Heat Induction of Prophage ϕ 105 in *Bacillus subtilis*: Replication of the Bacterial and Bacteriophage Genomes

RICHARD W. ARMENTROUT AND LARS RUTBERG

Department of Bacteriology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Received for publication 14 June 1971

A temperature-inducible mutant of temperate *Bacillus* bacteriophage ϕ 105 was isolated and used to lysogenize a thymine-requiring strain of *Bacillus subtilis* 168. Synthesis of phage and bacterial deoxyribonucleic acid (DNA) was studied by sucrose gradient centrifugation and density equilibrium centrifugation of DNA extracted from induced bacteria. The distribution of DNA in the gradients was measured by differential isotope and density labeling of DNA before and after induction and by measuring the biological activity of the DNA in genetic transformation, in rescue of phage markers, and in infectivity assays. At early times after induction, but after at least one round of replication, phage DNA remains associated with high-molecular-weight DNA, whereas, later in the infection, phage DNA is associated with material of decreasing molecular weight. Genetic linkage between phage and bacterial markers can be demonstrated in replicated DNA from induced cells. Prophage induction is shown to affect replication of the bacterial chromosome. The overall rate of replication of prelabeled bacterial DNA is identical in temperature-induced lysogenics and in "mock-induced" wild-type ϕ 105 lysogenics. The rate of replication of the bacterial marker *phe-1* (and also of *nia-38*), located close to the prophage in direction of the terminus of the bacterial chromosome, is increased in induced cells, however, relative to other bacterial markers tested. In temperature-inducible lysogenics, where the prophage also carries a *ts* mutation which blocks phage DNA synthesis, replication of both phage and bacterial DNA stops after about 50% of the phage DNA has replicated once. The results of these experiments suggest that the prophage is not initially excised in induced cells, but rather it is specifically replicated in situ together with adjacent parts of the bacterial chromosome.

The Campbell model (5) provides an elegant solution to the problem of integration and excision of temperate phage genomes. Integration is proposed to occur through a reciprocal recombinational event between a specific region on a circularized phage chromosome and a specific attachment site on the bacterial chromosome. Prophage excision is thought to represent a reversal of the integration event. There is strong genetic evidence for specific integration enzymes coded for by phages P2 (6), P22 (22), and lambda (8), as well as another enzyme, *xis*, in lambda (11) which together with the *int* product promotes excision of a resident prophage. Excision is believed to be the primary event in prophage induction. This is supported by experiments which show, for example, that genetic linkage between bacterial markers bracketing the prophage in-

creases after prophage induction (13) or that prophage deoxyribonucleic acid (DNA) can be recovered unreplicated in mature particles after heteroimmune superinfection (15). The primary DNA product after induction is thought to be a circularized DNA molecule equivalent in size to mature phage DNA. Presumably, excision of lambda prophage requires protein synthesis but not DNA synthesis (24).

DNA extracted from *Bacillus* bacteriophage ϕ 105 particles is a homogenous collection of molecules of low infectivity (3, 4, 18). The poor infectivity reflects some structural characteristic of the ends of the molecules (17). To study the structural basis for the biological activity of ϕ 105 DNA, we sought to isolate excised prophage DNA from lysogenic bacteria carrying a temperature-inducible mutant of ϕ 105 as prophage.

The results of these experiments indicate, however, that prophage replication rather than prophage excision is the primary event associated with heat induction of $\phi 105$ lysogenic bacteria. The experiments also suggest a common mechanism of control of replication of both bacterial and phage DNA in induced cells.

MATERIALS AND METHODS

Bacteria and phage. The bacterial strains employed are listed in Table 1. Some of these strains were used as recipients in transformation experiments, and the map order of the markers used on the bacterial chromosome is origin-*purA16-leu-2, leu-5, -ilvA1*-prophage $\phi 105$ -*phe-1-nia-38-metB5*-terminus (7, 16).

Phage $\phi 105$ and the temperature-sensitive (*ts*) and suppressor-sensitive (*sus*) mutants employed have recently been described (2). As a convenient marker of low background, *sus19* in gene C was used in marker rescue experiments. Mutation *tsN31* in gene K (2) is referred to here as *Kts31*. It has been previously shown that gene K is essential for phage DNA synthesis (2). The temperature-inducible mutant *cts23* was isolated after treatment of infected cells with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine (2). The *cts23* mutation maps close to *sus11* of gene J (*unpublished data*). Lysogenic derivatives of T^- bacteria were isolated from the central growth in plaques formed on these bacteria at 30 C. The growth rates of T^- and its lysogenic derivatives are identical in the media employed; in Min-CH (*see below*) at 30 C, the doubling time of these strains is about 60 min.

Media and growth of bacteria and phage. Bacterial strains were maintained on Tryptose Blood Agar Base (TBAB, Difco) plates. Bacteria were grown in Spizizen's minimal medium (23) supplemented with 0.05% casein hydrolysate (Difco) and 20 μ g (per ml) of any amino acid required and 10 μ g of thymidine per ml when required. We refer to this medium as Min-CH. Phage assays and preparation of phage stocks were done as described (2, 16, 18).

