JOURNAL OF VIROLOGY, Jan. 1977, p. 147-152
Vol. 21, No. 1

Printed in U.S.A.

Initiation and Termination Mutants of *Bacillus subtilis* Bacteriophage SPO1

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Received for publication 2 August 1976

Mutants affected in cistrons 21 and 32 of bacteriophage SPO1 are defective specifically in the initiation of DNA replication. Mutations in cistron 32 also specifically affect the termination of replication.

In several bacterial and viral systems, certain gene products are known to be necessary specifically for the initiation of DNA replication (see, for example, references 12, 13, 21, and 23). In this paper, we use density transfer analysis to demonstrate such a role for two gene products of *Bacillus subtilis* phage SPO1. In addition, we show that one of these gene products plays a specific role in the termination of DNA replication.

SPO1 has nine cistrons whose products are known to be necessary for DNA synthesis (16). We have previously analyzed temperature-sensitive (*ts*) mutations in eight of these nine cistrons by temperature shift experiments. We concluded that none of the mutations affected a gene product that was necessary only for initiation of the first round of replication. However, the products of five of the cistrons are candidates for a specific role in the initiation of each round of replication (8). To determine which, if any, of these five gene products are actually initiation proteins, we have combined the temperature shift with a density transfer, as follows.

B. subtilis, growing in heavy medium, is infected at permissive temperature with a *ts* mutant carrying light-density DNA. Midway through the first round of replication, the culture is shifted to the restrictive temperature. With a mutant that 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 mutations in cistrons 21 and 32.

MATERIALS AND METHODS

Bacterial strains. *B. subtilis* CB-10 has been described as SB-1 by Nester and Lederberg (15). CB-312, -313, and -314 were provided by M. Mandel and were described (22) as strains 129, 151, and 135, respectively. CB-10 is suppressor$^-$, *his*$^-$, *trp*$^-$; CB-312 is suppressor$^-$, *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, and CB-313 was used to grow most of the suppressor-sensitive (*sus*) phage mutants. Some of the *sus* mutants grew only on CB-314. CB-327 was created by transforming CB-312 to prototrophy. It grows at 20°C, whereas CB-10 does not.

Phage strains. All *sus* mutants were generously provided by E. P. Geiduschek and have been previously described by Okubo et al. (16). For clarity of presentation, each mutant is given a standardized designation, the first term of which indicates the phenotype, the second term the cistron number, and the third term the specific mutation within that cistron. Thus, *hs*30-2 refers to a heat-sensitive (*hs*) mutant, affected in cistron 30 and designated no. 2. Table 1 shows the correspondence between the nomenclature used in this paper and the original designations of the *sus* mutants used. All *hs* and cold-sensitive (*cs*) strains used have been described (8). Although these *ts* strains were originally isolated using permissive temperatures of 20 and 37°C, respectively, 30°C was also permissive for all strains.

Media used. Bacterial and phage growth was generally in VY medium. Platings used TBAB bottom and top agar. These media, the deuterated media, and Spizizen minimal medium have been described (17, 19). Deuterated algal extract and deuterated sugars were gifts of H. Crespi.

Preparation of ^{3}H-labeled 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 multiplicity of infection of about 10:1. Shortly thereafter, [6-H^3]uridine was added to about 10 μCi/ml. After lysis, the culture was centrifuged

TABLE 1. *sus mutants used*

Designation in this work	Original designation	Reference
*sus*1-1	F12	16
*sus*2-1	F47	16
*sus*3-1	F37	16
*sus*11-1	F6	16
*sus*14-1	F11	16
*sus*23-1	N5	16
*sus*27-1	HA20	16
*sus*30-2	O52	16
*sus*32-1	F38	16
*sus*33-1	F14	16
*sus*34-1	F4	16
*sus*35-1	F24	16
*sus*36-1	N34	16

at 3,000 × *g* for 10 min. The supernatant fraction was then recentrifuged at 13,300 × *g* for 90 min. The supernatant fraction was discarded, and the pellet was resuspended overnight in one-fifth volume of deuterated medium.

