and all the rest into class III. (Note that the longer time scale at 20 C means that the data for cs21-1 are roughly equivalent to those for the rapid shut-off hs mutants).

Discussion

The functions of the proteins affected by the class I and class II mutants are known (at least to some degree of probability). Cistron 28 codes for a transcription control protein which modifies the host RNA polymerase (Duffy *et al.*, 1975; Fox *et al.*, 1976). Cistron 29 codes for a dUMP hydroxymethylase (Okubo *et al.*, 1972; James Cregg, personal communication). Cistron 23 may code for a deoxynucleotide kinase. Hs23-1 and -2 are not complemented by an SP82

Fig. 1. *DNA synthesis of heat-sensitive mutants following a temperature shift-up.*

CB-312 was grown to a cell density of about 2.1 x 10^8 cells/ml at 37°C and a portion of the culture was shifted to 43°C. Another portion was shifted to 20°C and 30 minutes later infected with either wt or mutant SP01 at a M.O.I. of 5. Under these conditions, phage DNA synthesis began about 120 minutes after infection. 170 minutes after infection, 5 ml aliquots of the infected cultures were transferred to 250 ml flasks already at 43°C. At one, five, and nine minutes after this transfer, 0.5 ml aliquots from the infected cultures were assayed for DNA synthesis (see Materials and Methods) at 43°C for 2.5 minutes. At 172 minutes p.i., 0.5 ml aliquots from the portion of the cultures remaining at 20°C were assayed for DNA synthesis for 10 minutes at 20°C. This served to establish the level of DNA synthesis at permissive temperature and is seen in the figure as percent wt incorporation at time zero. The portion of the uninfected culture that was transferred to 43°C was subdivided and eight minutes later was infected with either wt or mutant SP01 at a M.O.I. of 5. Under these conditions, phage DNA synthesis began about 7 minutes after infection. 15 minutes p.i., 0.5 ml aliquots were assayed for DNA synthesis at 43°C. This served to establish the level of DNA synthesis when infection proceeded solely at 43°C. This level was less than 2% of the wt level for all mutants with the exception of hs29-3 (6.5%). The elevation in DNA synthesis at the 2.25 min. time point for hs23-1 was also seen in the other shift-up experiment in which this mutant was tested, but its significance is undertain. Symbols:

A. Δ-Δ, hs23-1; O-O, hs23-2; ●-●, hs30-1; □-□, hs22-1.

B. ▲-▲, hs29-3; Δ-Δ, hs29-1; ●-●, hs32-1; O-O, hs29-2; □-□, hs31-1.

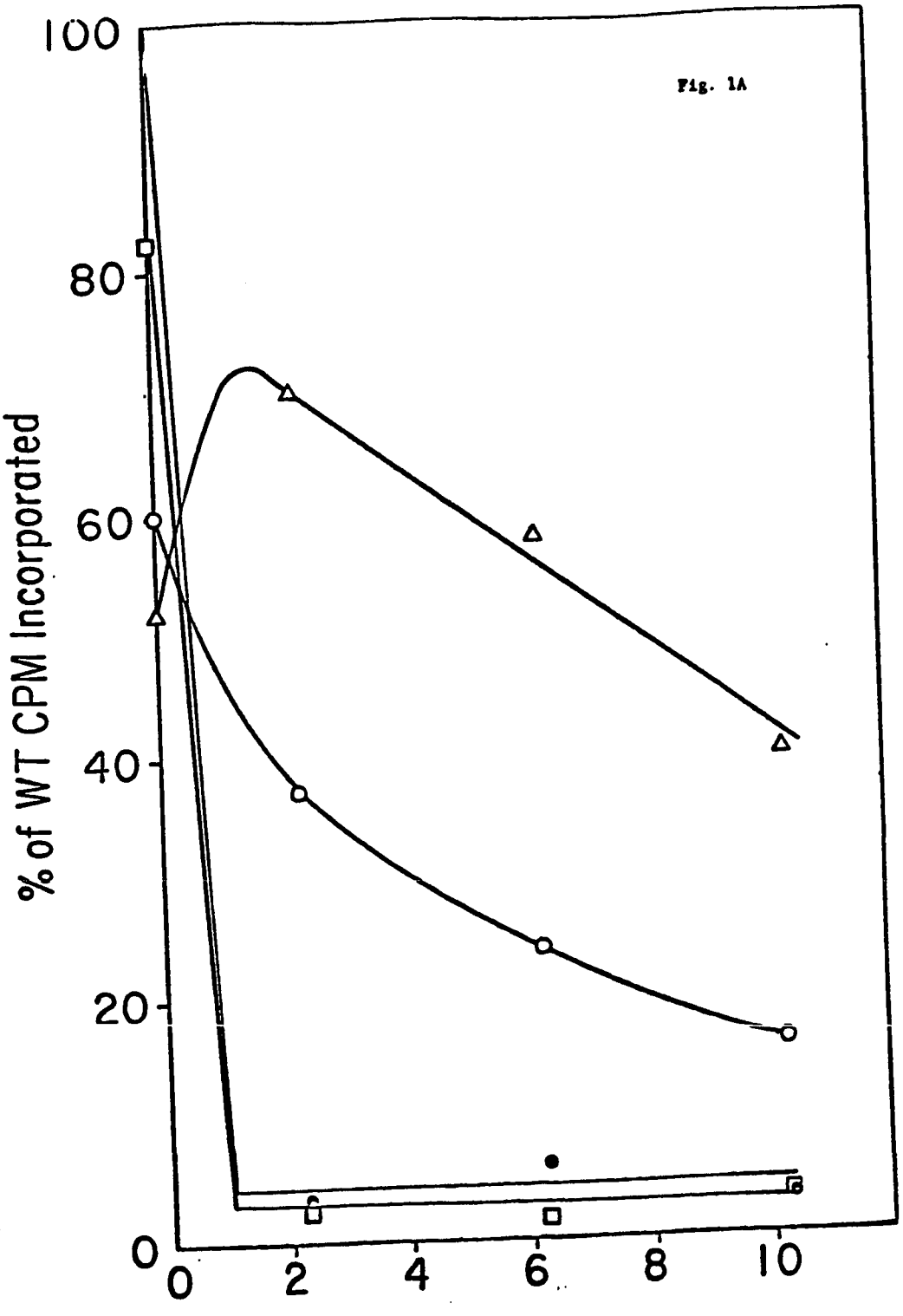


Fig. 1A
100
80
60
40
20
0
% of WT CPM Incorporated
0
2
4
6
8
10

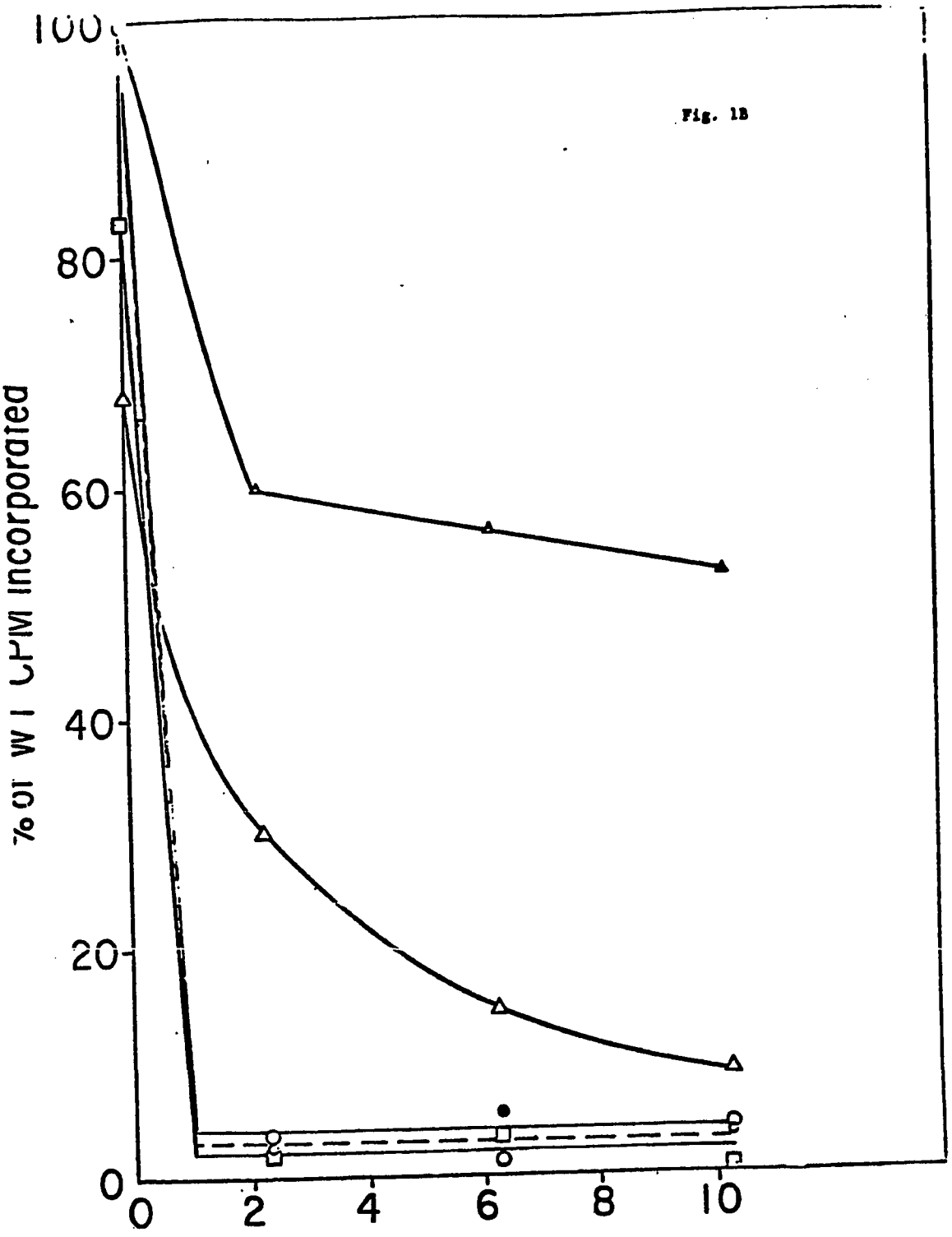


Fig. 1B

Fig. 2. *DNA synthesis of cold-sensitive mutants following a temperature shift-down.*

CB-312 was grown to a cell density of 2.1 x 10^8 cells/ml at 37°C, and a portion of the culture was shifted to 20°C. Another portion was shifted to 43°C and eight min. later infected with either wt or mutant SP01 at a M.O.I. of five. Under these conditions, SP01 DNA synthesis began about 7 minutes after infection. Eighteen min. later, 5 ml aliquots of the 43°C infected cultures were transferred to 250 ml flasks already at 20°C. At five, thirty and sixty min. after this transfer, 0.5 ml aliquots were removed and assayed for DNA synthesis (see Materials and Methods) at 20°C for 10 min. At 15 min. p.i., 0.5 ml aliquots from the portion of the culture remaining at 43°C were assayed for DNA synthesis at 43°C for 2.5 min. This served to establish the level of DNA synthesis at permissive temperature and is seen in the figure as the percent wt incorporation at time zero. The portion of the uninfected culture that was transferred to 20°C was subdivided and 19 min. later was infected with either wt or mutant SP01 at a M.O.I. of 5. Under these conditions, SP01 DNA synthesis began about 120 min. after infection. 200 min. p.i., 0.5 ml aliquots were assayed for DNA synthesis at 20°C for 10 min. This served to establish the level of DNA synthesis when infection proceeded solely at 20°C. These levels (expressed as percent wt incorporation) were: cs21-1--0.9%; and cs28-1--3.3%. The elevation in DNA synthesis at the 10 min. time point for cs28-1 was also seen in the other shift-down experiment in which this mutant was tested. Again, the significance of this is uncertain. Symbols: ●-●, cs28-1; O-O, cs21-1.