Assays of the "biological activity" of DNA samples. The relative amounts of bacterial genes, phage genes, and complete phage genomes present in DNA samples were measured by transformation, marker rescue,

and transfection, respectively. For these measurements, competent cells were obtained by the method of Anagnostopoulos and Spizizen (1). Transformants were assayed by spreading 0.1 ml of appropriate dilutions of the competent culture on selective media after exposure of the cells to a dilution of the DNA sample (2, 18). In marker rescue experiments, competent SR135 cells were exposed to a DNA sample, and, 20 min after addition of DNA, the cells were superinfected with an excess of mutant phage $\phi 105$ *Csus19*. *Bacillus subtilis* SR135 carries a suppressor gene which permits the superinfecting phage to grow and recombine with phage DNA taken up by competent cells from the DNA sample. Prior to lysis, the superinfected cells were diluted and 0.1-ml samples were plated with W168 as indicator; W168 does not permit growth of the superinfecting mutant phage. Details of the method have recently been described by us (2, 18). Assay of complete phage genomes was by transfection as described (2, 18). Competent cultures of SR135 were exposed to an appropriate dilution of DNA sample, and prior to lysis the bacteria were diluted and 0.1-ml samples were plated by using SR135 as indicator bacteria. The number of plaques that appeared on the plates was taken as a measure of the number of whole "active" phage DNA molecules taken up by competent cells.

By use of these assays, the distribution of transforming activity, marker rescue, and infectivity was determined over a given gradient. The competence of the different strains used in these assays varied, and the results obtained represent relative activities rather than specific activities (e.g., transformants per microgram of DNA corrected for constant competence). The data are presented on convenient scales to show the distributions of activities over the gradient, and the scales need not be the same for all graphs.

Induction of heat-inducible lysogenic bacteria. Lysogenic bacteria were grown in 20 ml of Min-CH at 30 C to a density of about 5×10^7 bacteria per ml. For prelabeling, 10 μ Ci of 3 H-thymidine (specific activity, 5 Ci/mmol; The Radiochemical Centre, Amersham) was added per 20 ml in addition to 10 μ g of cold thymidine per ml. The cells were centrifuged, washed once with Min-CH, and suspended in 0.5 volume of fresh, thymineless Min-CH at 45 C. After 5 min at

TABLE 1. *Bacterial strains*

Strain	Relevant properties ^a	Source
W168	Prototrophic, <i>su</i> ⁻	J. Spizizen
SR135	<i>spoA9, trp-7, su</i> ⁺³	J. A. Hoch
BR26	<i>trpC2, leu-2</i>	J. Spizizen
BR95	<i>trpC2, phe-1, ilvA1</i>	J. Spizizen
BR95 ($\phi 105$ <i>Ats15</i>)	<i>trpC2, phe-1, ilvA1</i> lysogenic for $\phi 105$ carrying mutation <i>tsN15</i>	Our collection
BD25	<i>purA16, leu-5, nia-38, metB5</i>	A. Lindahl-Adams
168T ⁻	<i>trpC2, thyA1, thyB1</i>	C. Anagnostopoulos

^a Properties: *su*⁺, carries the *su*³ suppressor gene; *su*⁻, nonsuppressing, *spo*, asporogenous; *trp*, tryptophan; *phe*, phenylalanine; *ilv*, isoleucine-valine; *pur*, purine (adenine); *leu*, leucine, *nia*, niacin, *met*, methionine, *thy*, thymine.

this temperature, the culture was shifted to 42 C and an equal volume of prewarmed Min-CH containing 20 μ g of thymidine per ml was added. For density labeling at 42 C, thymidine was omitted and instead the Min-CH contained 20 μ g of 5-bromodeoxyuridine (BUdR) per ml (Sigma Chemical Co., St. Louis, Mo.) and 14 C-thymidine (specific activity, 58 mCi/mmol; The Radioactive Centre, Amersham) to give a final concentration of 0.25 μ Ci per ml. The bacteria were incubated at 42 C with aeration, and samples were withdrawn for phage and bacterial assays and for extraction of DNA. T^- bacteria lysogenic for $\phi 105$ wild type are not induced by temperature shifts. When T^- ($\phi 105$) was treated as described above, it is referred to as mock-induced.

Extraction of DNA. Culture samples containing 2×10^8 to 5×10^8 bacteria were poured over frozen Min-CH and centrifuged. The pellets were suspended in 2 ml of 0.2 M NaCl-0.001 M ethylenediaminetetraacetic acid (EDTA) pH 8 (saline), lysozyme was added to a final concentration of 200 μ g/ml, and the suspension was incubated at 37 C for 5 to 10 min. When lysis occurred, sodium dodecyl sulfate was added to a final concentration of about 0.5%. After standing a few minutes at room temperature, 2 ml of washed [0.1 M tris(hydroxymethyl)aminomethane (Tris)-0.01 M EDTA, pH 8] phenol was added. DNA was extracted by gentle mechanical rolling of the sample at room temperature for 30 min (3). The sample was then centrifuged and the aqueous phase was removed with a "U"-shaped Pasteur pipette. The DNA solutions were dialyzed at 4 C for 16 hr against one change of about 1,000 volumes of saline. 14 C-labeled T7 DNA was prepared as described (3); 32 P-labeled P2 DNA was a gift from Jon Jonasson.

Sucrose gradients. All sucrose gradients were 5 to 20% sucrose in 0.05 M Tris(pH 8.0)-0.001 M EDTA, made up from boiled-stock solutions. Gradients were centrifuged at 14 C in a Spinco model L centrifuge. The SW50 rotor was run at 35,000 rev/min for 150 min, whereas the SW25.1 rotor was run at 24,000 rev/min for 180 min. Fractions were collected through a needle inserted into the bottom of the tubes. Ten-drop fractions were collected from the SW50 gradients, and 45-drop fractions were collected from the SW25.1 gradients. In the case of SW50 gradients, 0.05 ml from each fraction was added directly to 5 ml of dioxane-based scintillation fluid (3) and counted for radioactivity. Samples (0.1 ml) from the SW25.1 gradients were applied to squares of Whatman no. 3 filter paper, placed in cold 10% trichloroacetic acid, washed once with cold ethanol, and then dried under vacuum. The radioactivity on the filter papers was counted in 5 ml of toluene-based scintillation fluid (3). In all cases, the scintillation vials were precounted and the background radioactivity for each vial was subtracted from the sample counts. The biological activity of the fractions was assayed.