Preparation of deuterated *B. subtilis*. *B. subtilis* CB-327 was grown for about 16 h at 37°C in deuterated medium. Samples 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 1.2×10^8 cells/ml in deuterated medium. The culture was shifted to 43°C and then infected at a multiplicity of infection of about 3:1 with the appropriate *hs* mutant (light density, ^{3}H-labeled, and resuspended in deuterated medium). At 25 min postinfection, 5-ml samples of the infected culture were transferred to 250-ml flasks, precooled at 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 onto 10 ml of frozen Spizizen minimal medium or by pouring the culture into flasks already at 43°C (restrictive temperature) and incubating for 15 min before pouring onto the Spizizen minimal medium ice.

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

The DNA extraction procedure was essentially that of Okun (Ph.D. thesis, Stanford University, Stanford, Calif., 1969) as modified by Stewart et al. (20). 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, 0.015 M sodium citrate).

Shearing of DNA. DNA was sheared by passing three times through a 30-gauge hypodermic needle (10).

Equilibrium CsCl centrifugation. The procedure for equilibrium CsCl centrifugation was as described (18), with the following modifications. Centrifugation was in a Beckman L2-65B, using a type 65 rotor at 32,000 rpm for about 66 h. Each polyallomer tube contained 4.1 ml of sample plus water, plus 5.55 g of CsCl. This mixture produced 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 samples of each on Whatman glass-fiber filters. The filters were dried, toluene-based scintillation fluid was added, and the samples were counted in a Packard Tri-Carb scintillation counter.

Preparation of competent cells. Competent cells were prepared freshly each day as described (18).

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 (9). Each pair of adjacent fractions from the CsCl gradient was pooled. A 0.45-ml amount 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 SPO1 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 CB-10 (suppressor$^-$) (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 h, and PFUs were counted. CB-10 was used as the bacterial indicator because, unlike CB-312, it was insensitive to the concentrations of CsCl used. With the DNA concentrations used, there was no measurable transfecting activity, so plaque formation depended upon recombination between the *sus*$^-$ genome and DNA carrying its *sus*$^+$ allele.

Calculation of percentage of replication. For each CsCl gradient, it was determined, by inspection of the ^{3}H counts per minute profile, which fractions constituted the light, hybrid, and heavy regions of the gradient. For any given marker, the activity in each region was determined by marker rescue assay. The percentage of replication was then calculated as described by Copeland (2), using the formula [(HL/2)/(LL + HL/2)] × 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.

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

RESULTS

Initiation. Deuterated *B. subtilis*, growing in heavy medium, was infected at restrictive

temperature with temperature-sensitive, replication-deficient SPO1 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 min. (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 SPO1 suppressor-sensitive (*sus*) markers. For each marker, the proportion replicated was determined from the proportion of that marker's activity found in the hybrid region.

Figure 1 gives an example of the different density shifts of two marker rescue activities in the same CsCl gradient, showing that cistron 30 has replicated before cistron 3. By making similar comparisons for all markers, we have established the sequence of replication of the various markers on the SPO1 genome. An example of this is shown in Fig. 2, in which the percentage of replication for each marker is plotted against its position on the genetic map. The cistrons on the SPO1 map are arranged in numerical order, from 1 to 34 (16). Replication of the major portion of the genome begins near cistron 32 and proceeds leftward to a terminus in the vicinity of cistron 3 (Fig. 2).

At least one, and possibly two, additional origin is used for the replication of the leftmost and rightmost portions of the genome (cistrons 1, 2, 33, 34) (data to be published elsewhere). For convenience of presentation, this paper concerns itself only with the effect of mutations on replication of the major portion of the genome and 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, we 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 3 and 4 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 ex-

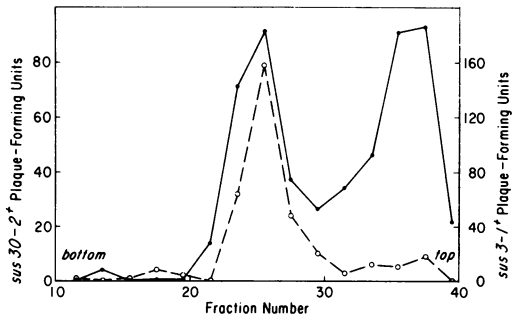

FIG. 1. *Differential density shifts of SPO1 markers in the same CsCl gradient. ^{3}H-labeled hs30-1 was allowed to replicate in heavy medium for 10 min at 20°C and then was shifted to 43°C for 15 min. The DNA was then extracted, sheared, and centrifuged to equilibrium in CsCl. Pairs of adjacent fractions of the gradient were pooled, and samples of the pooled fractions were used in marker rescue assays with either sus3-1 or sus30-2. The number of plaques due to reversion of the sus marker (19 for sus3-1 and 14 for sus30-2) has been subtracted from all values. Note that the hs30-1 mutation is irrelevant in the marker rescue assays, since these are done at permissive temperature. See Materials and Methods for details. Symbols: (○) sus30-2; (●) sus3-1.*