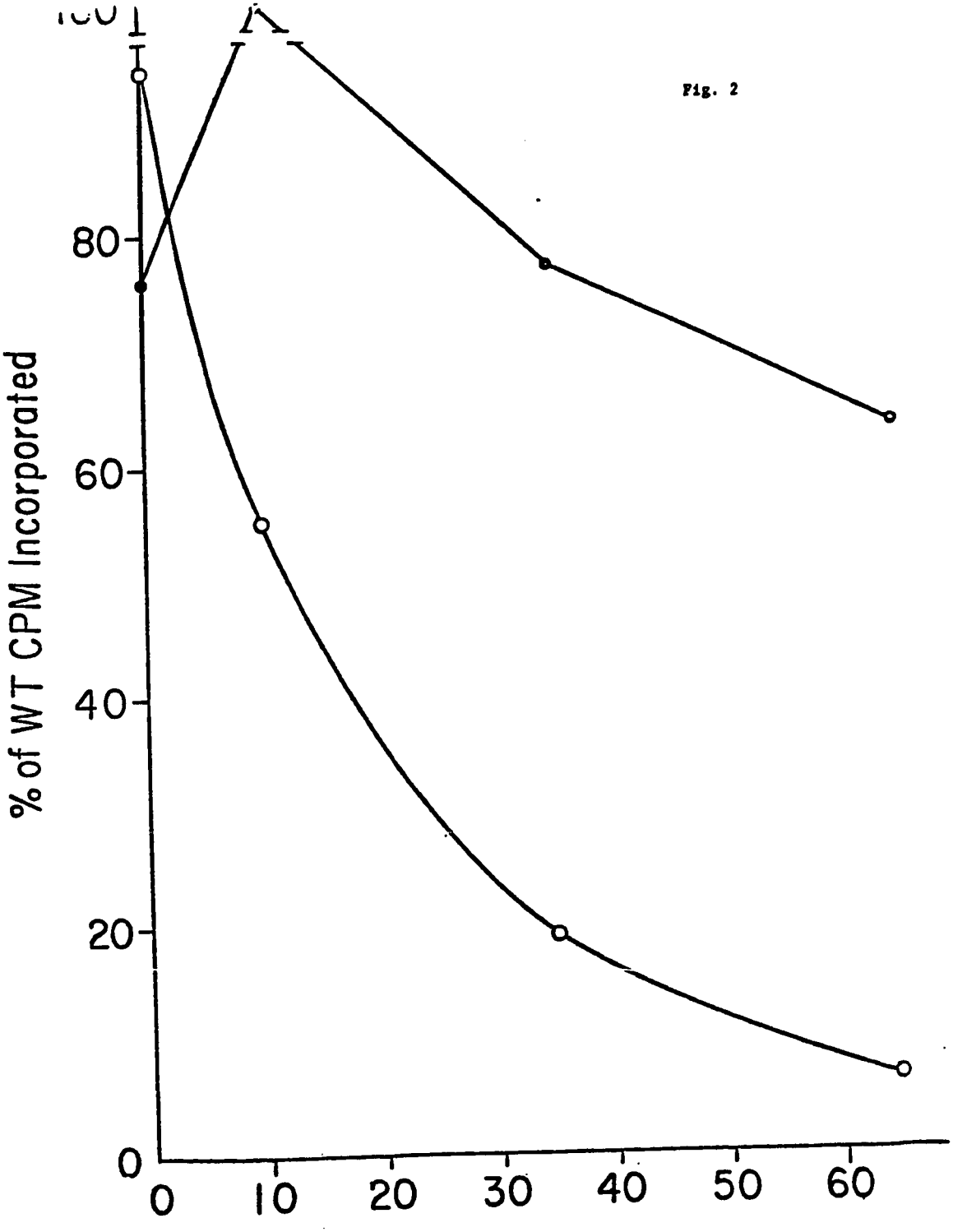


Fig. 2

TABLE V. DNA synthesis of hs mutants following a temperature shift-up.

Mutant	Time after shift-up (min.)	% of wt cpm incorporated
hs22-2	0	122
	2.25	8
	6.25	4.2
	10.25	6.8
hs30-2	0	82
	2.25	2.4
	6.25	1.7
	10.25	3.4
hs31-2	0	173
	2.25	4.2
	6.25	3.7
	10.25	4.5
hs32-2	0	83
	2.25	2.4
	6.25	3.2
	10.25	0.6

The procedure was identical to that described in the legend to Fig. 1. The level of DNA synthesis when infection proceeded solely at 43°C was less than 2% of the wt level for all mutants.

mutants affected in this cistron, but are complemented by an SP82 mutant affected in another cistron (Table VI).

Thus it appears that none of the class I or II mutants are initiator mutants. If an SP01-coded protein is necessary specifically for initiation of the first round of DNA replication, that protein is not affected in any of our more than 150 DO mutants. Presumably, all of the cistrons affected in class III mutants (21, 22, 30, 31, and 32) code for proteins directly involved in DNA replication.

TABLE VI. Liquid complementation of SP01 cistron 23 and SP82 gene 3 mutants.

Phage	Burst size
SP82 Z-2	0.53
SP82 Z-3	1.4
SP01 hs29-1	0.8
hs29-2	0.3
hs23-1	0.3
sus23-1	<0.05
Z-2 + hs29-1	1.3
Z-2 + hs29-2	1.6
Z-2 + hs23-1	36.4
Z-3 + hs23-1	1.8
Z-3 + sus23-1	1.6
Z-3 + hs29-1	8.3
SP82 wt	19.5
SP01 wt	5.6

B. subtilis CB-312, growing exponentially at 48°C, was infected with each phage at a M.O.I. of 5. At 7 min. p.i., anti-SP01 antiserum was added. At 12 min. p.i., infected cultures were diluted 1000-fold into fresh VY, pre-warmed to 48°C. At 120 min. p.i., lysates were plated on CB-312 at 20°C. Z-2 and Z-3 are hs (48°C is restrictive temperature) mutants of bacteriophage SP82 affected in the SP82 hydroxymethylase and deoxynucleotide kinase, respectively (Kahan, 1971).

SECTION III: The Origins and Directions of Replication of Bacteriophage SP01

Introduction

The previous sections described the isolation and characterization of replication-deficient mutants of SP01. Here I describe the analysis of the directions of replication and the positions of the origins for SP01 DNA replication, adapting the density-transfer techniques previously developed for *B. subtilis* (Yoshikawa and Sueoka, 1963; O'Sullivan and Sueoka, 1967; Copeland, 1974).

Results

Isolation of Partially Replicated Molecules

A culture of deuterated *B. subtilis*, growing in a deuterated medium, was infected at restrictive temperature with a temperature-sensitive, replication-deficient mutant, whose DNA was of light density. After the time at which DNA replication would normally have begun (but had not, due to the ts block), the culture was shifted to permissive temperature. This procedure permitted the partial synchronization of the initiation of SP01 DNA replication. Before the first round of replication had been completed, the infection was stopped, the DNA extracted and sheared, and then centrifuged to equilibrium in CsCl.

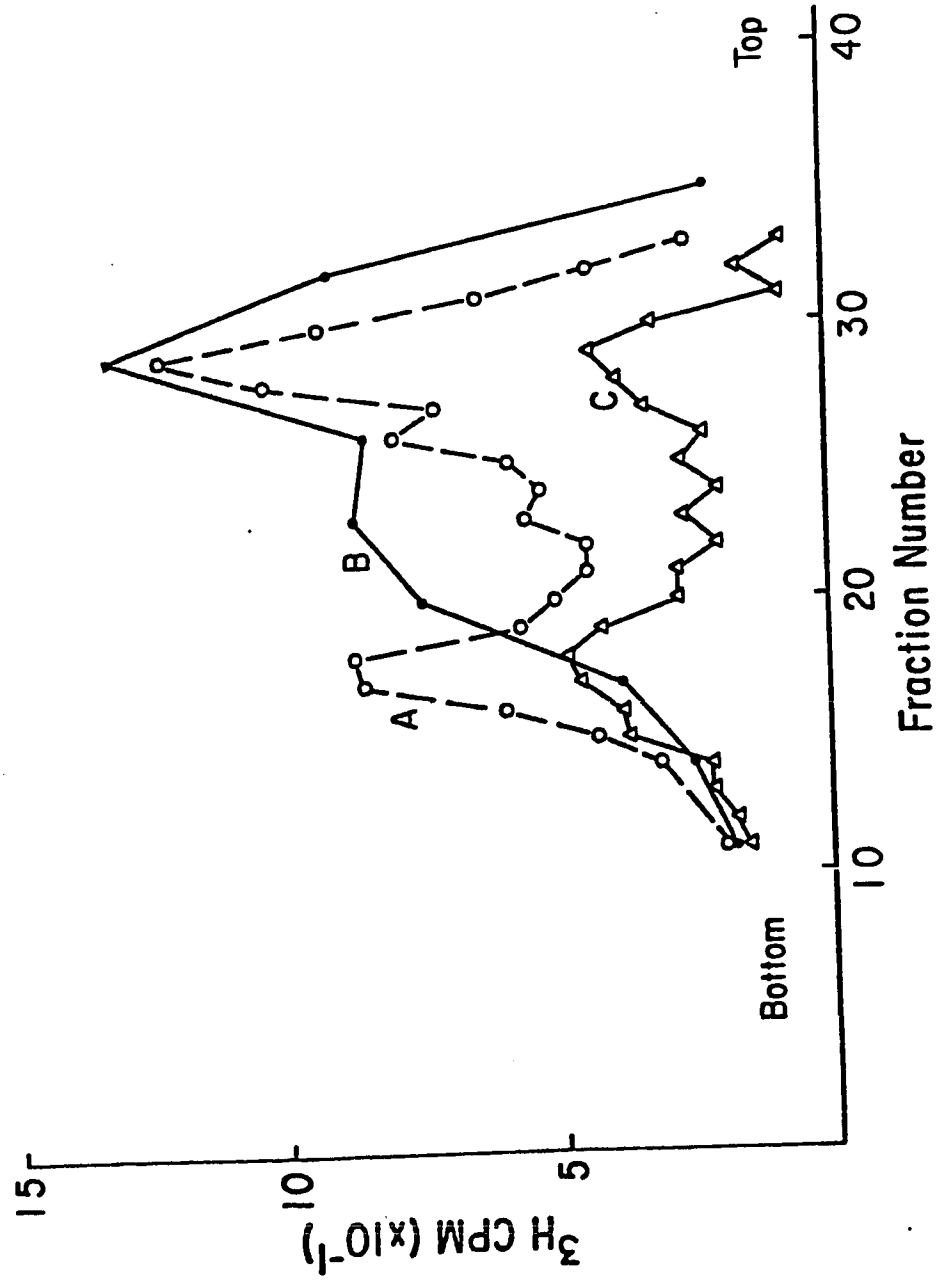
Efficacy of Shearing Procedure

Fig. 3, curves A and B, shows a comparison of the distributions of sheared and unsheared DNA extracts after such a centrifugation.

Fig. 3. *Equilibrium CsCl centrifugation of partially replicated SPO1 DNA.*

After a 25 min preincubation at 43°C, ^{3}H-labelled light density hs31-1 (an SPO1 heat-sensitive DO mutant) was allowed to replicate in deuterated *B. subtilis* CB-327 for 20 min at 20°C and DNA was extracted and either sheared (A) or not (B). The DNA extracts were centrifuged to equilibrium in CsCl, fractions were collected and aliquots were assayed for radioactivity. Gradients have been aligned by the positions of the light peaks. Fractions 17 to 25 from the CsCl gradient shown in (B) were pooled, dialyzed, sheared and re-centrifuged to equilibrium in CsCl (C). In all cases, background radioactivity has been subtracted.

Fig. 3



A substantial fraction of the unsheared DNA bands in a position indicating a buoyant-density intermediate between light and hybrid, while the shearing eliminated most of this intermediate density material and caused the appearance of a hybrid peak. I interpret this to mean that, in the unsheared extract, many of the molecules had been partially replicated, while in the sheared extract, the fragments were small enough that most contained only replicated or unreplicated DNA. When the fractions including partially replicated, unsheared molecules were pooled, dialyzed, sheared, and centrifuged again to equilibrium in CsCl, a light and a hybrid peak were seen (Fig. 3, curve C) as expected.

Gradient of Replication

By marker rescue assays, one can determine the distribution of particular markers in the CsCl gradient. For any particular marker, one can determine the proportion of its activity found in the hybrid region, and, thus, the proportion of genomes on which replication had proceeded at least as far as that marker. Fig. 4a shows that, in one particular gradient, most of the sus2-1^{+} activity is in the hybrid region of the gradient. Thus, we can calculate that, in this particular culture, a replication fork had reached the sus2-1 region of the genome in 62% of the genomes present (see Materials and Methods for the procedure used to calculate percent replication). A comparison of Figs. 4a through f, shows that regions of the SP01 DNA that contain various genetic markers have been replicated to differing extents.

Fig. 4. *Different density-shifts of SP01 genetic markers in the same CsCl gradient.*

After a preincubation at 43°C, ^{3}H-labelled, light density hs31-1 was allowed to replicate in deuterated *B. subtilis* CB-327 for 20 min at 20°C. DNA was then extracted, sheared, and centrifuged to equilibrium in CsCl. Adjacent fractions of the gradient were pooled, and aliquots of the pooled fractions used in marker rescue assays with the following sus mutants: (A) sus2-1, (B) sus27-1, (C)sus33-1, (D) sus14-1, (E) sus3-1, (F) sus35-1. Background PFUs due to reversion of the susmutant have been subtracted. See Materials and Methods for details.