Equilibrium density centrifugation. To 2 ml of DNA sample was added 1 ml of saline and 3.8 g of CsCl to give a 56% (w/w) solution. The samples were centrifuged at 14 C in an SW50 rotor at 31,000 rev/min for about 64 hr. Fractions of eight drops were collected as described above. The refractive index of every fifth

fraction was read on a Zeiss Abbe refractometer. In each case, a linear density gradient was obtained. To each fraction was added 1 ml of boiled saline, and 0.5 ml was added to 2 ml of cold 10% trichloroacetic acid. These samples were filtered through HA filters (Millipore Corp., Bedford, Mass.). The filters were dried in air and counted for radioactivity by using a toluene-based scintillation fluid (3). The biological activity of the samples was assayed.

RESULTS

Heat induction of $\phi 105$ *cts23*. When T^- bacteria lysogenic for the $\phi 105$ mutant *cts23* are grown at 30 C and then shifted to inducing temperature, an increased phage DNA synthesis is seen some 10 min after the shift and after an additional 20 to 30 min the bacteria lyse and liberate a burst of phage (Fig. 1).

The $\phi 105$ *cts23* mutant was crossed with $\phi 105$ *Kts31* and a double mutant carrying both of the *ts* mutations was isolated. This double mutant was then used to lysogenize T^- bacteria to give the lysogen $T^-(\phi 105$ *cts23*, *Kts31*). Mutation *Kts31* is located in gene K which has previously been shown essential for phage DNA synthesis (2). After a shift to inducing temperature, $T^-(cts23, Kts31)$ does not produce any phage as long as it is kept at the high temperature. Samples of DNA extracted from such a culture after the temperature shift show no or very little increase in specific infectivity when assayed by transfection of competent SR135 cells (Fig. 1). The *Kts31* mutation thus effectively blocks phage DNA synthesis also after temperature induction of lysogenic bacteria.

Irreversible induction of $\phi 105$ *cts23* lysogenic bacteria also occurs in the absence of DNA synthesis; $T^-(\phi 105$ *cts23*) is rapidly killed in the absence of thymine when shifted to inducing temperature. The viability of T^- lysogenic for wild-type $\phi 105$ is not affected under the same conditions (Fig. 2). When cells from temperature-induced and thymine-starved cultures are plated with indicator bacteria in the presence of thymine, there is no decline in the number of infectious centers formed during a 30-min incubation period without thymine (Fig. 2). Thus, the lack of thymine after temperature induction does not damage the ability of the cells to produce phage when returned to a thymine-containing medium. On the other hand, there is evidence that damage to the prophage does occur during incubation in the absence of thymine. After 35 min without thymine, there is about a 10-fold decrease in the specific infectivity of DNA extracted from induced bacteria (Fig. 2). Marker rescue, however, is not affected (*not shown*).

This damage to the prophage DNA must be

FIG. 1. Temperature induction of $T^-(cts23)$ and $T^-(cts23, Kts31)$. The bacteria were grown to about 5×10^7 cells per ml, centrifuged, and suspended in fresh medium at 42 C. Samples were taken for infectious centers and for DNA extraction. The purified DNA samples were assayed for their infectivity by using competent SR135; the infectivity is expressed as plaques per microgram of DNA. Symbols: ●—●, $T^-(cts23)$ infectivity of DNA; ■—■, $T^-(cts23, Kts31)$ infectivity of DNA; ○—○, $T^-(cts23)$ infectious centers; □—□, $T^-(cts23, Kts31)$ infectious centers.

reversible because the bacteria will produce phage when supplied with thymine. In mock-induced wild-type lysogenics, no or a very slight decrease in prophage DNA infectivity is observed when the cells are starved of thymine. Thus, during induction in the absence of DNA synthesis, prophage DNA suffers reversible damage which decreases its infectivity.

Sucrose gradients of DNA from induced or infected bacteria. To investigate whether the damage that prophage DNA suffers after induction in the absence of DNA synthesis (thymine starvation) reflects some excision process, DNA from induced lysogens was examined in sucrose gradients. It was expected that high-molecular-weight bacterial DNA could be separated from the smaller phage-sized piece, and the excision process might be examined.

