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Synthesis of the Unusual DNA of *Bacillus subtilis* Bacteriophage SP-15

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Cultures of *Bacillus subtilis* infected with phage SP-15 were examined to investigate the metabolic origin of two of the unique components of the phage DNA: the component responsible for the unusually high buoyant density in CsCl and the unusual pyrimidine, 5-(4',5'-dihydroxypentyl) uracil (DHPU). Newly synthesized pulse-labeled DNA was light in buoyant density and shifted to the high density of mature phage DNA upon further incubation. Parental DNA was converted to a light-density intermediate form prior to replication. When labeled uracil, thymidine, or DHPU were added to infected cells, it was found that only uracil served as the precursor to DHPU and thymine in phage DNA. Analysis of the bases from hydrolyzed DNA of labeled phage or infected cells indicated that the uracil was incorporated into the DNA as such (presumably via deoxyuridine triphosphate) and later converted to DHPU and thymine at the macromolecular level. The sequence of events after phage infection appeared to be: (i) injection of parental DNA; (ii) conversion of parental DNA to a light form; (iii) DNA replication, yielding light DNA containing uracil; (iv) conversion of uracil to DHPU and thymine; and (v) addition of the heavy component.

Bacteriophage SP-15 is a generalized transducing phage infecting sensitive strains (W23, Marburg, and ATCC 6633) of *Bacillus subtilis* (16, 26). The DNA of SP-15 possesses a number of highly unusual properties: (i) an unusually low thermal denaturation temperature in 0.195 M Na^+ (61.5 C); (ii) sensitivity to alkali, prolonged incubation in 0.3 M KOH, causing a gradual degradation of the molecule to fragments sedimenting at 5.5*S* in alkaline salt solutions; (iii) alkali-induced release of an unknown phosphorylated carbohydrate from the macromolecule, accompanied by a reduction in the buoyant density; and (iv) the replacement of thymine by the unusual pyrimidine base, 5-(4',5'-dihydroxypentyl) uracil (DHPU) (2, 16).

Our present model of the structure of the DNA molecule shows that DHPU is contained in both strands of the DNA. There is a sugar in a phosphodiester linkage which is presumably linked to the 4' or 5' hydroxyl group, or both, of the side chain of DHPU, and this sugar-phosphate, which is the component responsible for the high density, is removed by incubation in alkali. The identity of this sugar phosphate component is presently under investigation (C. Brandon, personal communication). The mechanism of alkali-induced fragmentation of the DNA remains unclear.

The presence of unusual pyrimidines in phage DNA has been reported. DNA of the T-even phage of *Escherichia coli* contains hydroxymethylcytosine (HMC) in place of cytosine (28), whereas *B. subtilis* phage PBS1 contains uracil in place of thymine (25). The pyrimidine 5-hydroxymethyluracil (HMU) replaces thymine in *B. subtilis* phages SP8 (9), SP5C (1), SP01 (19), SP82 (7), and ϕe (22), and the unusual pyrimidine 5-(4-aminobutylaminomethyl) uracil partially replaces thymine in the DNA of *Pseudomonas acidovorans* phage ϕ-W-14 (11). Both HMC (6) and HMU (22) are synthesized at the nucleotide level and incorporated into DNA via their deoxynucleoside triphosphates. 5-Hydroxymethyldeoxyuridine can also be incorporated into SP8 DNA when added to the growth medium (18).

Although sugar-phosphate components not in the backbone have not been previously reported in other nucleic acids, unusual sugars are present in some DNA species. The most notable example is the presence of glucose on the hydroxymethyl group of HMC residues in T-even phage DNA (8, 23, 24). The glucose is transferred by phage-induced glucosyl transfer-

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ase enzymes from uridine-diphosphate-glucose to HMC residues in polymerized DNA rather than to free nucleotides (10).

The present work, concerning SP-15 DNA synthesis, examines (i) whether the unusual sugar phosphate, probably responsible for the high density, is added to the unusual base before or after replication; (ii) the nature of the metabolic precursor to DHPU; (iii) whether DHPU is formed before or after the polymerization of newly synthesized DNA; (iv) whether DHPU can be incorporated as such into phage DNA; and (v) if there is any modification of parental phage DNA after infection.