Fig. 4A

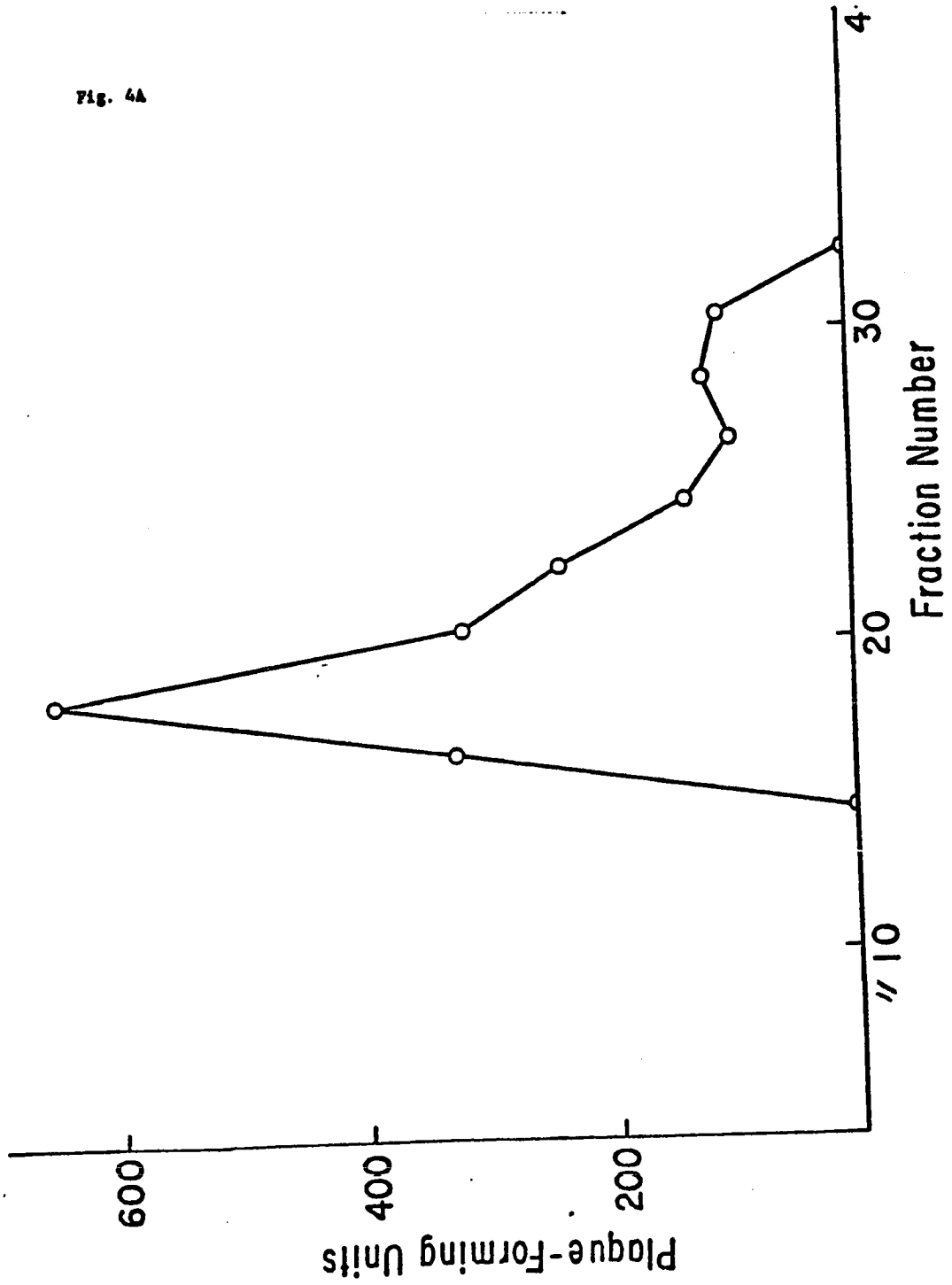


Fig. 4B

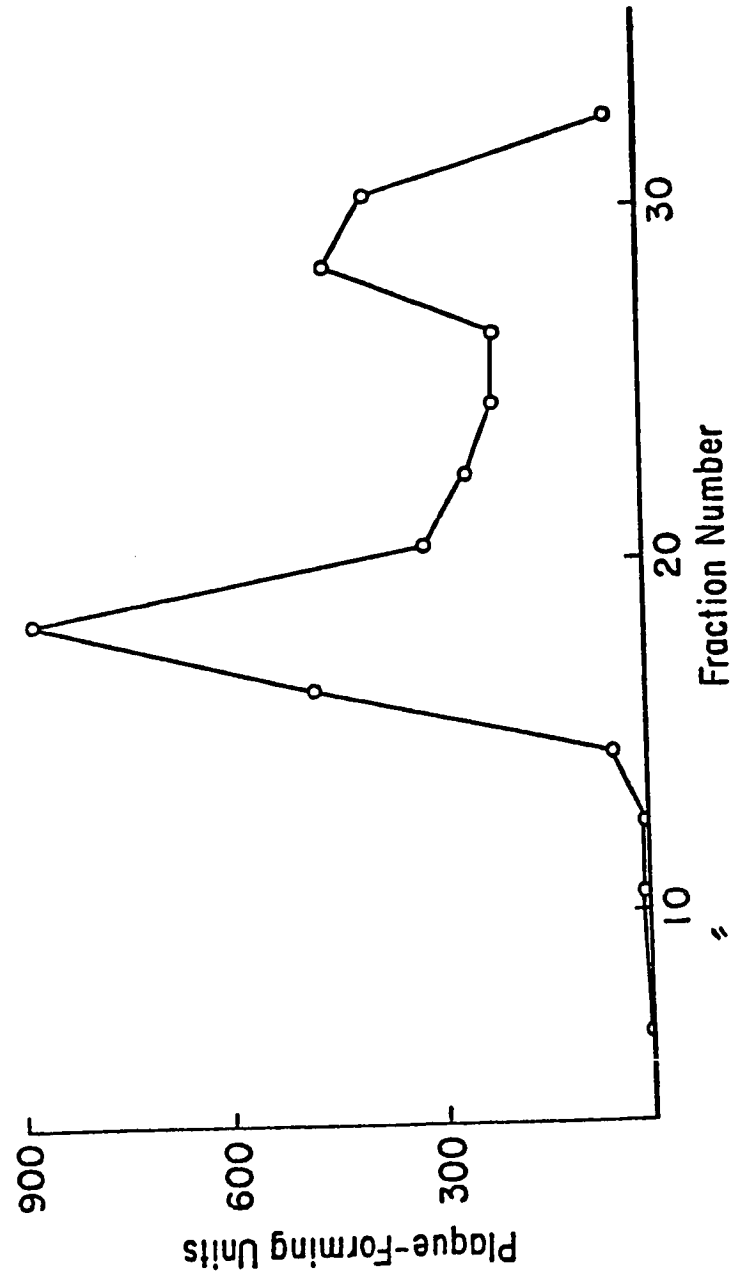


Fig. 4C

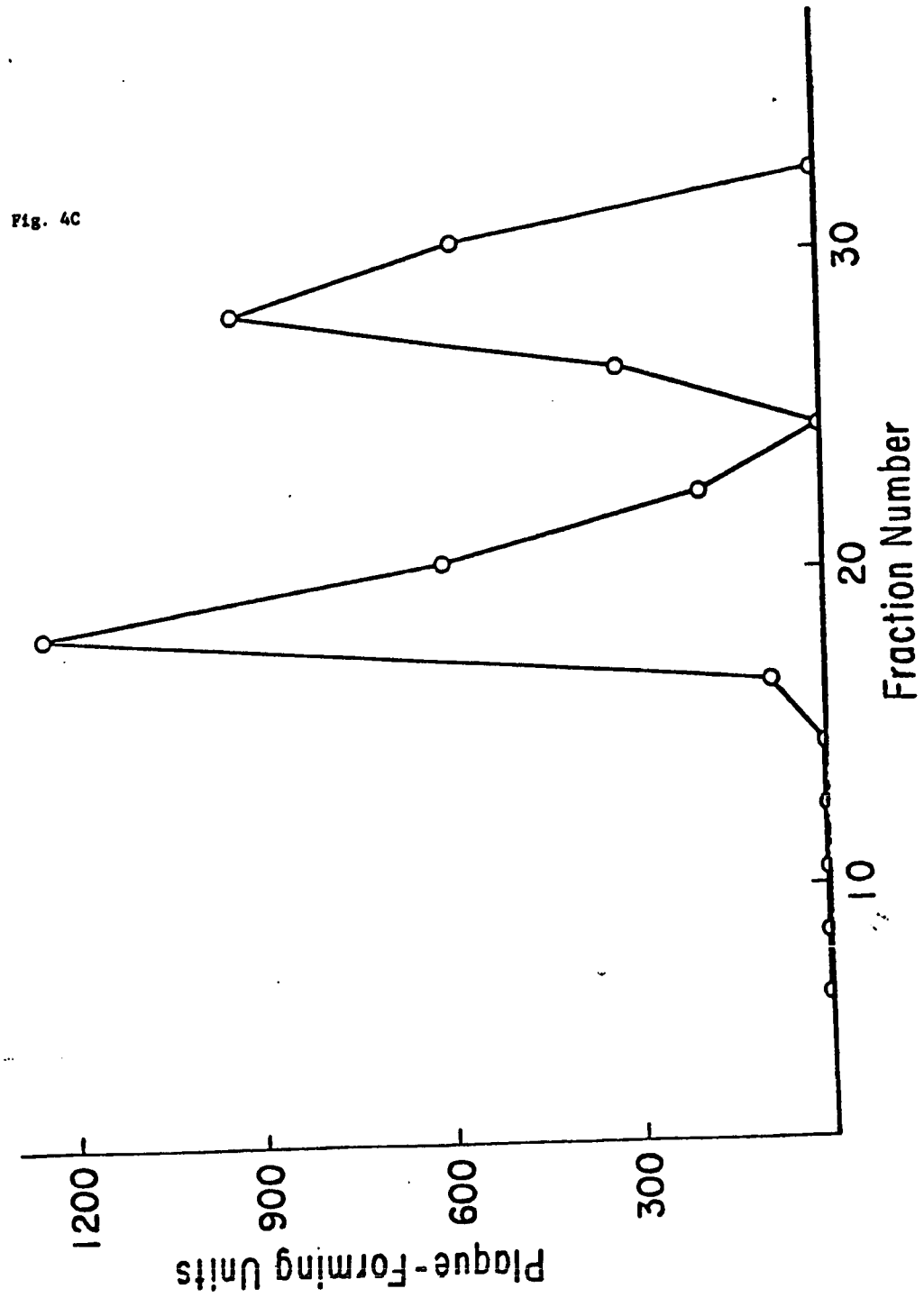
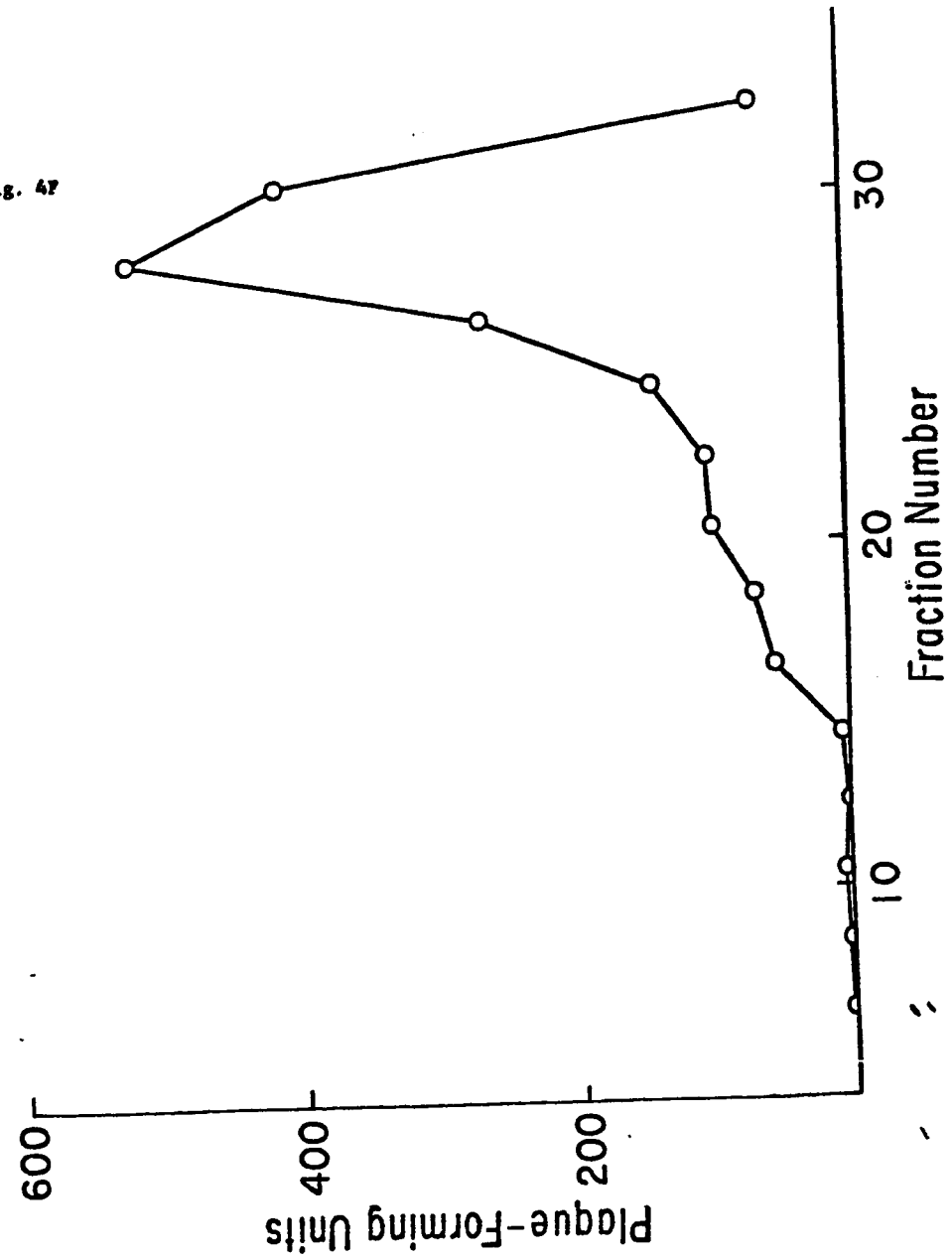

Plaque-Forming Units
1200
900
600
300
10
20
30
Fraction Number

Fig. 4F



In Fig. 5 I have plotted the percent replication of various markers against the SP01 genetic map. One can see that there is a gradient of replication from sus32-1 to sus3-1. This same gradient of replication was obtained regardless of which heat-sensitive or cold-sensitive, replication-deficient mutants were used to synchronize the initiation of DNA replication (although the form of the gradient was often non-linear). Since the closer a marker is to an origin of replication, the higher will be its percent replication, it appears that there is an origin of replication located between sus32-1 and sus35-1 and that replication proceeds unidirectionally to the left from this origin.

Rightmost segment of SP01 map

The sus35-1 and sus36-1 markers originally could not be placed on the genetic map because they recombine with high frequency with all other markers and with each other (Okubo *et al.*, 1972; my unpublished results). These markers have been tentatively placed between cistrons 32 and 33 on the basis of preliminary EcoRI restriction mapping (James Cregg, this laboratory). Fig. 5 and Table VII show a gradient of replication from sus34-1 through sus35-1, indicating that a second origin of replication lies in, or to the right of, cistron 34, with replication proceeding from sus34-1 through sus33-1 and sus35-1 and terminating somewhere near the first origin, the direction of replication through sus35-1 to sus36-1 is unclear (see Table X).