$T^-(cts23)$ was grown at 30 C in the presence of 3H -thymidine. The prophage was induced by heat, and the bacteria were incubated at 42 C with or without cold thymidine. Thirty minutes after induction, DNA was extracted and sedi-

FIG. 2. Effects of temperature induction on T^- lysogenic bacteria in the absence of thymine. The bacteria were grown to a density of about 5×10^7 cells per ml, centrifuged, and suspended in fresh medium at 45 C. Samples were taken for viable counts, infectious centers, and for DNA extraction. Symbols: □—□, $T^-(\phi 105)$ viable cells (v.c.); ■—■, $T^-(\phi 105)$ infectivity of DNA; ○—○, $T^-(cts23)$ v.c.; ●—●, $T^-(cts23)$ infectivity of DNA; △—△, $T^-(cts23, Kts31)$ v.c.; ▲—▲, $T^-(cts23, Kts31)$ i.c.; ▲—▲, $T^-(cts23, Kts31)$ infectivity of DNA.

mented in neutral sucrose gradients together with ^{14}C -labeled T7 DNA as a size marker. After a complete run, fractions were collected and assayed for radioactivity, for phage DNA activity, and for *phe-1* transforming activity. A similar experiment was also performed with DNA extracted from T^- bacteria infected with $\phi 105$ *cts23* at inducing temperature. The results of these experiments are shown in Fig. 3a-c. In the DNA sample extracted from lysogenic bacteria induced in the absence of thymine, tritium radioactivity and phage and bacterial DNA sediment together as high-molecular-weight species ($>200 \times 10^6$), indicating that prophage excision does not occur under these conditions (Fig. 3a).

When $T^-(cts23)$ lysogens are induced in the presence of thymidine, most of the tritium radioactivity is still associated with high-molecular-weight material at the time of sampling, indicat-

FIG. 3. Sucrose gradient centrifugation of DNA from induced *cts23* lysogens or *cts23*-infected cells. The bacteria were grown at 30°C with prelabeling of the bacterial chromosome with ^3H -thymidine to about 5×10^7 cells per ml. The bacteria were washed once and suspended in Min-CH (without any thymine) at 45°C for 5 min and then shifted to 42°C. After 30 min at inducing temperature, a sample was taken for DNA extraction. The purified DNA together with ^{14}C -labeled T7 DNA was centrifuged in the SW25.1 rotor as described in Materials and Methods. The fractions were tested for ^3H (●—●), ^{14}C (×---×), *phe*-1⁺ transforming activity (●---●), rescue of phage marker *sus19* (○---○), and for infectivity (○—○). (a) T-(*cts23*) with no thymine added during the experiment. (b) T-(*cts23*) with thymidine

ing no breakdown of preexisting DNA in the induced bacteria (Fig. 3b). Phage DNA active in the transfection or marker rescue assays is found in three main peaks in this sample. One peak of infectivity and marker rescue cosediments with the tritium peak. A second, more slowly sedimenting, peak (fraction 29) corresponds to the infectious vegetative DNA previously demonstrated in pulse-labeled DNA from mitomycin C-induced $\phi 105$ wild-type lysogenics (19). A third peak which exhibits marker rescue only is mature DNA (18, 19). Most of the *phe*-1 transforming activity cosediments with the tritium radioactivity, but a distinct peak is found at fraction 29. This peak of *phe*-1 activity is consistently reproducible and must represent newly synthesized bacterial DNA. In the DNA sample from infected bacteria, finally, tritium radioactivity and *phe*-1 transforming activity cosediment as high-molecular-weight material, whereas most of the phage DNA activity sediments more slowly and corresponds to vegetative and mature DNA (Fig. 3c).

Thus, 15 min after infection of T⁻ bacteria with phage *cts23*, there is little phage DNA present in the high-molecular-weight DNA from the bacterial chromosome. Therefore, the rapidly sedimenting phage DNA observed after prophage induction is not a very large form of vegetative phage DNA. The persistence of phage DNA activity among high-molecular-weight bacterial DNA even 30 min after prophage induction suggests that excision of prophage DNA has not occurred under these circumstances. Also, the experiments give no evidence for the existence of a uniquely phage-sized DNA early after prophage induction (see also Fig. 10a-c). Rather, the experiments suggest that excision may not be a necessary early event in induction and that after induction and subsequent replication, phage DNA may still be associated with bacterial DNA.

Replication of phage and bacterial DNA after prophage induction. Experiments were next performed to study DNA replication in temperature-induced T⁻(*cts23*). The bacteria were grown at 30°C in the presence of ^3H -thymidine. At a density of about 5×10^7 bacteria per ml, the cells were induced at 45°C in the absence of thymine. After 5 min at this temperature, the culture was shifted to 42°C and an equal volume of prewarmed medium containing BUdR and ^{14}C -thymidine was added. Samples were then taken at intervals from the culture, and DNA was extracted. The

added when cells shifted to 42°C. (c) T⁻ infected with *cts23* at an effective multiplicity of about 2.2; burst size was 48. Infectivity not shown in the figure. This sample was taken at 15 min after infection.

DNA was centrifuged to equilibrium in CsCl, and the fractions were assayed for radioactivity and for biological activity. In the presence of excess BUdR, phage DNA synthesis will occur, but no burst of infectious particles is produced.

In Fig. 4 is shown an experiment where DNA was extracted from $T^-(cts23)$ at 0 and 20 min after induction, respectively. In the 0-min sample, radioactivity and biological activity band at similar densities, about 1.703. After 20 min, about 11% of the prelabeled bacterial DNA has replicated as calculated from the shift of tritium to a position of increased density.