MATERIALS AND METHODS

Phage and growth media. Stocks of phage SP-15 were grown on *B. subtilis* Marburg in SP-15 medium as previously described (16). For labeling of DNA, *B. subtilis* W23 was used as the host. When DNA was to be radioactively labeled at high specific activity, infection was in NLM medium, which contained 0.1 M Tris-hydrochloride, 0.2% NaCl, 0.1% $(NH_4)_2SO_4$, 1.0% vitamin-free casein hydrolysate (Difco), 10^{-3} M $MgCl_2$, 10^{-3} M $CaCl_2$, 2×10^{-4} M $MnCl_2$, 2×10^{-6} M $FeCl_3$, 10^{-3} M KH_2PO_4, and 0.5% glucose, pH 7.4. Growth of infected cultures was rapidly stopped, when necessary, by adding frozen 0.15 M NaCl-0.1 M EDTA (pH 8.0) containing 0.01 M KCN.

Materials. ^3H- and ^{14}C-labeled biochemicals were purchased from New England Nuclear Corp. Unlabeled purine and pyrimidine bases were obtained from Calbiochem, and synthetic DHPU was kindly supplied by H. Hayashi and K. Nakanishi of Columbia University. Bacterial and SP-15 DNA, labeled with ^{14}C for use as density markers, were prepared from uninfected or infected W23 cultures, labeled in the presence of adenine-*8*-^{14}C for 1 h. 5-(*p*-Hydroxyphenylazo)-uracil (HPUra) was a gift of B. Langley of Imperial Chemical Industries, Ltd. CsCl was obtained from the Kerr-McGee Corp.

Assay of radioactivity in DNA. Samples containing labeled nucleic acids were made 0.5 M in NaOH and incubated at 60 C for 2 h or at 37 C overnight. Bovine serum albumin (0.1%) was then added, followed by sufficient HCl to neutralize the NaOH and trichloroacetic acid to a concentration of 5%. The chilled samples were filtered through GF/A glass-fiber filters (Whatman) which were then washed with 5% trichloroacetic acid and 95% ethanol, dried, and counted in a liquid scintillation counter by using Liquifluor (New England Nuclear Corp.) diluted with toluene.

Isopycnic banding in CsCl density gradients. DNA solution (7 ml) was brought to a density of about 1.73 g/cm^3 by using solid CsCl or by adding saturated CsCl solution. The solution was overlayered with light mineral oil and centrifuged at 33,000 rpm in the Beckman Spinco type 40 or 50 Ti rotor at 18 C for 65 h. Fractions were collected by slowly removing the solution from the bottom of the tube by using a peristaltic pump.

DNA isolation. DNA was purified from infected cells by the method of Marmur (15) and treated with pancreatic RNase and T1 RNase. DNA was isolated from purified phage preparations by shaking with neutralized, saline-EDTA-saturated phenol (15), followed by extensive dialysis.

Hydrolysis of DNA and separation of bases. A maximum of 1 mg of purified DNA was precipitated with 95% EtOH, the resulting DNA fibers were wound onto the tip of a sealed Pasteur pipette, and the tip was broken off and placed at the bottom of a 10- by 75-mm Pyrex tube. Formic acid hydrolysis was carried out at 175 C in 90% formic acid (0.5 ml) for 2 h in sealed tubes. The tubes were then opened and the formic acid was evaporated almost to dryness. The hydrolysate was applied to 20- by 20-cm or 10- by 20-cm Eastman Chromagram thin-layer chromatography (TLC) plates containing cellulose without indicator, and developed in the solvent systems described in Table 1. The six bases of the nucleic acid of phage-infected cells were completely resolved by two-dimensional chromatography using solvents 1 and 2, respectively. As markers, unlabeled bases were spotted on the plates prior to development, and the separated bases were observed under UV light.

Assay of radioactivity in separated bases. Spots containing the bases were cut out of the TLC plates. The spots were then cut into small pieces (or the cellulose powder was scraped from them) and placed in scintillation vials. Formic acid (0.5 ml) was added to elute the bases, and after 1 h at room temperature 10 to 15 ml of Aquasol (New England Nuclear Corp.) was added. The vials were allowed to stand in the cold for several hours and then were counted as above.