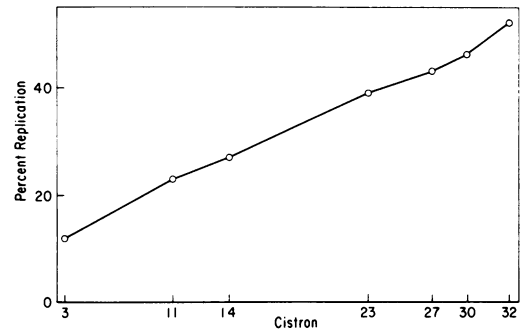

FIG. 2. *SPO1 replication gradient. Deuterated B. subtilis CB-327, growing in heavy medium, was infected with hs31-1 at 43°C. The culture was shifted to 20°C 25 min later (the preliminary incubation at restrictive temperature imposes some synchrony on the initiation of replication). After incubation for 20 min at 20°C, the culture was poured onto Spizizen 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 samples were assayed for marker rescue activity against sus markers in the indicated cistrons. The calculation of the percentage of replication for each marker was as described in Materials and Methods.*

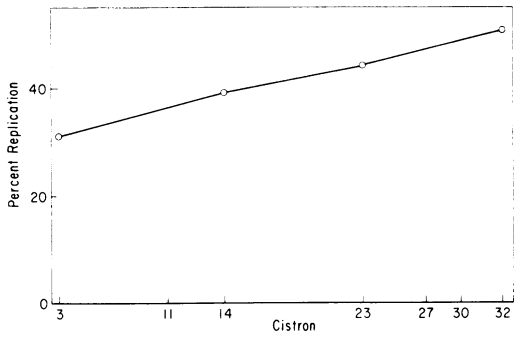

FIG. 3. *Replication of cs22-1. Deuterated B. subtilis CB-327, growing in heavy medium, was infected with cs22-1 at 20°C. The culture was shifted to 37°C 150 min later (the preliminary incubation at restrictive temperature imposes some synchrony on the initiation of replication). The culture was shifted back to 20°C 6 min later. After incubation for 60 min at 20°C, the culture was poured onto Spizizen 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 samples were assayed for marker rescue activity against sus markers in the indicated cistrons. The calculation of the percentage of 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 2 and 3, for example). This is probably due to the lesser degree of synchrony achieved with the cs mutants.*

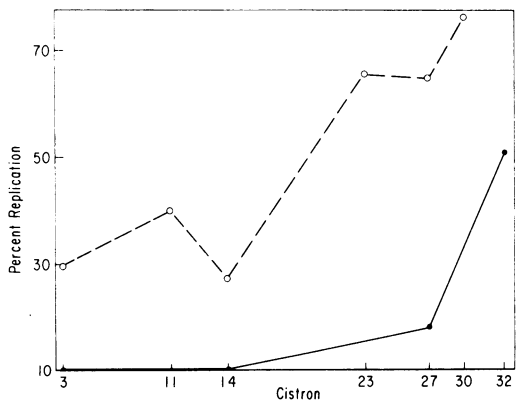

FIG. 4. *Replication of hs30-1 and hs31-2. Deuterated B. subtilis CB-327, growing in heavy medium, was infected with either hs30-1 or hs31-2 at 43°C. The culture was shifted to 20°C 25 min later (the preliminary incubation at restrictive temperature imposes some synchrony on the initiation of replication). Ten (hs30-1) or twenty (hs31-2) minutes later, the culture was shifted back to 43°C. After incubation for 15 min at 43°C, the culture was poured onto Spizizen 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 samples were assayed for marker rescue activity against sus markers in the indicated cistrons. The calculation of the percentage of replication for each marker was as described in Materials and Methods. Symbols: (○) hs30-1; (●) hs31-2.*

periment.) We 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 replication when replication was stopped by shifting to restrictive temperature, but gave the expected replication gradients when stopped by chilling (Tables 2 and 3).