Although only a fraction of the bacterial DNA has replicated at 20 min in the induced cells, about 80% of the marker rescue activity is found at a higher density than the major tritium peak of unreplicated DNA (the arrows in the figure de-

note the peaks of marker rescue in the gradient). In other words, phage DNA replicates more rapidly than the bulk of the bacterial DNA after induction. The gradient fractions were assayed for their contents of the bacterial markers *phe-1* and *leu-2* by transformation. Of these markers, about 45% of the *phe-1* transforming activity is associated with replicated material of increased density, whereas only about 10% of *leu-2* transforming activity is found outside the main tritium peak of unreplicated DNA. The *phe-1* marker is linked to prophage $\phi 105$ by transformation and transduction (14), whereas *leu-2* is located further away from the prophage. The *phe-1* and the *leu-2* markers are replicated at the same rate in mock-induced wild-type lysogenic T^- or in temperature-induced $T^-(cts23, Kts31)$ (Fig. 5a-b). Thus, if the prophage is not induced by the temperature shift employed or if the prophage

FIG. 4. Effect of temperature induction of $T^-(cts23)$ on the replication of the bacterial chromosome. The cells were grown and induced as described in Materials and Methods and in text. DNA was extracted as described and centrifuged to equilibrium in CsCl. The fractions obtained were tested for ^3H (○—○), ^{14}C (○---○), *phe-1*⁺ transforming activity (●—●), *leu-2*⁺ transforming activity (●---●), and rescue of phage marker *sus19*; the peaks of marker rescue are indicated by arrows. (a) DNA taken 0 min after shifting the cells to inducing temperature. (b) DNA taken 20 min after shifting the cells to inducing temperature.

FIG. 5. Effect of temperature-inducing treatment on replication of the bacterial chromosome in mock-induced $T^-(\phi 105)$ and in $T^-(cts23, Kts31)$. Cells were grown and treated as in Fig. 4. The fractions obtained from the CsCl gradient were assayed for ^3H (○—○), ^{14}C (○---○), *phe-1*⁺ transforming activity (●—●), and *leu-2*⁺ transforming activity (●---●). The DNA samples were taken 20 min after shifting of the cells to inducing temperature. (a), $T^-(\phi 105)$. (b), $T^-(cts23, Kts31)$. Data are expressed as per cent of total recovered from the gradients.

carries a mutation (*Kts31*), which prevents phage DNA replication, in addition to the *cts23* mutation, there is no preferential replication of the *phe-1* region over the *leu-2* region on the bacterial chromosome. Heat induction of $T^-(cts23)$ thus seems to be associated with preferential replication of phage genes and with an alteration in the pattern of replication of the bacterial chromosome. At least one marker, *phe-1*, which is linked to the prophage, is replicated at an increased rate compared to another marker, *leu-2*, after prophage induction.

The effects of prophage induction on replication of phage and bacterial DNA were next investigated in the following experiments. $T^-(cts23)$ and $T^-(cts23, Kts31)$ were induced in the presence of BUdR, and DNA was extracted from each culture at various times after induction. Each DNA sample was centrifuged in a CsCl density gradient. In Fig. 6a-c are shown the CsCl gradients for DNA from $T^-(cts23)$, and in Fig. 7a-c, the gradients for DNA from $T^-(cts23, Kts31)$. The DNA samples from $T^-(cts23)$ were

FIG. 6. DNA was extracted from temperature-induced $T^-(cts23)$ incubated with 5-bromodeoxyuridine, as described in Materials and Methods and centrifuged in CsCl. DNA was extracted at 10 min (a), 20 min (b), and 30 min (c) after shifting the cells to inducing temperature. Symbols: 3H (●—●); ^{14}C (○---○); unreplicated DNA (LL); hybrid DNA, replicated once (HL); heavy DNA, replicated twice or more (HH).

FIG. 7. Same experiment as shown in Fig. 6, but performed with $T^{-}(cts23, Kts31)$. Symbols identical to those of Fig. 6. Sample (c) was extracted at 40 min.

also centrifuged in linear sucrose gradients (Fig. 10a-c).

The fractions from the CsCl gradients were pooled into fully light (LL), heavy-light (HL), and heavy-heavy (HH) density material as indicated in Fig. 6a-c and 7a-c. The pooled fractions were then assayed for radioactivity, for transforming activity, for several bacterial markers, and for phage marker rescue activity.

It should be noted that the DNA was extracted so as to minimize fragmentation of the material. As a result of the large size of much of the DNA, e.g., Fig. 10a-c, some ^{14}C -radioactivity appears in the density gradients between LL and HL density.

In Fig. 8a-c are presented the data for the DNA

from induced $T^{-}(cts23)$. The curves represent the relative rates of replication of the particular markers as well as the rate of replication of DNA labeled with tritium before induction. For instance, in Fig. 8a (per cent activity in LL fraction), it is seen that phage marker rescue (the lowest curve) leaves the unreplicated, LL, DNA fraction at a much faster rate than, for example, the terminal *metB5* transforming activity. By scanning a fixed time such as the 10-min DNA sample, it is seen that only 40% of phage DNA activity (measured as marker rescue) remains unreplicated (LL) at 10 min after induction (Fig. 8a), and 60% of this activity appears in the once-replicated (HL) material (Fig. 8b), whereas no activity is found in the HH material (Fig. 8c). In Fig. 8d-f are presented the results obtained with DNA extracted from the induced $T^{-}(cts23, Kts31)$, to allow comparison between the patterns of DNA replication observed after induction of the two lysogens.

The results of these experiments, as summarized in Fig. 8a-f, show that prophage induction has a profound effect on DNA synthesis. Essentially all phage DNA has replicated at least once at 30 min after induction of $T^{-}(cts23)$, and, of the bacterial markers, at least 80% of the *phe-1* transforming activity has replicated at this time. About 25% of the *phe-1* activity is found in material replicated twice or more at 30 min (Fig. 8c). However, about 50% of *purA16*, *leu-2*, and *ilvA1* transforming activity is still in the LL material at 30 min after induction (Fig. 8a), and only 10% of the total activity of these markers is found in the HH material (Fig. 8c). The terminal *metB5* marker replicates very little (about 10%) during the experiment, and no activity of this marker is recovered in the HH material. Thus, preferential replication of phage DNA and the *phe-1* marker is confirmed.