RESULTS

Synthesis of DNA in SP-15-infected and HPUra-treated cells. Figure 1 shows the course of *B. subtilis* W23 DNA synthesis, as measured by the incorporation of adenine-*2*-^3H into alkali-stable, trichloroacetic acid-precipitable material. The drug HPUra has been shown to inhibit bacterial DNA synthesis, but not phage DNA synthesis in SP3-, SP02c-, and SP8-infected *B. subtilis* 168 (3) and phages P1- and 44 ADJD-infected *Staphylococcus au-*

TABLE 1. *R_f values of bases on cellulose TLC*

Base	Solvent 1[a]	Solvent 2[b]	Solvent 3[c]
Guanine	0.02	0.22	0.31
Adenine	0.08	0.34	0.34
Cytosine	0.03	0.49	0.48
Thymine	0.55	0.84	0.70
DHPU	0.13	0.82	0.59
Uracil	0.35	0.73	0.56

[a] Solvent 1: upper layer of ethyl acetate-water-formic acid (60:35:5).

[b] Solvent 2: 2-propanol-concentrated HCl-water (65:16.6:18.4).

[c] Solvent 3: *n*-butanol-glacial acetic acid-water (4:1:1).

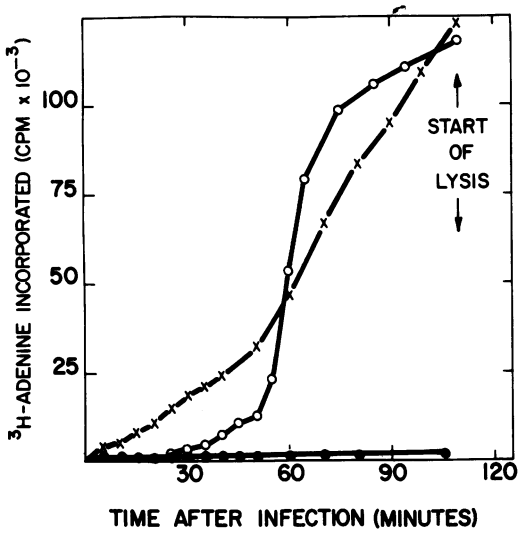

FIG. 1. *DNA synthesis in SP-15-infected and HPUra-treated cultures. Cells were grown in SP-15 broth and divided into three separate cultures. One culture was incubated without phage or HPUra (×), whereas 200 μM HPUra was added to the other two. Five minutes later, one of the HPUra-treated cultures was infected (○) and the other remained uninfected (●). At the same time, 10 μCi of adenine-2-^{3}H per ml was added to each culture. At the indicated intervals, samples were withdrawn and assayed for alkali resistant, trichloroacetic acid-precipitable radioactivity.*

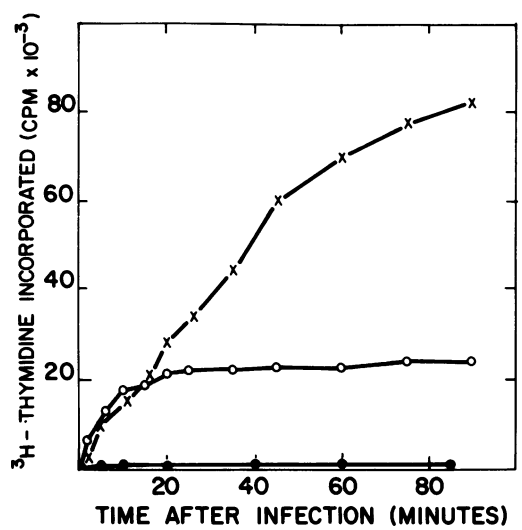

FIG. 2. *Incorporation of ^{3}H-thymidine into SP-15-infected and uninfected cultures. Experimental procedure was similar to Fig. 1. One culture was infected in the presence of HPUra (●), one was infected without HPUra (○) and one was uninfected (×). Labeling was with 5 μCi of thimidine-6-^{3}H per ml.*

reus (4). HPUra has been shown to act by inhibiting DNA polymerase III of *B. subtilis*, which is necessary for replication (5, 13). In the case of SP-15, 200 μM HPUra completely inhibits DNA synthesis in uninfected cells, as well as in infected cells prior to 30 min (Fig. 1). At 30 min, phage DNA synthesis begins, and it is insensitive to the action of the drug. Productive infection proceeds, and infective lysates are obtained in the presence of HPUra. Based on these results, subsequent experiments were performed with the drug present at 200 μM to permit specific labeling of phage DNA. At about 60 min after infection, the rate of phage DNA synthesis is maximal, and in subsequent experiments involving pulse labeling of DNA, label was added at this time. Similar results were obtained when uracil-6-^{3}H rather than adenine-2-^{3}H was used as a precursor. ^{3}H-thymidine, which was incorporated into the DNA of uninfected cells, was not taken up by infected cells in the presence of HPUra (Fig. 2). This indicates that uracil, and not thymidine, is the precursor to the DHPU and thymine in phage DNA. In infected cultures not treated with HPUra, thymidine incorporation proceeds for about 20 min, after which it ceases; this probably indicates the shutdown of host DNA synthesis.