Fig. 5. *Percent replication* vs. *genetic map.*

Deuterated *B. subtilis* CB-327, growing in deuterated medium, was infected with light density hs31-1 at 43°C. 25 min later, the culture was shifted to 20°C (the preliminary incubation at restrictive temperature imposes some synchrony on the initiation of replication). After shaking for 20 min at 20°C, the culture was poured onto Spizizen's minimal medium ice. The DNA was extracted, sheared and centrifuged to equilibrium in CsCl. After assaying fractions for radioactivity, adjacent fractions were pooled and aliquots were assayed for marker rescue activity against sus markers in the indicated cistrons. The calculation of the percent replication was as described in Materials and Methods. The percent replication for each marker is plotted against the position of that marker on the SP01 genetic map, taken from Okubo *et al.*, 1972.

Fig. 5

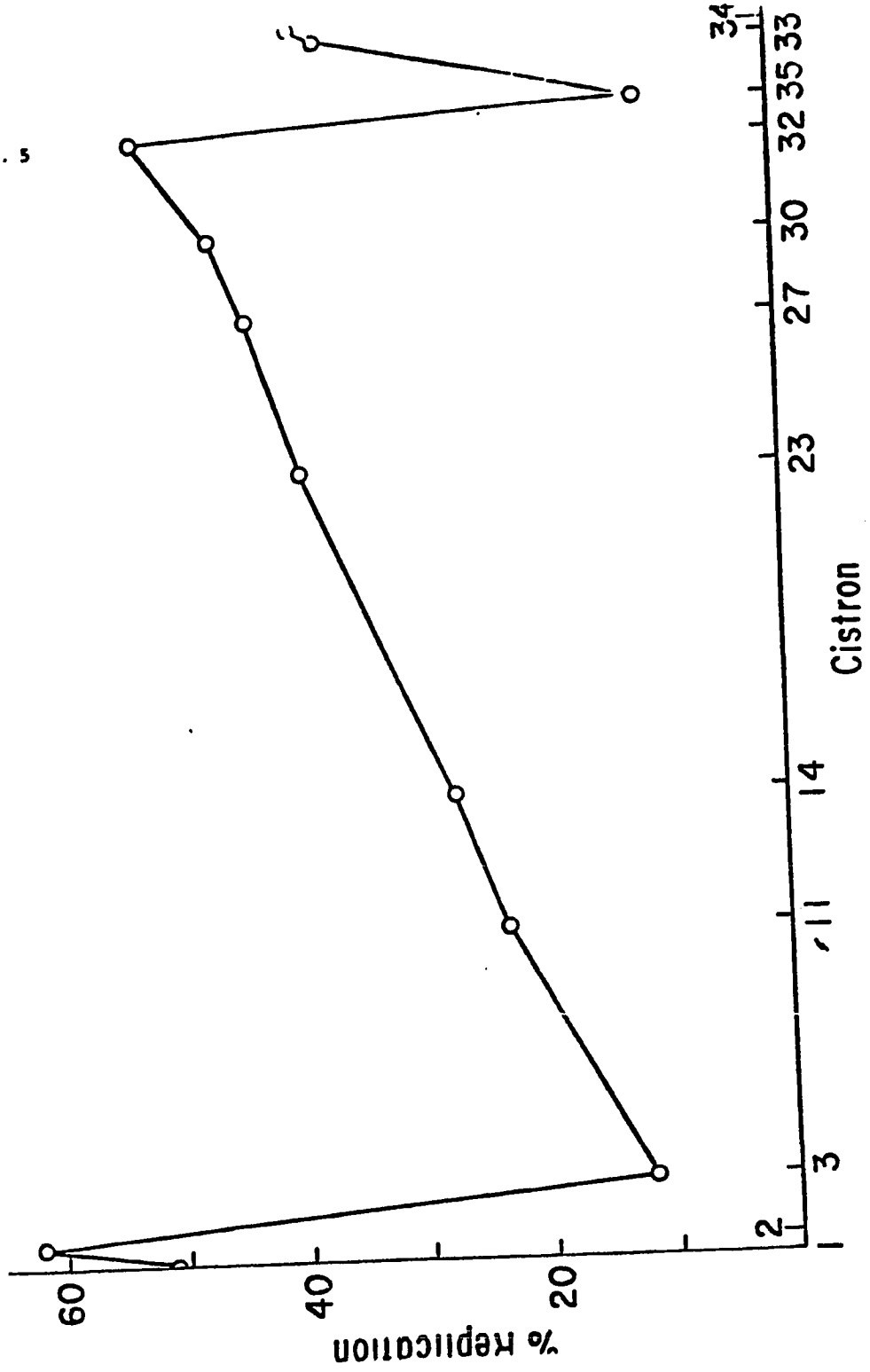


TABLE VII. Replication of right-hand end of genome

Experiment No.	Mutation carried by infecting phage	Percent of individual markers replicated			
		sus32-1	sus35-1	sus33-1	sus34-1
1	hs31-1[a]	52	11	37	39
2	hs32-1	66	13	22	29
3	cs22-1	51	32	36	55
4	cs21-1	43	13	16	24
5	cs21-1	30	11	11	17

[a]For hs mutants, the procedure was as described in the legend to Fig. 5 except that infection was with the hs mutants indicated.

For cs mutants, the indicated mutants were used to infect deuterated CB-327. After a 150 min preincubation at 20°C, the mutants were allowed to replicate at 37°C for 6 min and then stopped by chilling (expts. 4 and 5) or by shifting to 20°C for 1 hr before chilling (expt. 3). The rest of the protocol was as described in the legend to Fig. 5.

Leftmost segment of SP01 map

The direction of replication through the region from sus1-1 to sus3-1 is less clear. Table VIII shows that the relative percent replication for cistrons 1 and 2 was inconsistent from experiment to experiment. Thus, the data allow no conclusions as to the direction of replication through this region. However, they do force the conclusion that there is an origin to the left of sus3-1. This might be a third origin, or, if the SP01 DNA molecule replicates as a circle, it might be the same site at which leftward replication of the cistron 35-34 region originates.

Discussion

The data presented in this section support the following conclusions: (1) SP01 has at least two origins of DNA replication; (2) one of these origins is located about 8% from the right-hand end of the genetic map and replication proceeds from this origin unidirectionally to the left; (3) the replication of the extreme right-hand 8% of the genetic map originates in or to the right of cistron 34 and proceeds from right to left; (4) an origin of replication lies either in, or to the left of, the extreme left-hand 8% of the genetic map; and (5) a hot-spot for recombination exists at a terminus of replication. These are the simplest conclusions that the data permit. The data are also consistent with more complicated conclusions involving additional origins and/or more complicated directional patterns, but there is nothing in the data that suggests or requires such additional complexity.

TABLE VIII. Replication of left-hand end of genome

Expt. No.	Mutation carried by infecting phage	Percent of individual markers replicated		
		sus1-1	sus2-1	sus3-1
1	hs31-1[a]	51	62	12
2	hs31-1	82	79	ND[b]
3	hs31-2	24	29	9
4	hs32-1	51	57	3
5	cs21-1	17	16	15
6	cs21-1	44	37	32
7	cs22-1	35	40	31

[a]For hs mutants (except expt. 2), the procedure was as described in the legend to Fig. 5 except that infection was with the hs mutants indicated and in expt. 3 replication was stopped by shifting the infected culture to 43°C for 15 min. before chilling. In expt. 2, fractions from the CsCl gradient shown in Fig. 1 curve C, were assayed for marker rescue activity against the indicated sus markers.

For cs mutants, the indicated mutants were used to infect deuterated CB-327. After a 150 min preincubation at 20°C, the mutants were allowed to replicate at 37°C for either 6 min (expts. 5 and 7) or 8 min (expt. 6) and then stopped by chilling (expts. 5 and 6) or by shifting to 20°C for 1 hr before chilling (expt. 7). The rest of the protocol was as described in the legend to Fig. 5.

[b]ND - Not determined.

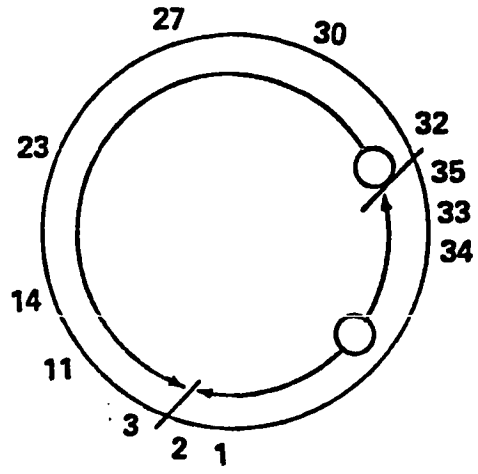
Replication of the extreme ends of a DNA molecule as separate replication units is aesthetically unappealing (although SP01 may be unaware of our feelings on this matter). Although there are obviously a number of possibilites, I favor the speculative replication scheme shown in Fig. 6. This model postulates that the SP01 DNA molecule circularizes prior to replication and that there exists a significant segment of the SP01 genome for which no genetic markers exist. No data exists to support the first postulate, but there are some relevant to the second.

From the data presented in Fig. 5, one can calculate that the average percent replication of the markers observed was 34%. (It was assumed that the average percent replication for each pair of markers was, in fact, the average percent replication for the length of DNA--measured in recombination units--subtended by those markers). The fractions of this same gradient were assayed for radioactivity, as usual. On the basis on the shift in cpm, from the light to the hybrid region, I calculated that the total DNA population had been 42% replicated. Thus the percent replication computed on the basis of the average shift in marker-rescue activity is only 0.81 (34%/42%) of that computed on the basis of the cpm shift. For eight gradients, this value (percent replication based on marker rescue/percent replication based on cpm shift) ranged from 0.50 to 0.87 with an average of 0.71. That this discrepancy between the two methods of calculating the average percent of DNA replication is not attributable to some anomaly in the way in which the two values were calculated can be concluded from the following

Fig. 6. *Possible replication scheme for SP01.*

→--indicates direction of replication. O--indicates an origin of replication. | --indicates a terminus of replication.

Fig. 6.



consideration. In Section IV, I describe mutants that are specifically affected in the initiation of replication. With these mutants, when replication is stopped by shifting to the restrictive temperature, a situation is created in which each DNA molecule is either completely replicated or completely unreplicated. In six gradients involving such DNA populations the ratio of the percent replication based on marker rescue to the percent replication based on the cpm shift varied between 0.91 and 0.97, with an average of 0.94.

Thus, in populations including partially replicated molecules, the regions represented by the genetic markers used (which span the known SP01 map) show a lower percent replication than the bulk of the DNA. One way to interpret this discrepancy is to postulate that there is a considerable segment of the genome for which no genetic markers exist, and that this segment of the genome is one of the first to be replicated, i.e., it contains an origin of replication within it. Fig. 6 shows the simplest model which combines this argument with the data presented above. The marker-less region connects the left and right ends of the known map. Replication is bidirectional from an origin within this region, and unidirectional, leftward, from the origin near sus32-1.

The fact that markers located near a terminus of replication show high frequencies of recombination with each other, and with all other markers, suggests that there is a hot spot for a specific sort of recombination in the terminus region. The recombinational event must somehow be limited to this region because there is a low recombination frequency between markers bracketing this region

(sus32-1 and sus33-1). Single-stranded gaps have been found near the terminii of newly replicated SV40 DNA (Chen *et al.*, 1976) and of replicating ØX174 DNA (Eisenberg *et al.*, 1975). It seems plausible that a similar gap in the SP01 terminus region could play an essential role in the recombination that occurs at high frequency in that region, but there are, of course, various other possibilities.

Multiple origins of replication are thought to be a general phenomenon among eukaryotes (Huberman and Riggs, 1968; Kriegstein and Hogness, 1974: Newlon *et al.*, 1974). However, most prokaryotic and viral systems have been reported to have single origins (Bird *et al.*, 1972; Masters and Broda, 1971; Harford, 1975; Schnös and Inman, 1970; Baas and Jansz, 1972; Dressler *et al.*, 1972; Danna and Nathans, 1972; Klotz, 1973). T4 is controversial (Mosig, 1970; Mosig *et al.*, 1972; Delius *et al.*, 1971; Kozinski and Doermann, 1975), and only recently have multiple origins been reported for other prokaryotic systems (Crosa *et al.*, 1975; Perlman and Rownd, 1976) and bacteriophage T5 (Bourguignon *et al.*, 1976). The addition of SP01 to this list suggests that, among viruses, multiple replication origins may be of more general occurrence than had previously been thought.

SECTION IV: Initiation and Termination Mutants

Introduction

In several bacterial and viral systems, certain gene products are known to be necessary specifically for the initiation of DNA replication (see, for example, White and Sueoka, 1973; Laurent and Vannier, 1973; Hirota *et al.*, 1972; Tegtmeyer, 1972; and Hartwell, 1971). In this section I use density transfer analysis to identify two such gene products for SP01. In addition, I show that one of these gene products plays a specific role in the termination of a round of SP01 DNA replication.