It should be noted that only 25% of the pre-labeled DNA has replicated at 30 min (Fig. 8b), whereas some phage DNA and *phe-1* transforming activity is found in the fraction replicated twice or more. This could not occur if there were not preferential local replication of the bacterial chromosome. We have also observed preferential replication of the bacterial marker *nia-38*, located close to *phe-1* in direction of the chromosomal terminus (Table 2). Thus, preferential replication of bacterial markers is not peculiar to the *phe-1* marker.

Induction of $T^{-}(cts23, Kts31)$ gives a totally different picture of DNA replication (Fig. 8d-f). About 50% of the phage marker rescue activity is found in the once-replicated (HL) fraction at 10 to 20 min after induction compared to about

FIG. 8. Rate of replication of bacterial markers, phage markers, and total DNA after temperature induction of $T^-(cts23)$ and $T^-(cts23, Kts31)$. The bracketed fractions in Fig. 6 and 7 were pooled, dialyzed overnight against saline, and assayed for biological activity. The data are given as per cent of total activity recovered from the gradient in a given time sample. On the left is shown $T^-(cts23)$ unreplicated (a), once replicated (b), and DNA replicated twice or more (c). On the right is shown $T^-(cts23, Kts31)$ unreplicated (d), once replicated (e), and DNA replicated twice or more (f). Symbols: 3H (○—○), *purA16*⁺ transforming activity (□—□), *leu-5*⁺ activity (□—□), *ilvA1*⁺ activity (×—×), *phe-1* activity (●—●), *metB5* activity (○---○), rescue of phage marker *sus19* (●---●).

15% for the bacterial markers *purA16*, *leu-5*, *ilvA1*, *phe-1*. Thus, in the presence of the *Kts31* mutation, preferential replication of phage DNA still occurs in the induced cells, although at a reduced rate. However, preferential replication of the *phe-1* marker is abolished, and *phe-1* is replicated at the same rate as other bacterial

markers (Fig. 8d). Twenty minutes after induction, replication of both bacterial and phage DNA virtually comes to a stop in $T^-(cts23, Kts31)$ and is not resumed during the remaining 20 min of the experiment (Fig. 8e-f).

Prophage induction has little effect on the rate of synthesis of total DNA, although it has a pro-

TABLE 2. Replication of bacterial and bacteriophage DNA in heat-induced $T^-(cts23)^a$

Time of DNA extraction (min)	Per cent marker activity in DNA replicated once or more				
	<i>purA16</i>	<i>leu-5</i>	<i>nia-38</i>	<i>metB5</i>	ϕ 105 <i>sus19</i> ⁺
10	20	23	42	7	59
20	37	30	74	14	81
30	41	43	71	14	97

^a DNA extracted from induced $T^-(cts23)$ and described in Fig. 6, 8, and 10 was used to transform BD25.

found effect on the rate of replication of individual portions of the bacterial chromosome (Fig. 9). $T^-(\phi 105)$, $T^-(cts23)$, and $T^-(cts23, Kts31)$ were induced, or mock induced, in the presence of BUdR. Samples were taken at intervals for DNA extraction, and the DNA was then centrifuged in CsCl. Pooled density fractions from the gradients were assayed as described above. In Fig. 9a is shown the rate of replication of DNA continuously labeled with 3H -thymidine for several generations before induction. In Fig. 9b is shown the rate of replication (as measured by shift to higher density) of the bacterial markers *leu-2* and *phe-1*. The results of these experiments are in full agreement with those presented in Fig. 8a-f. During the first 20 min after induction, the rate of replication of the prelabeled DNA is identical in the three lysogens. After this time, however, DNA replication stops in $T^-(cts23, Kts31)$. Only in $T^-(cts23)$ is there preferential replication of the *phe-1* marker. The *leu-2* marker is replicated at a higher rate in induced $T^-(cts23)$ compared to the wild-type lysogen, although their rates of replication of prelabeled DNA are identical, suggesting that reinitiation of DNA synthesis may occur after induction not only in the prophage and the *phe-1* region but also, less frequently, in other places on the bacterial chromosome.

The DNA samples taken from the induced $T^-(cts23)$ were assayed in transformation for linkage between the bacterial *phe-1* marker and the phage *Ats15* marker. A low degree of linkage could be detected even in DNA replicated twice or more (Table 3). No linkage was observed in DNA extracted from T^- cells infected with *cts23* phage under conditions similar to those used for induction. Some phage DNA thus remains covalently bound to bacterial DNA after induction and replication.

The DNA samples extracted from $T^-(cts23)$ and used in the experiments shown in Fig. 8a-c

FIG. 9. Rate of replication of the bacterial chromosome after temperature induction of $T^-(cts23)$, $T^-(cts23, Kts31)$, and mock induction of $T^-(\phi 105)$. Bacterial DNA was prelabeled with 3H -thymidine and induced in the presence of BUdR as described in Materials and Methods. DNA was extracted 10, 20, and 30 min after induction and centrifuged in CsCl. The gradient fractions were pooled as described for Fig. 6 and 7. Each fraction was assayed for its content of 3H -prelabel (a) and for *phe-1*⁺ and *leu-2*⁺ transforming activity (b). Results are presented as per cent activity of total, which is found in replicated DNA. (a) Per cent 3H -prelabel in replicated DNA: $T^-(\phi 105)$, $\circ$ — $\circ$; $T^-(cts23)$, $\bullet$ — $\bullet$; $T^-(cts23, Kts31)$, $\times$ — $\times$. (b) Per cent transforming activity in replicated DNA, *phe-1*⁺: $T^-(\phi 105)$, $\circ$ — $\circ$; $T^-(cts23)$, $\bullet$ — $\bullet$; $T^-(cts23, Kts31)$, $\times$ — $\times$; *leu-2*⁺: $T^-(\phi 105)$, $\triangle$ — $\triangle$; $T^-(cts23)$, $\blacktriangle$ — $\blacktriangle$; $T^-(cts23, Kts31)$, $+$ — $+$.