Presence of thymine in native SP-15 DNA. The failure of infected cells to incorporate thymine into phage DNA suggests that thymine may not be a component of the native DNA as previously reported (16) and that its presence in formic acid hydrolysates may be due to an artifact caused by breakdown of DHPU to thymine during the severe conditions of formic acid hydrolysis. When synthetic unlabeled DHPU or purified DHPU-6-^{3}H (from labeled phage DNA hydrolysates) was hydrolyzed in formic acid, it was indeed found that some of the DHPU was converted to thymine. However, in enzymatic digests of SP-15 DNA, thymidylic acid has been found, as well as an uncharacterized nucleotide which is probably that of DHPU (C. Brandon, personal communication).

Density of newly synthesized DNA. To determine whether SP-15 DNA is synthesized containing the high density component or whether it is synthesized in a light form and the dense component added later, the following experiment was carried out. Infected cells were pulse-labeled for 90 s with adenine-2-^{3}H, and the total infected cell-labeled DNA was isolated and banded in CsCl. Most (~85%) of the pulse-labeled DNA banded at a density slightly less than that of host DNA (1.703 g/cm^3) (Fig. 3A). A portion of the pulse-labeled culture was chased with 200 μg of unlabeled uracil per ml for

20 min. The labeled DNA isolated after this chase was of normal density (1.763 g/cm³) (Fig. 3B).

Fate of parental phage DNA in infected cells. The results shown in Fig. 2 indicate that newly synthesized DNA is of light density. Since, during semiconservative replication, newly synthesized DNA should consist of one strand of parental and one strand of progeny DNA, labeled DNA would be expected to be of hybrid density after a short pulse (17). Since light and not hybrid DNA was found, it appears that the parental DNA must be modified by removal of the dense component prior to replication. To test this possibility, phage was prepared with its DNA labeled with adenine-2-^3H at a high specific activity. Cells growing in SP-15 broth were infected with labeled phage, and 10 min later the cells were washed by centrifugation to remove unadsorbed phage. At intervals after resuspension, DNA was isolated and examined in CsCl density gradients similar to those shown in Fig. 3. The results (Table 2)

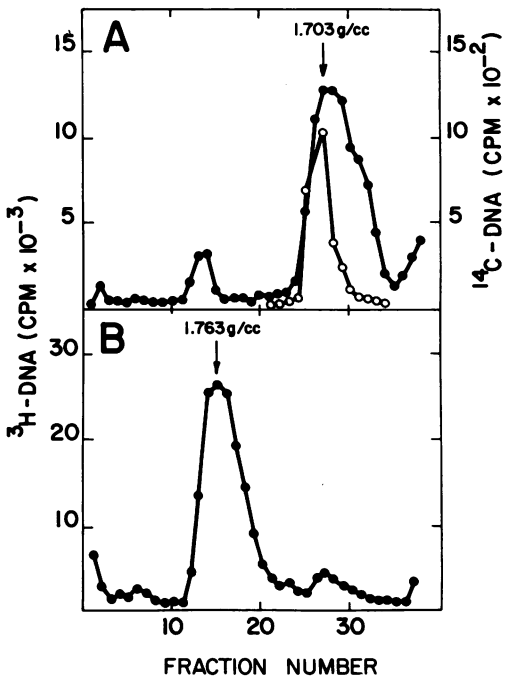

FIG. 3. *Buoyant density of pulse-labeled phage DNA in infected cultures. Infected cells growing in NLM medium were pulse labeled at 60 min with 200 μCi of adenine-2-^3H per ml. A, After 90 s, DNA was isolated from half the culture and banded in CsCl (●), in the presence of a marker of ^{14}C-labeled B. subtilis DNA (1.703 g/cm³) (○). B, The other half was chased with 200 μg of unlabeled uracil per ml for 20 min, and the DNA was banded in CsCl using ^{14}C-SP-15 (1.763 g/cm³) DNA as a marker (arrow).*