The lack of replication gradients in these mutant-infected cultures, after 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. We 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 *sus*35-1 and *sus*36-1 markers show a high frequency of recombination with each other and with all other known SPO1 mutants (16; our unpublished data), and their map location is, therefore, not known. They are located very near a terminus of rep-

TABLE 2. *Replication of a cistron 21 mutant*[a]

Marker	% Replication of various markers when replication was stopped by:			
	A		B	
	Chilling	Shift to restrictive temp	Chilling	Shift to restrictive temp
*sus*3-1	15	38	32	53
*sus*11-1	ND[b]	ND[b]	31	44
*sus*14-1	14	38	29	49
*sus*23-1	18	40	40	56
*sus*27-1	15	ND	47	51
*sus*30-2	25	32	59	54
*sus*32-1	30	34	56	45
*sus*35-1	11	33	28	46

[a] The procedure was the same as that described in the legend to Fig. 3, except that infection was with *cs*21-1 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.

[b] ND, Not determined.

TABLE 3. *Replication of cistron 32 mutants*[a]

Marker	% Replication of various markers when replication was stopped by:			
	A		B	
	Chilling	Shift to restrictive temp	Chilling	Shift to restrictive temp
*sus*3-1	3	46	39	55
*sus*11-1	18	48	39	50
*sus*14-1	20	49	46	58
*sus*23-1	37	48	68	56
*sus*27-1	31	41	62	54
*sus*30-2	38	49	89	56
*sus*32-1	66	42	ND[b]	50
*sus*35-1	13	24	29	27
*sus*36-1	11	29	ND	18

[a] The procedure was the same as that described in the legend to Fig. 4, except that infection was with *hs*32-1 (A) or *hs*32-2 (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, the *sus*1-1, *sus*2-1, *sus*33-1, and *sus*34-1 markers behaved like the bulk of the markers above.

[b] ND, Not determined.

lication, since in density transfer, temperature shift experiments like those above they are always the last, or nearly the last, markers to be replicated (see, for instance, Tables 2 and 3). Tentative results obtained by James Cregg in this laboratory suggest that they are near the terminus in the rightmost segment of the genome.

When initiation of a new round of replication is blocked by shifting *cs*21-1 to restrictive temperature, the *sus*35-1 marker is replicated to the same extent as all other markers (Table 2). In a similar experiment (data not shown), *sus*36-1 behaved in the same way. Thus, *cs*21-1, although prohibiting initiation, has no effect on the replication of these terminal markers. In contrast, when reinitiation is blocked by shifting either *hs*32-1 or *hs*32-2 to restrictive temperature, both the *sus*35-1 and the *sus*36-1 markers remain apparently less replicated than all other markers (Table 3). Thus, the cistron 32 product seems to play a specific role in the termination of replication.

DISCUSSION

Initiation. We have shown that mutants in cistrons 21 and 32 of SPO1 are affected specifically in the initiation of DNA replication. That these mutants are affected in the initiation of each round of replication can be deduced from the following. (i) When infection is allowed to proceed at restrictive temperature, there is no first round of replication (our unpublished data). Therefore, the proteins coded by cistrons 21 and 32 are necessary for the initiation of the first round of replication. (ii) 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. (iii) If cells infected with these mutants are allowed to replicate through many rounds of replication and then shifted to restrictive temperature, DNA synthesis is rapidly shut off (8). Thus, the affected proteins are necessary for later rounds of replication as well.

Elongation. Mutants in cistrons 22, 30, and 31 exhibit a rapid shut off of DNA synthesis when shifted to restrictive temperature (8) 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. By "termination," we mean the replication of the terminal region as well as any specific events occurring at the terminus itself. One might be tempted to turn this around and propose that reinitiation 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 21 prohibits reinitiation it allows replication of all tested markers, including those nearest to a terminus. We 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 we are 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 (14) did report, however, that replication of the *Escherichia coli* terminus required continued protein synthesis.

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.

Although the cistron 32 mutants prevent

much of the density shift of cistrons 35 and 36, some replication is still permitted (see Table 3). 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.

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 ϕX174, whose replication origin lies within cistron A, which specifies a protein that introduces a nick necessary for initiation (5, 6, 11). Although this protein differs from the SPO1 gene 32 protein in that it is *cis* active, it is possible that the gene 32 protein might also function in initiation by introducing a nick in the DNA molecule.

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant no. GM02221 from the NIH and Career Development Award no. GM70044 to C.R.S., both from the National Institute of General Medical Sciences.

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