were also sedimented in neutral sucrose with ^{32}P -labeled P2 DNA as a size marker (Fig. 10a-c). With increasing time after induction, there is a gradual decrease in the sedimentation rate of phage DNA, with the first appearance of mature phage DNA at 30 min after induction. In no experiment have we found an abrupt, early change in the sedimentation characteristics of prophage DNA after heat induction, such as might be expected from an excision event. The relative amounts of rescue and infectivity (Fig. 10c) differ from those shown in Fig. 3b, probably due to the effects of BUdR on the biological activity of the DNA (9).

DISCUSSION

Two conclusions emerge from the experiments presented. First, we do not observe excision of

TABLE 3. Linkage between bacteriophage and bacterial markers in DNA from $\phi 105$ lysogenic bacteria^a

Strain and treatment	Fraction	<i>ts</i> ⁺ / <i>phe</i> -I ⁺ Transformants
T ⁻ (<i>cts23</i>) heat induced (fractions shown in Fig. 6)	10 min LL	9/104
	HL	3/52
	Unfractionated	9/156
T ⁻ (<i>cts23</i>) heat induced	20 min LL	0/70
	HL	5/182
	HH	0/80
	Unfractionated	4/94
T ⁻ (<i>cts23</i>) heat induced	30 min LL	0/52
	HL	2/104
	HH	2/80
	Unfractionated	7/156
T ⁻ ($\phi 105$) mock induced	10 min	21/115
	Unfractionated	
T ⁻ ($\phi 105$) mock induced	40 min	17/104
	Unfractionated	
T ⁻ infected with <i>cts23</i>	15 min	0/298
	Unfractionated	

^a BR95 ($\phi 105$ *Ats15*) was grown for competence as described. DNA extracted from induced T⁻(*cts23*) and described in Fig. 6, 8, and 10 was used to transform the cells to *phe*-I⁺. The distribution of the *ts* and *ts*⁺ alleles of the *Ats15* marker was then determined among the transformants (14). Ten minutes LL, HL, and HH refers to the fractions described in Fig. 6. Unfractionated means that the DNA is used prior to any centrifugation.

prophage DNA in heat-induced $\phi 105$ lysogenic bacteria. That is, the experiments give no indication that both strands of the prophage are precisely cut out of the bacterial chromosome prior to autonomous replications of phage DNA. Second prophage induction has a profound, but covert, effect on the pattern of replication of the bacterial chromosome.

The first conclusion, that prophage excision need not be an early event in induction, is supported by several experimental findings. In sucrose gradients of DNA from induced bacteria, the bulk of phage genes and whole phage genomes cosediment with high-molecular-weight bacterial DNA, even when most of the phage DNA has replicated at least once (see Fig. 8b, 10a). With increasing time after induction, phage DNA is found in more slowly sedimenting material, with a concomitant decline in the amount of phage DNA associated with prelabeled bacterial DNA. In induced *E. coli* (λ), most of the early synthesized DNA sediments in neutral sucrose at a rate characteristic for mature phage DNA (12, 20). Later in infection, this DNA is converted to more rapidly sedimenting material, which most likely represents covalently closed, circular phage DNA (12, 25). The λ DNA

sedimentation pattern is thus the reverse of that found for $\phi 105$. With this phage, we have never observed DNA with sedimentation characteristics of covalently closed circles either in induction, in infection, or in superinfected, immune, lysogenic cells.

In DNA from $\phi 105$ lysogenics, genetic linkage between phage and bacterial markers can be demonstrated by transformation (14). The degree of linkage decreases after induction of lysogenic bacteria, but some linkage persists even in DNA replicated two or more.

When DNA from T⁻ bacteria infected with *cts23* phage, under conditions similar to those used for induction, is sedimented in sucrose, phage DNA sediments more slowly than, and well separated from, bacterial DNA. No genetic linkage between phage and bacterial markers is found in such DNA.

Phage genes do not leave the region of bacterial DNA in the sucrose gradients when DNA synthesis is blocked after induction (Fig. 3a). However, the lysogenic bacteria are fully committed to induction; when the cells are returned to conditions permissive for DNA synthesis, more than 90% of them produce phage.

Although the prophage state is maintained in

FIG. 10. Sucrose gradient centrifugation of the DNA samples from $T^-(cts23)$, shown in Fig. 6, in the SW50 rotor. To the gradients were added 0.15 ml of DNA solution and 0.05 ml of appropriately diluted ^{32}P -labeled P2 DNA. Symbols: ^3H (●—●), ^{32}P (x---x), rescue of phage marker *sus19* (○—○), infectivity of DNA (○—○). DNA samples were taken at 10 min (a), 20 min (b), and 30 min (c) after the cells were shifted to inducing conditions.

induced cells in the absence of DNA synthesis, the infectivity of prophage DNA decreases. The

damage suffered by the prophage in these experiments is repairable, because in practice all induced cells produce infectious phage when allowed to resume DNA synthesis. The thymine starvation damage might be the result of abortive DNA synthesis in the prophage region.