SP01 has nine cistrons whose products are known to be necessary for DNA synthesis (Okubo *et al.*, 1972). In Section I, I analyzed temperature-sensitive mutants in eight of these nine cistrons by temperature-shift experiments, and concluded that, while none of these mutants is affected only in initiation of the first round of replication, the products of five of the cistrons are candidates for a specific role in the initiation of each round of replication. To determine which, if any, of these five gene products are actually initiation proteins, I combined the temperature shift with a density transfer, as follows:

B. subtilis, growing in heavy medium, is infected at permissive temperature with a temperature-sensitive mutant carrying light density DNA. Midway through the first round of replication, the culture is shifted to the restrictive temperature. With a mutant which affects propagation of the growing point, replication will

arrest as soon as the ts protein has been inactivated, and the positions of the growing points will be frozen. Since synchrony is imperfect, growing points will be distributed throughout the genome. The closer a genetic marker is to the origin of replication, the higher will be the proportion of that marker to be found on replicated, and thus hybrid density, portions of the genome. On the other hand, if the mutant affects only initiation, all molecules on which replication has been initiated before the temperature shift, will replicate to completion. All molecules will be either completely replicated or completely unreplicated, and so, each genetic marker will be replicated to the same extent. The latter result is obtained with mutants in cistrons 21 and 32.

Results

Initiation

Deuterated *B. subtilis*, growing in deuterated medium, was infected at restrictive temperature with temperature-sensitive, replication-deficient SP01 whose DNA was of light density. After the time at which replication would normally have begun (but had not, due to the ts block), the culture was shifted to permissive temperature. (This preliminary incubation at restrictive temperature served to partially synchronize the initiation of DNA replication). While the first round of replication was still in progress, the culture either was rapidly chilled or was shifted to restrictive temperature for 15 or 60 minutes. (Stopping replication by chilling served as a control, since this would maintain the existing

distribution of growing points and thus should result in a higher proportion of replication for markers closer to an origin, no matter which type of mutant is used). The DNA was then extracted, sheared, and centrifuged to equilibrium in CsCl. Fractions from the CsCl gradient were assayed for marker-rescue activity with a series of SP01 sus markers. For each marker, the proportion replicated was determined from the proportion of that marker's activity found in the hybrid region.

Although, as described in Section II, the ends of the SP01 genome are replicated independently of the central portion of the genome, for convenience of presentation, this section concerns itself only with the effect of mutations on the replication of the major portion of the genome and the two unlinked markers. The effect of the mutations on the replication of the ends of the genome was no different from their effect on the major portion.

Using the experiment described above, I tested ts mutants affected in each of the five possible "initiator" cistrons, looking for the abolition of the cistron 32 to cistron 3 replication gradient. Figures 7 and 8 show that, for mutants affected in three of these cistrons, 22, 30 and 31, a gradient of replication is obtained even after a shift to restrictive temperature. (The variations in the details of these curves are not significant, since there is frequently considerable variation from experiment to experiment). I conclude that proteins coded by these cistrons probably are directly involved in DNA chain elongation (see Discussion). In contrast, mutants in cistrons 21 and 32 yielded no gradients of repli-

Fig. 7. *Replication of hs30-1 and hs31-2.*

Deuterated *B. subtilis* CB-327, growing in heavy medium, was infected with either hs30-1 or hs31-2 at 43°C. 25 min later, the culture was shifted to 20°C. 10 (hs30-1) or 20 (hs31-2) min later, the culture was shifted back to 43°C. After incubating 15 min at 43°C, the culture was poured onto Spizizen's minimal medium ice. The DNA was extracted, sheared and centrifuged to equilibrium in CsCl. After assaying fractions for radioactivity, pairs of adjacent fractions were pooled and aliquots were assayed for marker rescue activity against sus markers in the indicated cistrons. The calculation of the percent replication for each marker was as described in Materials and Methods. (O---O) hs30-1; (●---●) hs31-2.

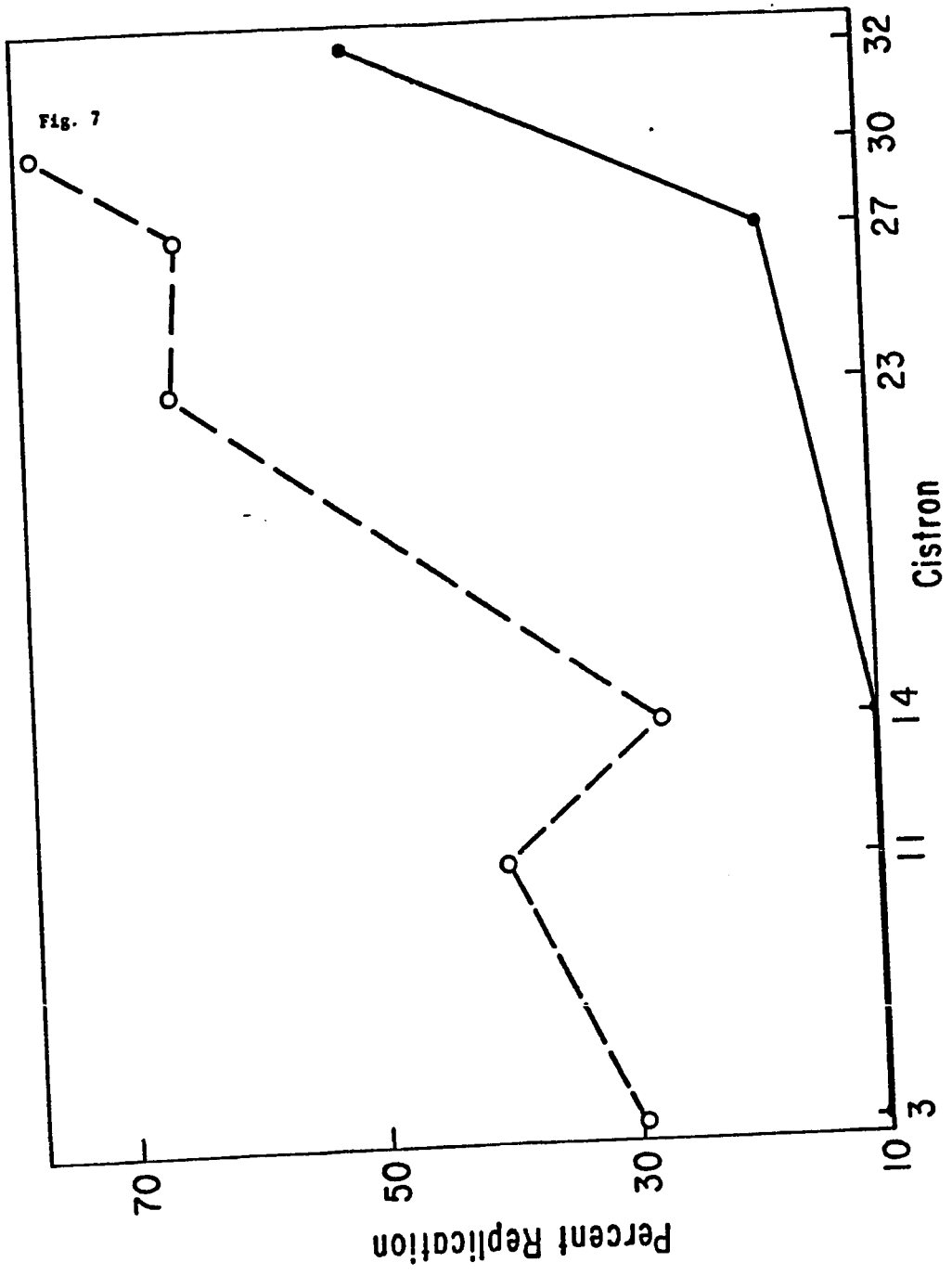


Fig. 7

Fig. 8. *Replication of cs22-1.*

Deuterated *B. subtilis* CB-327, growing in heavy medium, was infected with cs22-1 at 20°C. 150 min later, the culture was shifted to 37°C. 6 min later, the culture was shifted back to 20°C. After incubating 60 min at 20°C, the culture was poured onto Spizizen's minimal medium ice. The DNA was extracted, sheared and centrifuged to equilibrium in CsCl. After assaying fractions for radioactivity, pairs of adjacent fractions were pooled and aliquots were assayed for marker rescue activity against sus markers in the indicated cistrons. The calculation of the percent replication for each marker was as described in Materials and Methods.

The slope of the replication gradient when cs mutants were used as the infecting phage was generally less steep than when hs mutants were used (compare relevant data in Tables VII and VIII, for example). This is probably due to the lesser degree of synchrony achieved with the cs mutants.

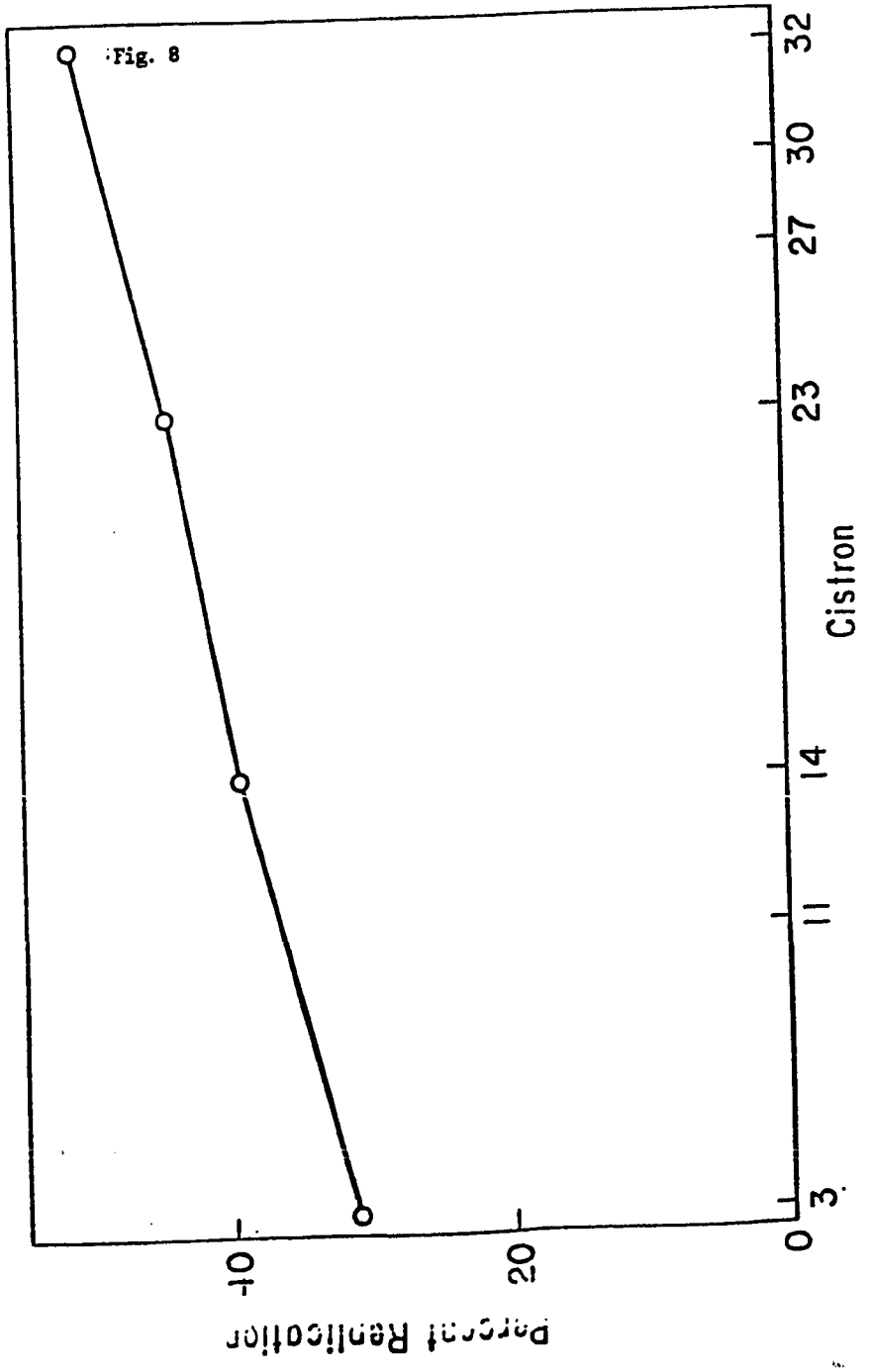


Fig. 8
Percent Replication
40
20
0
3
11
14
23
27
30
32
Cistron

cation when replication was stopped by shifting to restrictive temperature, but gave the expected replication gradients when stopped by chilling, as shown in Tables IX and X. Because of the scatter in the data shown in Table IX, the experiments with cs21-1 were repeated twice more, yielding essentially the same results.

The lack of replication gradients in these mutant-infected cultures, following a shift to restrictive temperature, is not due to some artifactual inability of the phage DNA to replicate further. In parallel-infected cultures in which replication was allowed to proceed for a greater length of time before the shift to restrictive temperature, the DNA was replicated to an appropriately greater extent (Figs. 9 and 10). I conclude that cistrons 21 and 32 code for proteins whose activities are required for the initiation of each round of replication, but not for polymerization.

Termination

The sus35-1 and sus36-1 markers show a high frequency of recombination with each other and with all other known SP01 mutants (Okubo *et al.*, 1972; my unpublished results), and their map location is, therefore, not known. By the criterion of the above sort of density transfer, temperature shift experiments, they are located very near to a terminum of replication, since they are always the last, or nearly the last, markers to be replicated (see for instance, Figure 5 and Tables IX and X). As mentioned before, tentative results obtained by James Cregg in this laboratory suggest that they are at the terminus in the rightmost segment of the genome.

TABLE IX. Replication of a cistron 21 mutant

	Marker	Percent replication of various markers when replication was stopped by			
		A		B	
		Chilling	Shift to restrictive temperature	Chilling	Shift to restrictive temperature
Major portion of the genome	sus3-1	15	38	32	53
	sus11-1	ND[a]	ND[a]	31	44
	sus14-1	14	38	29	49
	sus23-1	18	40	40	56
	sus27-1	15	ND[a]	47	51
	sus30-2	25	32	59	54
	sus32-1	30	34	56	45
Unlinked marker	sus35-1	11	33	28	46

The procedure was the same as that described in the legend to Figure 8, except that infection was with cs21-1 and, after 6 min. (A) or 8 min. (B) at 37°C, replication was stopped by either rapid chilling or shifting the culture to 20°C (restrictive temperature) and keeping it there for 60 min. before chilling and extracting DNA.