TABLE 2. *Fate of parental phage DNA after injection into the bacterial host*

Source of DNA	Time after infection (min)	Treatment	Buoyant density	
			1,763 g/cm³	1,700 g/cm³
Purified phage			98[a]	2
Infected cells	10	None	98	2
Infected cells	30	None	69	31
Infected cells	45	None	89	11
Infected cells	60	None	98	2
Infected cells	30	KCN	93	7
Infected cells	30	Chloramphenicol	91	9

[a] Numbers represent the percent of the total DNA radioactivity of the CsCl gradient appearing in the respective peak. In each case at least 20,000 counts/min were recovered from the gradients. Background, amounting to less than 0.5% per fraction, was subtracted.

indicate that early in infection, when the parental DNA begins to be replicated (Fig. 1), a fraction of the parental DNA is indeed of light density. The density of this light species is identical to that of the newly synthesized DNA shown in Fig. 3A. It thus appears that, prior to replication, the dense component is removed, new DNA is synthesized, and the heavy component is then attached to both strands. Similar results were obtained with uracil-6-^3H-labeled parental phage and with cultures washed free of unadsorbed phage at the end of the incubation time. Preliminary experiments indicate that when the infected cells are incubated in the presence of 0.005 M KCN or 100 μg of chloramphenicol per ml beginning at 10 min, the conversion of parental DNA to the light species is prevented, suggesting that protein synthesis is required for this conversion to take place.

Origin of the DHPU of mature phage DNA. Since nascent phage DNA is synthesized without the dense component, it was of interest to see whether the DNA is polymerized containing DHPU or whether uracil (which is the precursor) is incorporated into the polymer and later converted to DHPU at the macromolecular level. To determine the sequence of events infected cells were pulse labeled with uracil-6-^3H for 90 s. DNA was prepared from a portion of the culture, while another portion was chased with 200 μg of cold uracil per ml for 20 min prior to DNA isolation. A third portion was chased until the culture lysed, and DNA was isolated from purified phage. The DNA was extensively treated with RNase and precipitated several times with 95% ethanol. Precipitated DNA was

hydrolyzed in formic acid, and the bases were separated by two-dimensional TLC, using solvents 1 and 2 (Table 1). The radioactivity in the various pyrimidines (Table 3) indicated that uracil is indeed the precursor of DHPU and that pulse-labeled DNA contains ^{3}H-uracil, which is subsequently chased into the thymine and DHPU which appear in phage DNA of infected cells and mature phage.

DHPU-6-^3H was isolated by scraping the cellulose from the DHPU spot of a similar two-dimensional TLC plate, eluting the base with 50% formic acid and concentrating the eluate by evaporation. The DHPU was further purified by TLC in solvent 3. Like thymidine, labeled DHPU was not incorporated into the DNA (or RNA) of infected cells or mature phage. Assuming that DHPU can be converted to the nucleotide, this indicates that uracil is the obligatory precursor of the DHPU of SP-15 DNA. The isolation of the nucleoside and nucleotides of DHPU is presently being undertaken.

Sequence of events in SP-15 DNA synthesis. The above results have shown that SP-15 DNA is synthesized in a light density form containing uracil, and that the dense component is subsequently attached and uracil is converted to DHPU. Do these events occur simultaneously, or does one occur before the other? To answer this question and to investigate the kinetics of these two modifications of the newly made polymer, infected cultures were labeled with uracil-6-^3H, and portions were removed at various times after the addition of the label. DNA was prepared and each sample was examined in CsCl gradients to determine the relative amounts of dense and light DNA, and by TLC to determine the relative amounts of labeled DHPU, thymine, and uracil. Figure 4 shows that uracil in newly synthesized DNA is rapidly converted to DHPU and thymine, and thereafter the light DNA is converted to a dense species. Addition of KCN to a culture 1 min after addition of the label prevents the conversion of uracil to DHPU and thymine, indicating that this conversion is energy dependent.

TABLE 3. *Radioactivity of pyrimidines in SP-15 DNA labeled by uracil-6-^3H*

Source of DNA	Pyrimidine			
	Cytosine	Uracil	Thymine	DHPU
Pulse-labeled cells	1,140[a]	7,522	851	1,020
Pulse-chased cells	2,256	2,215	36,914	21,628
Purified phage	2,410	2,544	40,683	23,540

[a] Numbers represent counts per minute of tritium in the respective spots on TLC plates.