When the lysogen $T^-(cts23, Kts31)$ is induced under conditions nonpermissive for gene K activity, phage DNA is preferentially copied once (Fig. 8d, e), whereas the bulk of the bacterial DNA remains unreplicated even 40 min after induction. The preferential replication of phage DNA indicates that some mechanism operates in the induced cells, which can specifically initiate replication of the prophage.

No extensive breakdown of bacterial DNA occurs either upon induction of lysogenic bacteria or after infection of sensitive bacteria. After infection, replication of the bacterial chromosome seems to proceed in a normal fashion at least during the first 30 min. Preliminary experiments indicate no preferential effect on replication of *phe-1* in infection.

It should be pointed out that the rate of replication of the bacterial chromosome, as measured by the transfer of prelabeled DNA from light to hybrid material, is identical in induced and in mock-induced T^- lysogenic bacteria, whereas the rate of replication of phage DNA, and at least the bacterial *phe-1* marker, is more rapid in induced bacteria (Fig. 9).

After induction of prophage *cts23*, bacterial markers closer to the origin of the bacterial chromosome than the prophage, replicate at similar rates. Marker *phe-1*, situated just distal to the prophage, exhibits a different pattern. It is initially replicated at a faster rate than other bacterial markers, and it appears earlier in fully heavy DNA. Induction of the prophage thus leads to preferential replication not only of phage DNA but also of an adjacent part of the bacterial chromosome. A new initiation site for replication must then be exposed in induced cells at or close to the prophage, with replication proceeding from this site in direction of the terminus, but stopping short of the terminal *metB*. Fragments of a size exceeding that of phage DNA are produced, as shown by sucrose gradient sedimentation as well as by preserved linkage between phage and bacterial markers in replicated DNA from induced $T^-(cts23)$. The integrated state of the prophage, in itself, generates an important element of the mechanism controlling replication, for induction alters the pattern of host DNA synthesis, whereas infection appears to have no such effect.

Preferential synthesis of adjacent regions of the bacterial chromosome after prophage induction

is not unique to the $\phi 105$ system. In lambda lysogenic *E. coli*, there is evidence from DNA-DNA hybridization studies that replication of the prophage may occur in situ after induction and proceed into the *gal* operon (10). Such replication in induced lambda lysogenics has been implied also in other types of experiments (13). This regional replication appears to be under control of the O and P cistrons.

Induction of integration-deficient (L) mutants of phage P22 gives few infectious particles but a substantial increase in *proC* and *purE* transducing particles (21). These markers are located close to one end of the prophage. The distribution of phage genes in the defective particles is strongly polar with genes distal to *proC* appearing with diminishing frequency. After induction of L mutants of P22, replication thus seems to be initiated at or close to the prophage and to proceed in the direction of *proC*. The mechanism which ultimately produces phage-size DNA is unknown.

Of particular interest in the present context is the defective phage PBSH carried by *B. subtilis* 168 (9). PBSH is inducible by mitomycin C. After induction, the relative amount of *purA16* transforming activity increases manyfold due to preferential replication of this marker (9). However, it cannot be decided whether preferential replication of *purA16* is due to repeated reinitiations at the bacterial initiation site at the origin or to the appearance of a new initiation site associated with the phage. Other bacterial markers also replicate after PBSH induction albeit at a slow rate compared to *purA16*. Breakdown of the bacterial chromosome does not occur in the induced cells; even late after induction, *purA16* containing DNA exists in pieces at least twice the size of the DNA found in the mature phage particles (9).

Our experiments indicate that there is more than one step involved in separating bacterial and phage genes after heat induction of $\phi 105$ *cts23* lysogens. After induction, phage genes replicate although they are still genetically linked to bacterial genes and appear in large-size DNA pieces. Apparently, there is a gradual decrease in the size of the DNA molecules which harbor phage genes during the course of induction (Fig. 10a-c). Rather than an early excision event, the process of separating phage and bacterial genes in induced bacteria seems to be gradual, with final definition occurring with the mature phage DNA.

In summary, excision does not seem to be the only mechanism by which a prophage can enter the phase of vegetative growth after induction of lysogenic bacteria. Whether induction in-

volves nucleases, recombinases, or polymerases, it requires recognition of the prophage region on the bacterial chromosome. Our data indicate that a DNA replication system may recognize the $\phi 105$ prophage after induction. It would appear, then, that in our system the initial events in lysogenic induction are fundamentally similar to the process of normal replication of the bacterial chromosome, in that some control apparatus directs a DNA replication system to a specific chromosomal site for initiation of replication.

On the basis of the data presented, this control apparatus cannot be defined. It could involve the unveiling of an initiation site at the prophage, which is blocked in the uninduced lysogenic bacteria, followed by binding of the DNA-polymerizing system already present in the cell to this site. Or, the primary event in induction may involve some interaction between this DNA-polymerizing system and some (phage-coded) product such that the specificity of the polymerizing system is altered. Further experiments are now in progress trying to resolve these possibilities.

ACKNOWLEDGMENTS

Kerstin Nilsson provided excellent assistance throughout this work.

The work was supported by grants from The Swedish Medical Research Council, Karolinska Institutets Forskningsfonder, and Emil och Wera Cornell's stiftelse. One of us (R.W.A.) holds a postdoctoral fellowship from the American Cancer Society.

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