[a] - not determined.

TABLE X. Replication of cistron 32 mutants

	Marker	Percent replication of various markers when replication was stopped by A: Chilling	A: Shift to restrictive temperature	B: Chilling	B: Shift to restrictive temperature
Major portion of the genome	sus3-1	3	46	39	55
	sus11-1	18	48	39	50
	sus14-1	20	49	46	58
	sus23-1	37	48	68	56
	sus27-1	31	41	62	54
	sus30-2	38	49	89	56
	sus32-1	66	42	ND[a]	50
Unlinked markers	sus35-1	13	24	29	27
	sus36-1	11	29	ND[a]	18

The procedure was the same as that described in the legend to Figure 7, except that infection was with hs32-1 (A) or hs32-2 (B) and after 20 min. at 20°C, replication was stopped by rapid chilling, or, after 15 min. at 20°C, replication was stopped by shifting the culture to 43°C (restrictive temperature) and keeping it there for 15 min. In similar experiments (see Tables XI and XII), the sus2-1, sus33-1 and sus34-1 markers behaved like the bulk of the markers above.

[a] - not determined.

Fig. 9. *Increasing percent of replication of a cistron 21 mutant with increasing time at permissive temperature.*

The procedure was as described in the legend to Fig. 8 except that infection was with cs21-1 and, after 8 min. (O-O) or 12 min. (X--X) at 37°C, replication was stopped by shifting the culture to 20°C and incubating for 60 min. before being rapidly chilled. The DNA was extracted, sheared, and centrifuged to equilibrium in CsCl and the fractions assayed for radioactivity. The 6 min. culture is the same as shown in Table IXb.

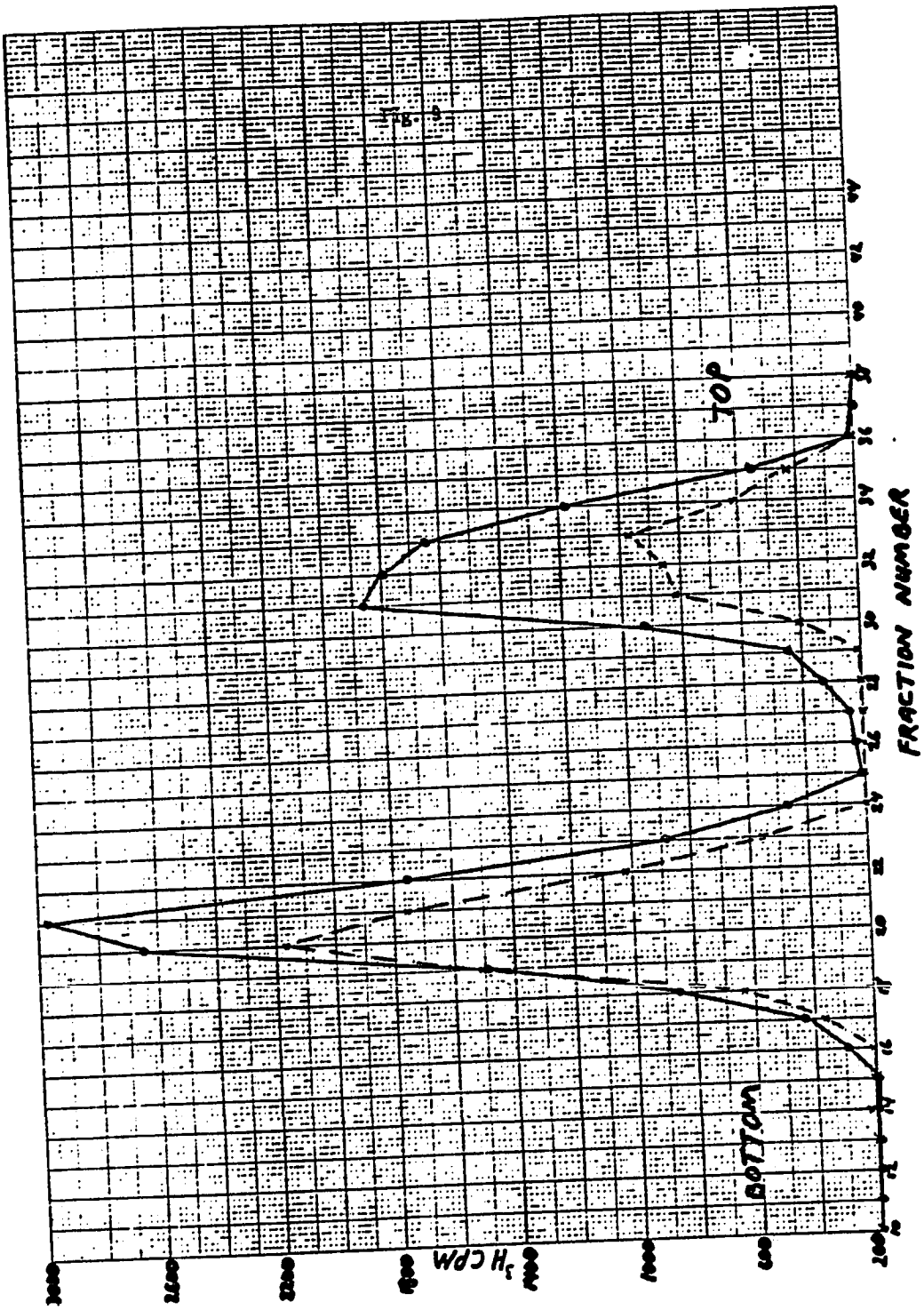


FIG. 3
TOP
BOTTOM
FRACTION NUMBER
^3H CPM

When initiation of a new round of replication is blocked by shifting cs21-1 to restrictive temperature, the sus35-1 marker is replicated to the same extent as all other markers (Table IX). In a similar experiment (data not shown), sus36-1 behaved in the same way. Thus, cs21-1, while prohibiting initiation, has no effect on termination. In contrast, when re-initiation is blocked by shifting either hs32-1 or hs32-2 to restrictive temperature, both the sus35-1 and the sus36-1 markers remain substantially less replicated than all other markers (Table X). Thus, the cistron 32 product seems to play a specific role in the replication of the terminus region.

Discussion

Initiation.

I have shown that mutants in cistrons 21 and 32 of SP01 are affected specifically in the initiation of *each* round of replication can be deduced from the following: (1) When infection is allowed to proceed at restrictive temperature, there is no first round of replication (see Tables IX and X). Therefore, the proteins coded by cistrons 21 and 32 are necessary for the initiation of the first round of replication. (2) If cells infected with these mutants are shifted to restrictive temperature while still in the first round of replication, the second round of replication is not initiated. Therefore, these proteins are necessary for the initiation of the second round of replication. (3) If cells infected with these mutants are allowed to replicate through many rounds of replication and then shifted to restrictive temperature, DNA synthesis is rapid-

TABLE XI. Replication of the right-hand end of the genetic map with a cistron 32 mutant.

Marker	Percent replication
sus3-1	51
sus11-1	57
sus30-2	61
sus35-1	25
sus33-1	52
sus34-1	54

The procedure was the same as that described in the legend to Fig. 7, except that infection was with hs32-2 and after 20 min. at 20°C replication was stopped by shifting the culture to 43°C and incubating for 15 min.

TABLE XII. Replication of a marker on the left-end of the genetic map with a cistron 32 mutant

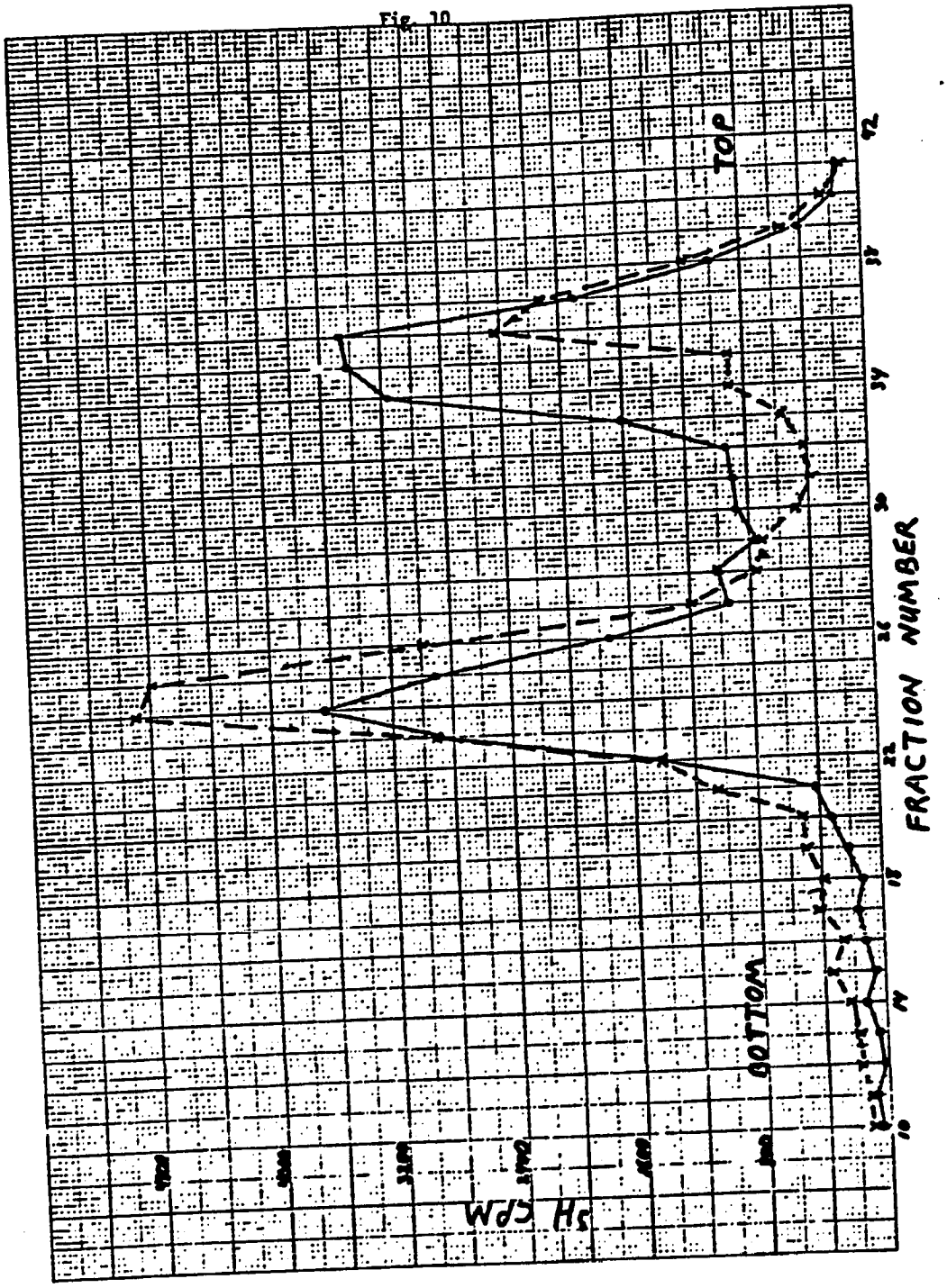
Marker	Percent replication
sus2-1	65
sus30-2	60
sus35-1	28

The procedure was the same as that described in the legend to Fig. 7 except that infection was with hs32-1 and after 15 min. at 20°C replication was stopped by shifting the culture to 43°C and incubating for 15 min.

Fig. 10. *Increasing percent of replication of a cistron 32 mutant with increasing time at permissive temperature.*

The procedure was as described in the legend to Fig. 7 except that infection was with hs32-1 and after 15 min. (O-O) or 40 min. (X--X) at 20°C, replication was stopped by shifting the culture to 43°C and incubating for 15 min. before being rapidly chilled. The DNA was extracted, sheared, and centrifuged to equilibrium in CsCl and the fractions assayed for radioactivity. The 15 min. culture is the same one as shown in Table Xa.

Fig. 10



ly shut off (Fig. 1). Thus the affected proteins are necessary for later rounds of replication as well.

Elongation.

Mutants in cistrons 21, 30, and 31 exhibit a rapid shut-off of DNA synthesis when shifted to restrictive temperature (Figs. 1 and 2), but fail to abolish the replication gradient. Taken in conjunction, these data indicate that the products of these cistrons are probably directly involved in the elongation of nascent DNA chains.

Termination.

Most interestingly, mutants in cistron 32 are affected in the termination of a round of replication as well as in its initiation. One might be tempted to turn this around and propose that re-initiation depends upon termination. However, this is probably not so, because, under restrictive conditions, cistron 32 mutants fail to initiate the *first* round of replication (for which, presumably, no termination event is necessary). It can also be seen that termination of one round of replication is not dependent *per se* upon initiation of the next, because, under conditions in which a mutant in cistron 1 prohibits re-initiation, it allows termination. I therefore conclude that the activity of the cistron 32 protein is necessary both for initiation and for some process associated with termination. As far as I am aware, this is the first mutant in any system in which the termination of a round of DNA replication is specifically affected. Marunouchi and Messer (1973) did report, however, that replication of the *E. coli* terminum required contin-

ued protein synthesis.

That special mechanisms may be required for the termination event may be inferred from the following consideration. If the replication terminus is membrane-bound, some mechanism may be required to yield this region accessible to the replication machinery. For a linear replication DNA molecule, some special mechanism is required to replicate the 3'-terminal nucleotides (Watson, 1972). If the replicating DNA molecule is circular, some special mechanism may be required to segregate the two daughter molecules (Gefter, 1975) and the segregation event may be necessary for termination.