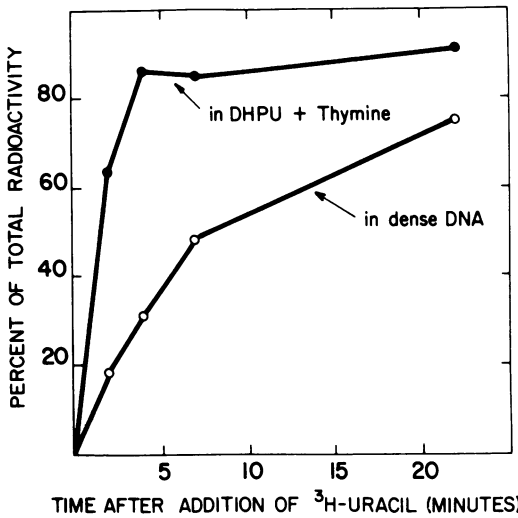

FIG. 4. *Conversion of labeled DNA from light to heavy form and of uracil to DHPU plus thymine. Samples of infected cultures were removed at various times after labeling with uracil-6-^3H, and the fraction of the label appearing in the heavy peak (○) in CsCl, and in DHPU plus thymine on TLC (●) was determined.*

DISCUSSION

The data presented show that, upon infection of HPUra-treated *B. subtilis* with SP-15, the following sequence of events take place. A protein(s) is synthesized which causes the removal of the dense component from parental DNA. At about 30 min after phage addition, replication begins. New DNA strands are synthesized, incorporating uracil (deoxyuridylic acid) into the polymer. Uracil is then converted to DHPU (and perhaps thymine) by an energy-requiring process after which the dense (sugar phosphate) component is added to both strands.

An interesting feature of this scheme is that parental DNA is found in the light intermediate form only early in infection, before replication has reached its maximal rate (Table 2). This could be due to either of two reasons. (i) Possibly parental DNA is replicated only early in infection, and progeny DNA is replicated later. This implies a mechanism for identification of DNA containing parental strands. (ii) Possibly only a fraction of the DNA is engaged in replication at a given time, the remainder being engaged in transcription or withdrawn for packaging into phage heads. As replication

progresses, an increasingly large number of phage equivalents of DNA is present in the infected cells, and the fraction having been irreversibly withdrawn for packaging increases while the fraction engaged in replication decreases. Since, in addition, only a small fraction of the DNA molecules contain parental DNA by this time, the radioactivity in the light peak might be too small to detect.

The postreplication conversion of uracil to DHPU differs from the synthesis of HMC (6) and HMU (22), which are synthesized as the nucleoside monophosphates from deoxycytidylate and deoxyuridylate, respectively, before their polymerization into DNA. Unlike the case of phage SP8, where labeled 5-hydroxymethyldeoxyuridine can be incorporated (18), DHPU cannot be incorporated into SP15-DNA. Isolation and purification of the labeled deoxynucleoside of DHPU will allow us to determine whether the nucleoside can be incorporated.

The above work does not clarify the physiological role of these unusual components. The presence of glucose in T-even phage DNA appears to play a role in protecting the DNA from host nucleases, since phage grown on hosts which are unable to glucosylate DNA are unable to infect restricting hosts (21). However, this may not be the function of the sugar phosphate of SP-15 DNA, since phage restriction does not appear to operate in *B. subtilis*, at least in strain 168 (J. Marmur, unpublished results). Two preliminary observations indicate that the sugar phosphate may play a role in transcription. Mature phage DNA is a very poor template for RNA polymerase isolated from host cells, *E. coli*, or from *Caulobacter*. Furthermore, extracts of SP-15 infected (but not uninfected) cells can remove the sugar phosphate from SP-15 DNA (I. Bendis, personal communication). Taken together, these observations point to the possibility that the sugar phosphate may control the extent of phage transcription at different stages of the infectious cycle.

The function of DHPU likewise remains unclear. In amber mutants of T4, phage DNA can be produced containing up to 20% replacement of HMC by cytosine. Such DNA is extensively degraded by phage-induced nucleases. In addition, transcription of some late genes does not occur from cytosine-containing DNA (12, 20, 27). However, mutants of the virulent *B. subtilis* phage ϕe, in which 10 to 25% of the HMU can be replaced by thymine, appear to carry out normal infection (14). Mutants of SP-15 lacking DHPU and mutants lacking the sugar phosphate will have to be isolated before the function of these components becomes clear. It is conceivable that DHPU may influence transcription, since it is known that the presence of this base lowers the denaturation temperature of phage DNA (16).

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