Although the cistron 32 mutants prevent much of the replication of cistrons 35 and 36, some replication is still permitted (see Table X). All, or part, of this may be replication that takes place during the time at permissive temperature. However, it is possible that the situation is more complicated and that a portion of the normal replication of the terminus region can take place even without the participation of the gene 32 protein. For example, one possibility would be that the terminus region is entirely replicated, *except* for a gapped region, so that the normal density shift occurs, but the replicated DNA of the terminal region has a decreased marker rescue activity because of its single-stranded region.

It is of interest that an origin of replication lies either within, or very close to, cistron 32, and that cistron 32 codes for a protein necessary for initiation and termination. This is reminiscent of the situation with ØX174, whose replication origin lies within cistron A, which specifies a protein that introduces a nick

which is necessary for initiation (Francke and Ray, 1971; Francke and Ray, 1972; Henry and Knippers, 1974). Although this protein differs from the SP01 gene 32 protein in that it is cis-active, it is possible that the gene 3 protein might also function in initiation by introducing a nick in the DNA molecule. This nick might then result in the removal of a steric block to the termination of the previous round of replication.

APPENDIX

In this section are included miscellaneous data and observations which, while of interest, would break the flow of the narrative if included in the main body of the text.

CB-313 and 314 support of sus mutant growth

All sus mutant strains grew on CB-314 at 37 C but since CB-314 is more difficult to handle than CB-313, most strains were routinely grown and plated on CB-313. Table XIII lists strains which grow only on CB-314 or require other special considerations.

Differential distributions of heat- and cold-sensitive mutations

The heat- and cold-sensitive mutants that were isolated seem to affect different groups of cistrons. This is shown graphically in Figure 11. In seven of nine cistrons, there are either heat-sensitive or cold-sensitive mutations but not both. This could be due to chance alone (although this is extremely improbable) or either of at least two other possibilities. First, it is possible that the difference in the distributions stems from various biases in the screening procedures. It is probably true that not all tsDO mutants will be equally able to survive the screening procedures. For example, if a ts mutation affects a protein whose synthesis is normally shut-off some time before lysis, then this protein would have to be renatureable for the mutant carrying it to survive the screening procedure. The data in Table XIV indicate that the great majority of hs mutants surviving the screen carry

TABLE XIII. CB-313 support of sus mutant phage growth

Strain	Growth on CB-313
sus2-1	+[b]
sus13-1	+[a]
sus21-2	-
sus22-1	+[b]
sus23-2	-
sus27-1	+[c]
sus29-1	-
sus31-1	+[b]
sus32-1	-
sus33-1	+[a]

[a]Normal top agar concentration is 16 g/l. For these mutants 12 g/l should be used due to small plaque size.

[b]Cold-sensitive on CB-313 at 20°C.

[c]Somewhat heat-sensitive on CB-313 at 43°C.

Fig. 11. *Distribution of heat- and cold-sensitive mutants among DO cistrons.*

Shown as the percent of total heat-sensitive or cold-sensitive mutants (isolated by the general screening procedure) that are affected in each DO cistron. The tot numbers included 84 heat-sensitives and 26 cold-sensitives.

Fig. 11

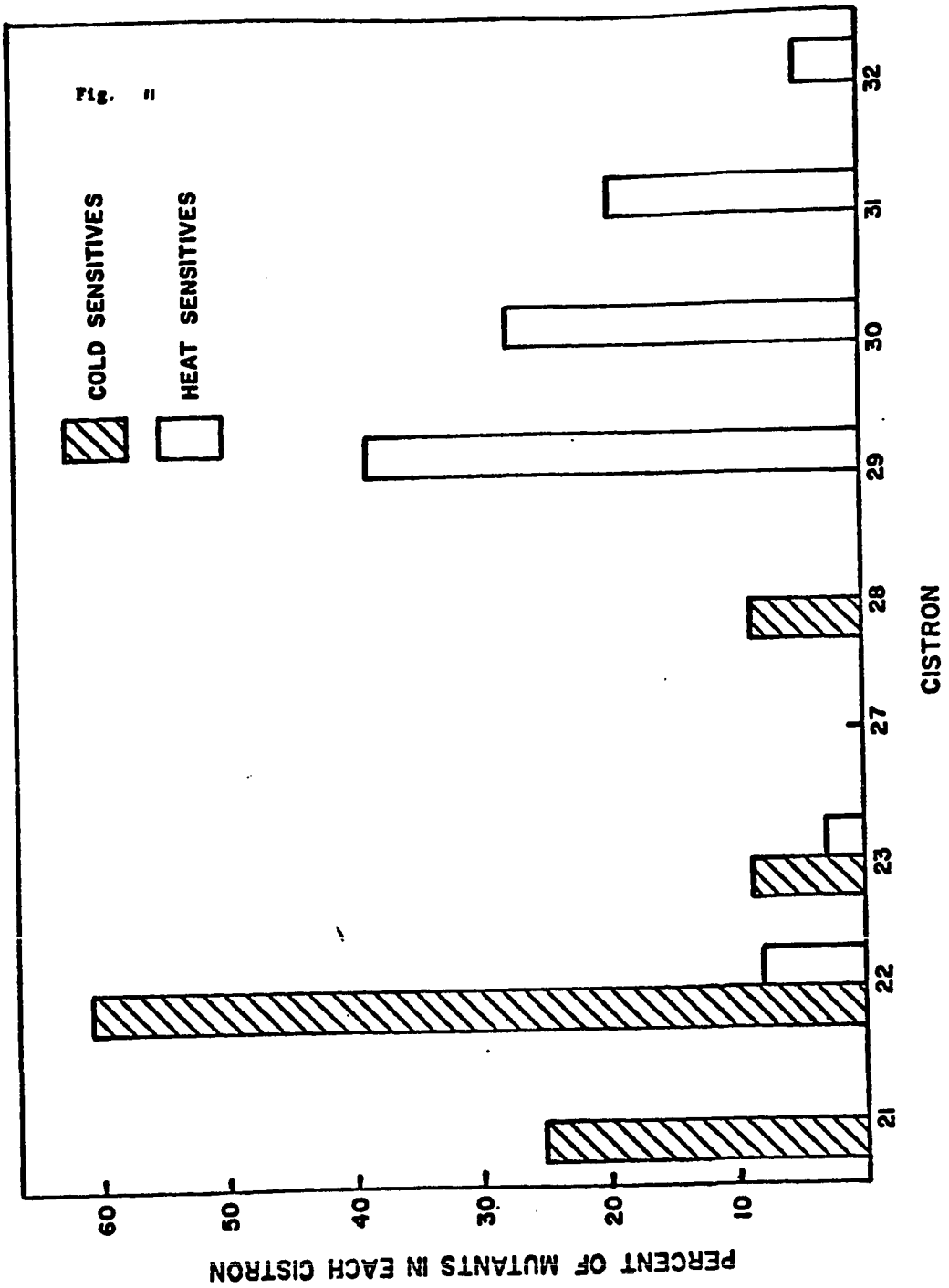

COLD SENSITIVES
HEAT SENSITIVES
32
31
30
29
28
27
23
22
21
CISTRON
60
50
40
30
20
10
PERCENT OF MUTANTS IN EACH CISTRON

TABLE XIV. Renaturability of proteins affected in SP01 hs mutants.

Phage	Time p.i. of chloramphenicol addition	$\frac{\text{DNA synthesis (+ chloramphenicol)}}{\text{DNA synthesis (- chloramphenicol)}} \times 100\%$
hs30-1	5	3.2
hs22-1	15	102.2
hs22-2	15	83.8
hs30-1	15	48.5
hs30-2	15	66.0
hs31-1	15	21.1
hs31-2	15	51.4
hs32-1	15	53.4
hs32-2	15	60.3

Duplicate cultures of *B. subtilis* CB-312, growing exponentially at 43°C, were infected with the indicated phage at a M.O.I. of about 5. At either 5 or 15 min. p.i., chloramphenicol was added (to a final concentration of 130 µg/ml) to one of each pair of cultures. At 125 min. p.i., cultures were assayed for DNA synthesis (see Materials and Methods) at 20°C for 30 min.

hs proteins that are, in fact, renatureable. However, I can think of no likely way in which this sort of effect would cause the differential distributions of cold- and heat-sensitives observed. A second possibility is that the different distributions of heat- and cold-sensitive mutants reflect a real difference in the nature of heat- and cold-sensitive mutations and/or a difference in the susceptibility of different classes of protein to a heat- or cold-sensitive phenotype. In *E. coli* (Guthrie *et al.*, 1969) and *S. typhimurium* (Tai *et al.*, 1969), ribosome assembly mutants are very frequent among cold-sensitives. In T4 (Scotti, 1968) and Lambda (Cox and Strack, 1971), cs mutations are found to primarily affect phage morphogenesis. From the available data (Jarvik and Botstein, 1973), this also seems to be the case for P22. Perhaps the assembly of complex protein or protein-nucleic acid aggregates is particularly susceptible to a cold-sensitive phenotype. If so, it may be that the SP01 cistrons showing a predominance of cold-sensitive mutations specify proteins involved in such complexes, for instance a complex of replication enzymes. Two of the SP01 cistrons affected by csD0 mutations have known functions. One, cistron 21, codes for an initiation protein, while the other, cistron 28, codes for a protein modifier of the host RNA polymerase.

Cistron 29

As expected, mutants affected in the same cistron generally behaved in a similar manner in the temperature shift experiments (Section I). However, cistron 29 (dUMP hydroxymethylase) mutants

were an exception, since the three hydroxymethylase (HMase) mutants examined fell into each of the three phenotypic classes (see Figure 1). It seems reasonable to expect HMase mutants to fall into classes I or II if a sufficient pool of HMdUMP remained after inactivation of the HMase to allow DNA synthesis to continue. However, hs29-2 is in class III (stops DNA synthesis immediately upon shifting to restrictive temperature). It does not seem likely that this could be explained simply by invoking a smaller HMdUMP pool size for hs29-2, since the HMase activity of extracts of hs29-2 infected cells was as great at both 30 and 43 C as that of hs29-1, which shuts off replication slowly (James Cregg, personal communication). Greenberg and co-workers (Tomich *et al.*, 1974; Chiu *et al.*, 1976) have argued that dCMP hydroxymethylase, the equivalent enzyme in T4 infection, is part of a complex which functions both for nucleotide synthesis and directly in DNA polymerization. The existence of a similar complex in SP01-infected *B. subtilis* could explain the varying phenotypes of cistron 29 mutants, with those in class I and II affecting only the nucleotide synthesis function, while those in class III affect the polymerization function.

Cistron 27

As noted in Section I, a cistron 27-specific screen yielded several hs mutants which failed to complement sus27-1 (Table XV). However, I hesitate to assign these mutants to cistron 27 since they differ phenotypically from sus27-1. Although sus27-1 is DO (Okubo *et al.*, 1972; my unpublished results), both of the hs mu-

TABLE XV. Liquid complementation and recombination between K13, K18, and sus27-1

Mutant(s)	Burst size[a]	% Recombination[b]
K13	0.45	
K18	1.2	
sus27-1	0.05	
K13 x K18	1.7	<0.003
K13 x sus27-1	1.1	<0.003
K18 x sus27-1	1.4	<0.003
hs22-1	0.11	
K13 x hs22-1	16.1	12.9
K18 x hs22-1	27.0	12.8
sus27-1 x hs22-1	32.3	6.0

[a]Procedure for liquid complementation was as described in legend to Table IV.

[b]CB-312 was grown to a cell density of 2 x 10^8 cells/ml at 37°C then shifted to 30°C for 10 min. The desired phage were added at a M.O.I. of 5 for each phage strain, incubation was continued at 30°C with shaking, and, at 15 min. p.i., anti-SP01 antiserum was added. At 50 min. p.i. cultures were diluted 1000-fold into fresh VY prewarmed to 30°C. At 200 min. p.i. lysates were plated on CB-312 at 43° and 30°C.

tants studied (K13 and K18) synthesize 50 to 100 percent of wt levels of DNA at the restrictive temperature. This DNA is phage DNA as judged by its buoyant density in equilibrium CsCl centrifugation. These mutants do not, however, cause lysis, a characteristic which explains their selection by the screening procedure and which is otherwise true only of replication-deficient strains. If CB-312 is infected at 43 C with these strains and then plated for surviving cells shortly thereafter, it is found that about 10% of the cells survive but that the resulting colonies are phage-eaten in the center, giving them a "doughnut" appearance. It is possible that these mutations are located in a cistron on which sus27-1 has a polar effect. However, as shown in Table XV, they are located so close to sus27-1 that no recombination can be detected between either of them and sus27-1.

SUMMARY AND CONCLUSION

An elaboration and modification of a screening procedure for the isolation of tsDO mutants was developed. It is hoped that this procedure will prove to be applicable to other viral systems and thus increase knowledge of the various ways in which DNA can be relicated.

The screening procedure was used to isolate both hs and cs, DO mutants of SP01. It was found that the distributions of the cs an hs mutants differed markedly. It may be that there is a difference in the nature of cs and hs mutations and that these mutants will allow one to gain insights into what these differences might be.

The tsDO mutants were found to be affected in at least 8 of the 9 known SP01 DO cistrons. On the basis of their ability to synthesize DNA following a shift to restrictive temperature, the mutants were classified into 3 groups as follows: (I) DNA synthesis continues, (II) DNA synthesis slowly stops, and (III) DNA synthesis rapidly stops. Mutants affected in cistrons 23, 28, and 29 fell into classes I or II. This was not unexpected since these cistrons are thought to code for a deoxynucleotide kinase, a transcriptional control protein, and a hydroxymethyltransferase, respectively, and so are involved only indirectly in DNA replication. What was unexpected, however, was that one cistron 29 mutant shut-off DNA synthesis immediately following a shift to restrictive temperature. This suggests that the SP01 hydroxymethyltransferase may be directly involved in DNA replication. Mutants in cistrons 21, 22, 30, 31, and 32 fall into class III and so are directly involved in replication. Two of

these cistrons, 21 and 32, code for proteins involved specifically in the initiation step of DNA replication, while the other 3 cistrons are presumably involved in the elongation step. Furthermore, mutants in cistron 32 are also affected in the termination of a round of replication.

The mutants also were used to map the origins and directions of replication of SP01. It was found that SP01 possesses at least two sites at which replication originates and that replication proceeds unidirectionally to the left from an origin near cistron 32. The simplest model consistent with the data presented has only two origins of replication for SP01, one near cistron 32 and one in a region of the genome in which no genetic markers exist. The model postulates that the SP01 DNA molecule circularizes prior to replication and that replication proceeds bidirectionally from this second origin.

It is hoped that the availability of these mutants will prove to be helpful in answering questions involving the mechanisms and regulation of initiation, questions such as: Why does replication sometimes proceed unidirectionally and sometimes bidirectionally? Does replication involve the covalent attachment of a new DNA chain to an old one? Is RNA priming used to initiate replication on both strands or just one (or none)? If an RNA primer is used, how does the DNA polymerase recognize the primer as different from mRNA? If an RNA primer is used, how does the DNA polymerase recognize when to start adding dNTPs to the chain? How is the timing of these events regulated and coordinated? In viral systems, is there any regula-

tion of the time between initiation events? What is necessary to terminate a round of replication? Finally, what role (if any) does the membrane play in all of this?

REFERENCES

Baas, P.D., and H.S. Jansz. 1972. J. Mol. Biol. *63:* 569-576.

Baas, P.D., H.S. Jansz, and R.L. Sinsheimer. 1976. J. Mol. Biol. *102:* 633-656.

Beyersmann, D., W. Messer, and M. Schlicht. 1974. J. Bacteriol. *118:* 783-789.

Bird, R.E., J. Louarn, J. Martuscelli, and L. Caro. 1972. J. Mol. Biol. *70:* 549-566.

Bourguignon, G.J., T.K. Sweeney, and H. Delius. 1976. J. Virol. *18:* 245-259.

Brutlag, D., R. Schekman, and A. Kornberg. 1971. Proc. Natl. Acad. Sci. (USA) *68:* 2826-2829.

Carl, P. 1970. Mol. Gen. Genet. *109:* 107-122.

Chen, M.C.Y., E. Birkemeier, and N.P. Salzman. 1976. J. Virol. *17:* 614-621.

Chiu, C.-S., P.K. Tomich, and R. Greenberg. 1976. Proc. Natl. Acad. Sci. *73:* 757-761.

Copeland, J.C. 1974. Genetics *78:* 1015-1034.

Cox, J.H., and H.B. Strack. 1971. Genetics *67:* 5-17.

Crosa, J.H., L.K. Luttropp, F. Heffron, and S. Falkow. 1975. Mol. Gen. Genet. *140:* 39-50.

Danna, K.J., and D. Nathans. 1972. Proc. Natl. Acad. Sci. USA *69:* 3097-3100.

Delius, H., C. Howe, and A. Kozinski. 1971. Proc. Natl. Acad. Sci. USA *68:* 3049-3053.

Dressler, D., J. Wolfson, and M. Magazin. 1972. Proc. Natl. Acad. Sci. USA *69:* 998-1002.

Dubnau, D., C. Goldthwaite, I. Smith, and J. Marmur. 1967. J. Mol. Biol. *27:* 163-185.

Duffy, J.J., L. Petrusek, and E.P. Geiduschek. 1975. Proc. Natl. Acad. Sci. USA *72:* 2366-2370.

Edgar, R.S., and I. Lielausis. 1964. Genetics *49:* 649-662.

Eisenberg, S., B. Harbers, C. Hours, and D.T. Denhardt. 1975. J. Mol. Biol. *99:* 107-123.

Eisenberg, S., J.F. Scott, and A. Kornberg. 1976. Proc. Natl. Acad. Sci. USA *73:* 1594-1597.

Fox, T.D., R. Losick, and J. Pero. 1976. J. Mol. Biol. *101:* 427-433.

Fralick, J.A., and K.G. Lark. 1973. J. Mol. Biol. *80:* 459-475.

Francke, B., and D.S. Ray. 1971. J. Mol. Biol. *61:* 565-586.

_____, and _____. 1972. Proc. Natl. Acad. Sci. USA *69:* 475-479.

Freifelder, D., and I. Kirschner. 1971. Virology *44:* 223-225.

Fujita, D.J., B.M. Ohlsson-Wilhelm, and E.P. Geiduschek. 1971. J. Mol. Biol. *57:* 301-317.

Gefter, M.L. 1975. Ann. Rev. Biochem. *44:* 45-78.

Gage, L.P., and E.P. Geiduschek. 1971. J. Mol. Biol. *57:* 279-300.

Geisselsoder, J. 1976. J. Mol. Biol. *100:* 13-22.

Green, D.M. 1966. J. Mol. Biol. *22:* 1-13.

Gudas, L.J., R. James, and A.B. Pardee. 1976. J. Biol. Chem. *251:* 3470-3479.

Guthrie, C., H. Nashimoto, and M. Nomura. 1969. Proc. Natl. Acad. Sci. USA *63:* 384-391.

Harford, N. 1976. J. Bacteriol. *121:* 835-847.

Harris-Warrick, R.M., Y. Elkana, S.D. Ehrlich, and J. Lederberg. 1976. Proc. Natl. Acad. Sci. USA *72:* 2207-2211.

Helland, D., and A.P. Nygaard. 1975. Febs Letters *50:* 13-16.

Hemphill, H.E., and H.R. Whiteley. 1975. Bacteriol. Rev. *39:* 257-315.

Henry, T., and R. Knippers. 1974. Proc. Natl. Acad. Sci. USA *71:* 1549-1553.

Hiraga, S., and T. Saitoh. 1974. Mol. Gen. Genet. *132:* 49-62.

Hirota, Y., J. Mordoh, and F. Jacob. 1970. J. Mol. Biol. *53:* 369-387.

Huberman, J.A., and A.D. Riggs. 1968. J. Mol. Biol. *32:* 327-341.

Jacob, F., S. Brenner, and F. Cuzin. 1963. Cold Spring Harbor Symp. *28:* 329-347.

Jarvik, V., and D. Botstein. 1973. Proc. Natl. Acad. Sci. USA *70:* 2046-2050.

Kahan, E. 1966. Virology *30:* 650-660.

_____. 1971. Virology *46:* 634-637.

Kallen, R.G., M. Simon, and J. Marmur. 1962. J. Mol. Biol. *5:* 248-250.

Karamata, D., and J. Gross. 1970. Mol. Gen. Genet. *108:* 277-287.

Klein, A., and F. Bonhoeffer. 1972. Ann. Rev. Biochem. *41:* 301-332.

Klotz, G. 1975. Mol. Gen. Genet. *120:* 95-100.

_____, and H. Ch. Spatz. 1971. Mol. Gen. Genet. *110:* 367-373.

Kohiyama, M., D. Cousin, A. Ryter, and F. Jacob. 1966. Ann. Inst. Pasteur *110:* 465-486.

Kornberg, A. 1974. DNA Synthesis. W.H. Freeman and Co., San Francisco.

_____. 1976. In "RNA Polymerase". pp. 331-352. Cold Spring Harbor Laboratory, Cold Spring Harbor.

_____, I.R. Lehman, M.J. Bessman, and E.S. Simms. 1956. BBA *21:* 197-198.

Kozinski, A.W., and A.H. Doermann. 1975. Proc. Natl. Acad. Sci. USA *72:* 1734-1738.

Kriegstein, H.J., and D.S. Hogness. 1974. Proc. Natl. Acad. Sci. USA *71:* 135-139.

Laurent, S.J., and F.S. Vannier. 1973. J. Bacteriol. *114:* 474-484.

Levner, M.H. 1972. Virology *48:* 417-429.

_____, and N.R. Cozzarelli. 1972. Virology *48:* 402-416.

Lin, N. S.-C., and D. Pratt. 1972. J. Mol. Biol. *72:* 37-49.

McAllister, W.T., and D.M. Green. 1973. J. Virol. *12:* 300-309.

Marsh, R.C., A.M. Breschkin, and G. Mosig. 1971. J. Mol. Biol. *60:* 213-233.

Marunouchi, T., and W. Messer. 1973. J. Mol. Biol. *78:* 211-228.

Masters, M., and P. Broda. 1971. Nature New Biol. *232:* 137-140.

Matthews, C.K. 1971. Bacteriophage Biochemistry. Von Nostrand Reinhold Co., New York.

Mendelson, N.H., and J.D. Gross. 1967. J. Bacteriol. *94:* 1603-1608.

Messer, W., L. Dankwarth, R. Tippe-Schindler, J.E. Womack, and G. Zahn. In "DNA Synthesis and Its Regulation," (Goulian, M., and P. Hanawalt, eds.), W.A. Benjamin, Inc., Menlo Park, California.

Mosig, G. 1970. J. Mol. Biol. *53:* 503-514.

_____, D.W. Bowden, and S. Bock. 1972. Nature New Biol. *240:* 12-16.

Nester, E.W., and J. Lederberg. 1961. Proc. Natl. Acad. Sci. USA *47:* 52-55.

Newlon, C.S., T.D. Petes, L.M. Hereford, and W.L. Fangman. 1974. Nature *247:* 32-35.

Okubo, S., T. Yanagida, D.J. Fujita, and B.M. Ohlsson-Wilhelm. 1972. Biken J. *15:* 81-97.

Okun, L.M. 1969. Ph.D. Thesis, Stanford University, Stanford, California.

O'Sullivan, M.A., and N. Sueoka. 1967. J. Mol. Biol. *27:* 349-368.

_____, and _____. 1972. J. Mol. Biol. *69:* 237-248.

Parker, D.L., and D.A. Glaser. 1974. J. Mol. Biol. *87:* 153-168.

Perlman, D., and R.H. Rownd. 1976. Nature *259:* 281-284.

Portalier, R., and A. Worcel. 1976. Cell *8:* 245-255.

Prescott, D.M., and P.L. Kuempel. 1972. Proc. Natl. Acad. Sci. USA *69:* 2842-2845.

Riva, S., C.V. Sluis, G. Mastromei, C. Attolini, G. Massa, M. Polsinelli, and A. Falaschi. 1975. Mol. Gen. Genet. *137:* 185-202.

Schekman, R., J.H. Weiner, A. Weiner, and A. Kornberg. 1975. J. Biol. Chem. *250:* 5859-5865.

Schnös, M., and R.B. Inman. 1970. J. Mol. Biol. *51:* 61-73.

Scotti, P.D. 1968. Mutation Res. *6:* 1-14.

Shapiro, B.M., A.G. Siccardi, Y. Hirota, and F. Jacob. 1970. J. Mol. Biol. *52:* 75-89.

Shuster, R.C., and A. Weissback. 1969. Nature *223:* 852-853.

Siccardi, A.G., B.M. Shapiro, Y. Hirota, and F. Jacob. 1971. J. Mol. Biol. *56:* 475-490.

Siegel, P.J., and M. Schaechter. 1973. Ann. Rev. Microbiol. *27:* 261-282.

Spizizen, J. 1958. Proc. Natl. Acad. Sci. USA *44:* 1072-1078.

Stewart, C.R. 1969. J. Bacteriol. *98:* 1239-1247.

_____, M. Cater, and B. Click. 1971. Virology *46:* 327-336.

_____, B. Click, and M.F. Tole. 1972. Virology *50:* 653-663.

Studier, F.W. 1972. Science *176:* 367-376.

Tai, P.-C., D.P. Kessler, and J. Ingraham. 1969. J. Bacteriol. *97:* 1298-1304.

Talavera, A., F. Jimenez, M. Salas, and E. Viñuela. 1971. Virology *46:* 586-595.

Tegtmeyer, P. 1972. J. Virol. *10:* 591-598.

Tevethia, M.J., J.N. Baptist, and M. Mandel. 1974. J. Bacteriol. *119:* 961-975.

Tomich, P.K., C.-S. Chiu, M.G. Wovcha, and G.R. Greenberg. 1974. J. Biol. Chem. *249:* 7613-7622.

Van der Vliet, P.C., and J.S. Sussenbach. 1975. Virology *67:* 415-426.

Wada, C., and T. Yura. 1974. Genetics *77:* 199-220.

Watson, J.D. 1972. Nature New Biol. *239:* 197-201.

_____, and F.H.C. Crick. 1953a. Nature *171;* 737-738.

_____, and _____. 1953b. Cold Spring Harbor Symp. Quan. Biol. *18:* 123-131.

Wechsler, J.A., and J.D. Gross. 1971. Mol. Gen. Genet. *113:* 273-284.

_____, and M. Zdzienicka. 1975. In "DNA Synthesis and Its Regulation," pp. 624-637, (Goulian, M., and P. Hanawalt, eds.), W.A. Benjamin, Inc., Menlo Park, California.

Weintraub, S., and S. Dales. 1974. Virology *60:* 96-127.

White, K., and N. Sueoka. 1973. Genetics *73:* 185-214.

Yehle, C.O., and A.T. Ganesan. 1972. J. Virol. *9:* 263-272.

_____, and _____. 1973. J. Biol. Chem. *248:* 7456-7463.

Yoshikawa, H., and N. Sueoka. 1963. Proc. Natl. Acad. Sci. USA *49:* 806-813.